ADRENAL CORTICAL FUNCTION IN MURINE LEUKEMIA

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ABSTRACT
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Utilizing an ultramicro radioassay for corticosteroids, the level of adrenocortical function was significantly higher in AKR mice with acute lymphatic leukemia than in all other groups investigated. Mice with incipient leukemia, however, had below normal plasma corticosterone (PC) levels than controls. There was no such significant difference between healthy mice of the two strains, but healthy females of each strain had reliably higher functional levels than healthy males. Female AKR mice bearing the transplantable BW5147 tumour also had higher PC levels than tumour-bearing males. However, no such sex differences were observed in the other groups of sick mice.

PC levels of both strains showed a marked decline between 2 and 4 months which was similar in females of both strains, but which was significantly greater in C57 than in AKR males. A second peak occurred at 6 and 8 months in males and females respectively with little change thereafter except for a second decline in C57 females.

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IN MURINE LEUKEMIA

by

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To my Parents,
Brothers and Sisters,
for their constant encouragement
and kind thoughts

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An ultramicro radioassay based on the principle of competitive protein-binding was adapted for use in the determination of plasma corticosterone and urinary corticosteroids of high-leukemia AKR and low-leukemia C57 mice. Tests of precision, accuracy, specificity and of stimulation with ACTH revealed that the technique was suitable for this purpose. Mice of both sexes, a wide range of ages, healthy or sick with spontaneous lymphatic leukemia in its incipient or advanced stages or ill with a transplanted lymphatic leukemia, or sick with diseases other than leukemia were investigated. One series of animals was investigated under conditions not involving stimulation by exogenous ACTH while another was studied before and after stimulation by the hormone.

The results showed that there was no significant difference in adrenocortical function between healthy mice of the AKR or C57 strain, but that healthy females of each strain had reliably higher levels of adrenocortical function than healthy

males. Female AKR mice bearing the transplantable BW5147 tumour for one week also had higher plasma corticosterone levels than males bearing the same tumour. However, no significant difference in plasma corticosterone levels was observed in males and females ill with spontaneous lymphatic leukemias. Mice ill with acute lymphatic leukemia had significantly higher levels of adrenocortical function not only than healthy mice of either strain, but also reliably higher than those with incipient lymphatic leukemia as well as those sick from causes other than leukemia. The latter group of mice did not appear to have a reliably higher level of adrenocortical function than healthy mice, but there was a suggestion from the plasma corticosterone levels that mice with incipient leukemia had a lower than normal level of adrenocortical function. However, further studies of the urinary corticosteroids are indicated in this group.

Both AKR and C57 mice showed a decline in PC levels between 2 and 4 months of age which was similar in degree in the females of both strains, but which was steeper in C57 than in AKR males. In males of both strains, the PC level rose until 6 months of age whereas in females the

peak occurred at 8 months. After the maximum was reached, the values remained more or less at the same level in C57 males and in both sexes of the AKR strain but the values declined further in C57 females.

In general, differences between groups in plasma-corticosterone levels and urinary corticosteroids amounts existing prior to ACTH stimulation remained afterwards also.

Finally, thymic and splenic weights were significantly higher in mice with ALLk than in all other groups including mice with ILLk that in turn had significantly higher lymphatic tissue weights than non-leukemic AKR controls.

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LIST OF ABBREVIATIONS

a	Alpha
ACE	Adrenocortical extracts
ACTH	Adrenocorticotropic hormone
Adrex	Adrenalectomy
adrexed	Adrenalectomized
AGLk	Acute granulocytic leukemia
Aldo	Aldosterone
ALk	Acute leukemia
ASD	Delta 4-androstenedione
B	Corticosterone
BW	Body weight
Ca	Calcium
CBG	Corticosteroid-binding globulin
CC	Circulating corticosterone
CCl_4	Carbon tetrachloride
CCS	Circulating corticosteroid (corticoid)
CGLk	Chronic granulocytic leukemia
Ch	Cholesterol
CH_2Cl_2	Dichloromethane (methylene dichloride)
$CHCl_3$	Chloroform
CHO	Carbohydrate
Cl	Chloride
CLk	Chronic leukemia
CLLk	Chronic lymphatic leukemia
CNS	Central nervous system
CPB	Competitive protein binding
cpd	Compound
CRF	Corticotrophin releasing factor
CS	Corticosteroid
CSF	Cerebrospinal fluid
DHEA	Dehydroepiandrosterone
DOC	11-desoxycorticosterone
E	Cortisone
EDTA	Ethylenediamine tetra accetate
EEG	Electroencephalogram
F	Cortisol (hydrocortisone)
GCC	Glucocorticoid
GH	Growth hormone
GLk	Granulocytic leukemia
G-6-P	Glucose-6-phosphate
H_2O	Water
HCl	Hydrochloric acid

Continued...

HCO_3	Bicarbonate
H_2SO_4	Sulphuric acid
HGH	Human growth hormone
HMP	Hexose monophosphate shunt
hypox	Hypophysectomy
hypoxed	Hypophysectomized
ILk	Incipient leukemia
ILLk	Incipient lymphatic leukemia
K	Potassium
Lk	Leukemia
LLk	Lymphoid (lymphatic) leukemia
LSF	Lymphocytosis-stimulating factor
MCC	Mineralocorticoid
MER-29	Triparanol
Mg	Magnesium
mgs	Milligrams
MLk	Myeloid (myelogenous) leukemia
N	Nitrogen
Na	Sodium
NHS	Normal human serum
NPB	Non-protein bound
P	Phosphorus
P^{32}	Radio phosphorus
PB	Protein binding
PC	Plasma corticoid
PMN	Polymorphonuclear
Prog	Progesterone
RAS	Renin-angiotensin system
S	11-deoxycortisol
SD	Standard deviation
SNLk	Sick non-leukemic
T_3	Triiodothyronine
T_4	Thyroxine
TBG	Thyroxine-binding globulin
TBPA	Thyroxine-binding pre-albumin
TEM	Triothylenemelamine
thymy	Thymectomy
TLC	Thin-layer chromatography
TPNH	Reduced triphosphopyridine nucleotide
TSH	Thyroid-stimulating hormone
UCS	Urinary corticosteroid (corticoid)
UV	Ultra violet
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticularis

PART I

CHAPTER I

THE ADRENAL CORTEX

1. HISTORICAL BACKGROUND

The first reported description of the adrenal gland was that of Bartholomaeus Eustachius in 1563 (Ibanez, 1952), who called it "Glandulae renibus incumbentes". Since then it has been called by several other names, such as "Glandulae renales", "capsulae renales", and more familiar name of "supera-renal capsules".

In the 17th century the idea was prevalent that the adrenals functioned only in fetal life, although Sampson probably recognized, in 1697, that the adrenals may be associated with the production of tumours. However, in 1803, Tilesius (Soffer, Dorfman and Gabrilove, 1961) reported the first recorded case with autopsy findings of a tumour of the left adrenal in a four-year old girl, who was enormously obese with a marked precocious development of the brests. In 1811, Cooke described a similar case, also a four-year old obese girl, who had enlarged external genitalia, extensive hirsutism of the

genitalia and face, and low-pitched voice. She died at the age of seven.

Gulliver, in 1840, suggested that the superarenals pour a peculiar matter into the blood which doubtless has a special use.

The latter half of the 19th century saw a significant advance in our knowledge, both of morphology and function of the adrenal. In 1855, Thomas Addison described the disease which bears his name, and stimulated by Addison's clinical observations, Brown-Séquard (1856) showed that dogs, cats and rabbits die soon after bilateral adrenalectomy (adrex). In the same period, Claude Bernard (Jones, 1957) introduced the idea of internal secretion, that is the secretion into the circulation by a ductless organ. Also, in 1856, Vulpian reported the first significant observation on adrenal medulla. He noted that when the adrenal medulla was moistened with dilute ferric chloride solution, a green coloration was produced. This observation led to the thought that the adrenal medulla secreted some unknown substance or substances which had a catechol nucleus but the exact significance and nature of which were not known at the time. Later, Oliver and Schaefer (1894) demonstrated the remarkable rise in blood pressure

following the injection of an extract of the adrenal medulla, thus reporting a potent pressor substance as an active principle of the medulla. Some eight years later, Abel (1902) succeeded in isolating a crystalline compound from the adrenal gland, which he considered to be its active principle and which he called "epinephrine". Following that, the chemical configuration of adrenaline became known and a method for its synthesis was established (Aldrich, 1901; Takamine, 1901; Stolz, 1904).

Early in the 20th century, Biedl (1913) provided evidence for functional division of the adrenal gland by showing that the removal of the cortex in dogs and rabbits, leaving the medulla intact, always resulted in death, while destruction of the medulla had little effect. Animals would survive with only one-eighth of their adrenal tissue, provided it was cortical tissue. This was the first evidence that the adrenal cortex played a vital role in the maintenance of life. Later he proposed the concept of an "endocrine integration" which, together with the notion of internal secretion, promoted by Claude Bernard (Jones, 1957), marks the dawn of modern endocrinology.

Since the recognition of the disease, Addisonian patients had been treated with various adrenal

preparations and it was observed that better therapeutic results were obtained with whole adrenal extracts than those from purely medullary source (Biedl, 1913). As more evidence accumulated (Wheeler and Vincent, 1917; Houssay and Lewis, 1923; and Zwemer, 1925), it became obvious that it is the cortical portion of the supera-renal complex that is essential for life, and consequently scientists began their quest for the active principle which, in 1928, Hartman, Brownell, Hartman, Dean and MacArthur, named "cortin". A few years later, Swingle and Pfiffner (1930) demonstrated adrenalectomized (adrexed) animals can be maintained by injections of adrenocortical extracts (ACE), and simultaneously with other workers, traced the activity to the lipid fractions (Hartman and Brownell, 1930; Swingle and Pfiffner, 1930a, 1930b, 1931). At about the same time, Smith (1927, 1930) reported that the adrenal cortex is under the influence of pituitary gland. Later, Ingle, Higgins and Kendall (1938) observed that the administration of ACE resulted in adrenalcortical atrophy, a condition that could be reversed by the administration of pituitary extracts.

Between 1930 and 1940 a number of steroids were isolated from adrenal extracts by Reichstein, Kendall and Wintersteiner (Reichstein and Schoppee, 1943). In 1943,

Reichstein and Schoppee summed up the work on the adrenal cortex, as follows: the adrenals are vital organs in nearly all animals and the complete adrex leads to death. The vital function is connected with the adrenal cortex, and appears to operate by delivery into the blood of a mixture of substances, since by injection of suitable ACE, adrexed animals can be kept alive, and numerous insufficiency symptoms prevented or cured. Furthermore, the investigations of active ACE show that the activity can be concentrated in those fractions which contain principally a mixture of relatively heavily oxygen-substituted steroids.

The first quantitative studies on the biochemical composition of the stimulated adrenal cortex were reported by Sayers' group in 1944 (Sayers, Sayers, Fry, White, Lewis and Long, 1944a, 1944b). These authors showed that the administration of adrenocorticotropic hormone (ACTH) to rats resulted in a decrease of both the ascorbic acid and the cholesterol concentrations of the adrenals. Stress was shown to induce similar biochemical changes in normal rats (Sayers, Sayers, Lewis and Long, 1947).

It is remarkable that it has been known for more than a century, since the time of Addison

and of Brown-Séquard, that the adrenal glands are essential for life, and it has been well established for over 50 years that they may be associated with the production of human virilization. As mentioned earlier, it is even probable that the latter circumstance was first recognized in 1697 by Sampson, in association with a tumour of an adrenal gland. Yet it is only within the last four decades that any knowledge has become available of the steroid hormones secreted by the adrenal cortices, and their chemical synthesis achieved. Moreover, there is a very great difference that exists between the detailed knowledge of the chemistry and biochemistry of these hormones on the one hand, and of our comparative ignorance of their mode of action, and even their functions under physiological conditions.

2. MORPHOLOGY OF THE ADRENAL GLAND

2.1. EMBRYOLOGY

The adrenal gland consists of two parts, cortex and medulla, each with a separate origin, structure and endocrine function. The cortical anlagen develops in the mesodermal coelomic epithelium near the genital ridges at about the 12th day, the medullary

anlagen from sympathetic nervous system ganglia at the 13th day, and the first sign of union of the two occurs at about the 14th day (McPhail and Read, 1942).

In the adult, accessory units of cortical and medullary (chromaffin) tissue may be present in scattered small groups near the left renal vein (Coupland, 1960), and as cortical nodules on both sides near the kidneys and adrenals (Hummel, 1958). The adrenal cortex makes up 75-90% of the gland in most mammalian tissues (Jones, 1957).

In evolution, chromaffin tissue, at first separate, becomes intermingled with cortical tissue forming islets and finally coalesces as the medulla, a central mass in the adrenal glands of metatherian and eutherian mammals. At the same time, scattered cortical and chromaffin cells aggregate and come to lie in or upon some part of pronephros or mesonephros. With the emergence of the metanephros in the Amniota-reptiles, birds and mammals - the chromaffin and cortical tissue enjoined into a discrete encapsulated mass, vascularized separately from the renal system; the adrenal gland as such was formed (Jones, Phillips and Bellamy, 1962).

2.2. ANATOMY

Symington (1960) pointed out that the adrenals of various species can be classified into "non-fatty" and "fatty" types. The hamster and most herbrivora fall in the non-fatty group, while other mammals, including man, fall in the fatty group.

The mouse adrenal glands are a pair of small ovoid structures situated one on either side of the midline near the anterior pole of the kidney. The right and left adrenals differ in respect to closeness to the kidney, renal vessels and inferior vena cava.

The absolute weight of the adrenal increases rapidly in young mice, but the rate of growth declines with increasing age until it eventually ceases. This pattern is similar to that found in rats (Donaldson, 1924).

Sex differences in adrenal gland weight have been well established by now (Bourne and Jayne, 1961). The glands of males and females differ in size and appearance, those of the female being consistently larger and more opaque due to the presence of more lipid. The adrenals are smaller and dark red in males (Chai and Dickie, 1966).

There is considerable evidence for the existence of strain differences in the absolute and relative weight of the adrenal glands of adult mice (Jones, 1955; Hummel, 1958; Thiessen and Nealey, 1962; Badr, 1965; Meckler and Collins, 1965; Spickett and Badr, 1965; Chai and Dickie, 1966). These strain differences are, in large part, a consequence of genetic differences between strains, as has been shown by diallele analysis (Meckler and Collins, 1965), by the biometrical analysis of F1, F2 and backcross generations, and the demonstration of significant heritabilities in a successful directional selection experiment (Badr, 1965; Badr and Spickett, 1965a). Strain differences in adrenal weight are not confined to mice, having been described in guinea pigs (Elliot and Tuckett, 1906), rabbits (Robb, 1929), and rats (Rogers and Richter, 1948).

Badr, Shire and Spickett (1968) studied three strains of mice, and observed two phases of adrenal growth. In the early phase the rate of growth was much slower than later. There was also a sex and age interaction as in the early phase, adrenal growth was faster in females than in males. Female mice had larger adrenals than males in all the three strains, and adrenal weight bore a linear relationship to body weight (BW) in female mice. Also, phenotypic differences between female mice of different genotypes became more marked as

development proceeded. In the male, the observable phenotype produced by particular genotype changed as development proceeded. The pattern of differences in phenotype among male mice of different genotypes depended on the particular stage of development chosen for comparison. The authors reported the existence of genetic variation, not only in quantitative parameters of the growth of adrenal glands, but also in qualitative aspects of their development.

2.3. HISTOLOGY

While rapid advances were being made in the investigation of adrenal function, advances in the knowledge of the finer structure of the adrenal glands was slower. By staining the first adrenal with carmine, Harley (1858) made the earliest step in this direction. Arnold (1866) used teased or macerated sections to study the reticular framework of the gland and showed that in the outer part of the adrenal, the reticular framework was in the form of a basket in which lay the adrenal cells. He called this layer the "zona glomerulosa" (ZG). Deeper in the cortex, he found that the basket arrangement changed abruptly to long reticular columns between which lay the adrenal cells.

This layer he named the "zona fasciculata" (ZF), and the inner portion of the cortex he called the "zona reticularis" (ZR).

Gottschau (1883) also divided the cortex into three zones similar to those described by Arnold (1866). It is revealing that the terms suggested by Arnold and Gottschau are still used and until recently, little has been added to our knowledge of adrenal morphology as expounded by them.

Lever (1955), and Braunsteiner, Fellinger and Pakesch (1955), were the first to describe the fine structure of the adrenal cortex in mouse, rat, hamster and rabbit. Later, Zelander (1957, 1959) also described the fine structure of the mouse adrenal cortex, in detail.

In man and most mammals, the three zones are visible in the adrenal cortex, but in the mouse only two zones are clearly defined. The outer glomerulosa (ZG) is a narrow zone consisting of small cells arranged in arches. The cells have relatively large nuclei, basophilic cytoplasm, and a rich capillary blood supply. The ZF is composed of long regular columns of cells separated by fine connective tissue septa bearing

capillaries. The nuclei are vesicular and the cytoplasm acidophilic and foamy due to the presence of finely distributed lipid droplets. Although some investigators describe an inconspicuous third zone, the reticularis, others question its existence (Jones, 1950; Miller, 1950).

In young nulliparous females and in male mice prior to sexual maturity, there is a zone of variable width between cortex and medulla. This juxta-medullary zone, the X-zone, disappears with sexual maturity in the male and with first pregnancy in the female. It persists in castrated males and in virgin females for periods varying with strain (Delost and Chirvan-Nia, 1958). In the immature male mouse, castration is followed by hypertrophy of the X-zone, although the significance of this change is obscure (Hall and Korenchevsky, 1938).

While the three classical zones can be seen in many human glands, the ZG is by no means always prominent and may be seen in some part of a section and not in the others. The ZG has been shown to contain abundent mitochondria (Symington, 1962), variable lipid content (Carr, 1961), and greatest cell proliferation of the three zones (Ford and Young, 1963). Ford and Young (1963), using the technique of tritiated

thymidine, have also shown that the cell proliferation is least in the ZR, and the cell generation time was the shortest in the ZG.

There is no sharp dividing line between ZG and ZF; the cells of the latter merge with the ill-defined ZG, and where this zone is absent, the ZF constitutes most of the cortex, its cells are arranged in long regular columns, and, in the resting phase, are filled with large, lipid globules. The clear vacuolated cytoplasm is also named as "clear" cells. Only a few mitochondria are seen in the cytoplasm (Culling, 1957).

The ZR can be easily identified as there is a change from the clear cells of the ZF to "compact" cells of the ZR. These compact cells are poor in lipid content, but rich in mitochondria (Carr, 1961). The presence and extent of each zone depends on the functional status of the gland, as will be described later (Deane and Greep, 1946; Deane, Shaw and Greep, 1948; Greep and Deane, 1949a).

Symington (1962a) compared the histological structure of the adrenal cortex of a wide variety of species, laying considerable stress on the differences

both in amount and in distribution of lipid. He pointed out, as did others, that there is no storage of hormone in the adrenal cortex in contradistinction to other endocrine glands.

There is considerable evidence to indicate that new cortical cells are constantly being formed in the inner part of the ZG and outer part of the ZF (Jones and Spalding, 1954; Jones and Roby, 1954), and as they age, they migrate towards the ZR from which they are finally removed (Crowder, 1957). However, it has been observed that cell division occurs throughout the cortex. Similarly, studies with intravital dyes have failed to demonstrate any cell translocation. Ingle (1949) demonstrated the regeneration of adrenal cortex cells from the enucleated capsule following the continuous parenteral administration of ACTH in the rat. Greep and Deane (1949a) have confirmed these observations and demonstrated that the regenerated cortex actually includes all three zones. However, other evidence would tend to throw some doubt upon this view.

The probabilities are, that both views are correct, depending essentially upon the species, with

particular regard to the presence or absence of a capsular blastema. In the human adrenal cortex, where the capsular blastema is conspicuous, regeneration presumably occurs in good part from the capsule, while in the rat, and in those species where the capsular blastema is poorly developed, regeneration from the capsule may play a subordinate role (Elias and Pauly, 1956). In this regard, it is interesting to note that the functional integrity of such regenerated cortices occurring after enucleation, may be impaired (Jones and Roby, 1954; Jones and Wright, 1954), and indeed hypertension has been observed to occur during adrenal capsular regeneration in the rat (Skelton, 1955, 1959; Macchi and Wyman, 1963; Birmingham, MacDonald and Rochefort, 1968; De Nicola, Oliver and Birmingham, 1968).

The tri-zonal arrangement of cortical cells holds true for most mammals, although a well-defined division into several layers is by no means universally true. In the rat, there is an additional reasonably well-defined zone, the zona intermedia, located between the ZG and the ZF, and readily identified with the usual staining techniques. This zone has also been identified in other mammals, such as the

horse, cattle, sheep, pig, dog, cat, rabbit, guinea pig, mouse and hamster, although in these animals, it is generally not as clearly defined as it is in the rat (Nicander, 1952). Other investigators, while recognizing this zone, consider it either as the inner part of the ZG or the outer part of the ZF (Lever, 1954).

Ashworth, Race and Mollenhaver (1959) suggested that the major sites of hormone synthesis are the deep layers of the ZF and the ZR since these areas are richer in mitochondria and vesicular cytoplasmic reticulum. The importance of the mitochondria in the synthesis of the adrenal cortical hormones is supported by independent biochemical studies demonstrating the association of enzyme systems with these bodies (Hayano, Saba, Dorfman and Hechter, 1956; Luft and Hechter, 1957).

Zelander (1957, 1959) studied the mouse adrenal cortex in detail and showed that the administration of cortisol (F) resulted in the appearance of large lipid vacuoles but with few mitochondria. The areas most strikingly affected were the deep fascicular and reticular zones.

Later, evidence will be presented demonstrating a biochemical as well as a morphological zonation in the adrenal cortex.

2.4. BLOOD SUPPLY

The adrenal cortex is richly supplied with blood. The adrenal arteries branch into multiple tiny vessels that penetrate the connective tissue capsule. The cortical arterioles arising from these vessels branch and rebranch as they penetrate the cortex, finally to drain into the medullary vessels and thus into the central adrenal vein. The capillary vessels of the cortex have classically been termed sinusoids. It is interesting that apart from minor subsidiary channels nearly all the blood from the cortex traverses the medulla (Pauly, 1957; Jones, 1957).

2.5. NERVOUS INNERVATION

All endocrine glands are highly dependent for their functional activity on their vascular supply, and for some of the endocrine glands, sympathetic and parasympathetic innervation has been satisfactorily demonstrated thus far only to the blood vessels. Both

parasympathetic and sympathetic nerve fibres have been traced to the region of the adrenal gland. The parasympathetic fibres transmitted by way of the vagus, traverse the celiac and the aorticorenal plexuses to the region of the gland (Kuntz, 1953). Not all investigators have found vagal fibres, and no fibre degeneration has been found in the adrenal after section of the vagus (Kiss, 1951). Evidence for the region of the synapse is not available, although it may be in the ganglia of the adrenal capsule. If vagal fibres reach the adrenal gland, they probably supply blood vessels of the cortex. The preganglionic fibres for blood vessel innervation probably synapse in the aorticorenal and adrenal plexuses. The postganglionic fibres supply the blood vessels of the cortex (Lever, 1953), and the medulla (Hollinshead, 1936; Swinyard, 1937).

Opinions differ on the question of a sympathetic innervation of the gland cells of the adrenal cortex. Some investigators have demonstrated terminal fibres on cortical cells (Kiss, 1951; Stöhr, 1957); while Lever (1953) has demonstrated terminal fibres on cells of the ZF as well as the more superficial layers in rats. According to other workers, however, satisfactory evidence for any innervation of the secretory cells of the adrenal cortex is lacking

(Hollinshead, 1936; Swinyard, 1937; McFarland and Davenport, 1941; Kuntz, 1953; Gillilan, 1954).

The innervation of the adrenal medulla differs from that of other glands. There is no parasympathetic innervation (Kuntz, 1953; Gillilan, 1954), and the preganglionic sympathetic fibres to the secretory cells of the adrenal medulla pass through the adrenal plexus and the adrenal cortex to terminate directly upon these cells (McFarland and Davenport, 1941). Some investigators (Stöhr, 1957; Botar, 1958) consider the terminal fibres to be in the form of a net. Studies of the nerve fibres of the adrenal medulla show changes in them with advancing age and severe illness (Botar, 1958, 1959).

3. FUNCTIONS OF THE ADRENAL CORTEX

The remarkable advances made in recent years in the regulation of secretory activity of the adrenal cortex such as delineation of neural pathways and centres, isolation of corticotrophin-releasing factors (CRFs), isolation of ACTH, histochemical characterization of the hypothalamo-hypophyseal link, characterization of the intermediates and enzyme system concerned with corticosteroid (CS) synthesis,

discoveries of the agents which block CS synthesis, analyses of androgen synthesis by normal and by abnormal adrenocortical tissue — are yielding insight into the complex mechanisms concerned with the secretory activity of the adrenal cortex.

3.1. THE EFFECTS OF ADREX

Extirpation of the adrenals has been carried out by a host of workers since the pioneer work of Brown-Séquard, who demonstrated the rapidly fatal outcome of the operation in the laboratory animals. Many factors determine the period of survival of animals following adrex. This accounts for the diversity of the results reported by different investigators. Certain animals (e.g., the mouse, rat, goat and frog) survive adrex much longer than others (e.g., the dog or guinea pig), due to their greater ability to withstand injuries in general.

Adrexed animals are extremely sensitive to trauma and hemorrhage; hence, the finesse of the operative technique employed in removing the glands is an important factor in determining the period of survival. Young animals survive a much shorter period than do adult animals (Grollman, 1941).

In completely adrexed animals, no obvious abnormalities are noted immediately, if the operation has been done competently. The animal is alert, eats, drinks, and appears to be in normal condition. The blood pressure is normal; the constituents of the blood are present in their normal concentrations. Gradually, however, the symptoms of insufficiency supervene. The animal becomes apathetic, refuses food, and may vomit. Muscular movements become slow and uncertain. Weakness of the hind legs develops, causing the animal's gait to become unsteady. Eventually the animal lies prostrate; the body temperature fails; the skin becomes cold and the mucosae pale. Muscular twitches and convulsions may occur. The respiration is at first rapid, then slow. Anuria is present. The pulse becomes feeble, the animal becomes comatose, and though the heart is still beating, respiratory paralysis sets in and leads to death.

Animals in adrenal cortical insufficiency are hypersensitive to many extraneous influences. They manifest an abnormal sensitivity to toxins, are very prone to infections, and frequently succumb to influences which have only trivial effects in normal animals (Grollman, 1941).

Porges (1910); Bauman and Kurland (1927); Marine and Bauman (1927); Britton and Silvette (1932); Simpson (1932); Rubin and Krick (1934); Long, Katzin and Fry (1940) contributed to the understanding of the mechanisms behind the most important deficiency symptoms which follow adrex, and Swann (1940) wrote a comprehensive review of the early work concerning the physiological effects of adrex.

In summary, the effects of adrex are:

i) disturbance of the sodium (Na^+), potassium (K^+), chloride ($C\bar{l}$) ions and water balance,

ii) increased extretion of Na^+ and $C\bar{l}$ ions as well as water while retention of K^+,

iii) increase of urea content of blood,

iv) disturbance of carbohydrate (CHO) metabolism with decrease in liver glycogen and decreased resistance to insulin,

v) reduction of resistance to various trauma such as cold, shock or an injury.

Experimentally, Yates and Urquhart (1962) demonstrated that a combination of corticosterone (B), F and aldosterone (Aldo) is sufficient to reverse the fatal effects of adrex.

3.2. FUNCTIONAL ZONATION OF THE ADRENAL CORTEX

Based on histo-physiological correlational studies of the rat adrenal cortex, Swann (1940) put forward the concept of functional zonation. This hypothesis stated that the outer part of the adrenal cortex, the ZG, which is not profoundly altered after hypophysectomy (hypox), is responsible for the regulation of electrolyte metabolism, whereas the inner cortical zones, ZF and ZR, which rapidly atrophy in the absence of the pituitary, is responsible for the elaboration of hormones primarily concerned in carbohydrate (CHO) and protein metabolism.

Later, Sarason (1943), and Deane, Greep and Shaw (1946, 1948) confirmed that CHO metabolism is controlled by the ZF — ZR, while the electrolyte balance is under the influence of the ZG. Bergner and Dean (1948) reported that ACTH has very little or no glomerulotrophic activity.

Ayres, Gould, Simpson and Tait (1956) incubated tissue from the three zones of ox adrenals and found that compound (cpd) F was produced in the ZF, Aldo in the ZG, and B in both zones. Giroud, Stachenko and Venning (1956) incubated the rat ZG

separately from the other zones and established that only glomerulosa tissue produces Aldo, while ZF and ZR produce mainly cpd B but a small amount of B is also secreted by ZG. Symington (1960) considered the ZF merely as a storage zone for steroid precursors and that the ZR is the actual site of C-21 steroids production with the exception of Aldo.

Later work by Stachenko and Giroud (1959, 1962), Lucis, Dyrenfurt and Venning (1961), and Birmingham and Ward (1961) has shown that 18-hydroxy-corticosterone (18-OH-B) is also produced exclusively by the ZG of the rat, while 18-OH-11-deoxycorticosterone (DOC), like B, is produced by all zones but mainly by the ZG and ZF. Indeed, Stachenko and Giroud (1959) have provided evidence of an enzymatic zonation with the same steroid substrates being handled in radically different ways depending on the zone in which they are located.

Sheppard, Swenson and Mowles (1963) did further work on functional zonation in the rat adrenal cortex by using labelled precursor, progest-erone-4-^{14}C (Prog-4-^{14}C). They concluded that Aldo and DOC originate almost entirely from the ZG. In

summary, there is a functional zonation of the adrenal cortex paralleling the morphological one.

3.3. ADENOHYPOPHYSEAL-ADRENOCORTICAL RELATIONSHIPS

The early recognition of the relation of the adrenal cortex to the hypophysis was essentially a clinical one. Falta (1913) observed extensive atrophy of the adrenal cortex in association with destructive lesions of the anterior lobe of the hypophysis. Subsequently, hypoplastic adrenals were noted in pituitary dwarfism (Erdheim, 1916), and in Simmond's cachexia.

The experimental demonstration that the size of the adrenal cortex was dependent on pituitary activity was made early in the 1920's by Smith (1927), who described a good method for extirpating the pituitary in the rat and also reported a rapid adrenocortical atrophy which follows this operation.

Further studies by Smith (1930) demonstrated that such atrophic phenomenon, however, were not seen in the adrenal cortex when only the posterior lobe was removed, indicated the specific role of the anterior lobe in the pituitary-adrenal system. The same investigator further demonstrated that

substitution therapy, consisting of daily homotransplants of fresh pituitary tissues, could reverse the degenerative changes observed in the adrenal cortex after hypox. During the 1930's, additional support was provided to establish firmly the functional relationship between the pituitary and the adrenal cortex (Houssay, Biasotti, Mazzocco and Sammartino, 1933; Collip, Anderson and Thomson, 1933).

Smith (1930), and Houssay and Sammartino (1933a) demonstrated that the atrophy is limited essentially to the cortex, the medulla remaining unaffected. The cells of all three zones of the cortex show a diminution in the amount of cytoplasm. The atrophic process begins in the ZR and eventually involves the ZF and to a much lesser extent, ZG. When the process is complete, the cells are small and distorted, and the ZR is unrecognizable, while the ZF has completely lost its cord-like arrangement of cells.

Greep and Deane (1949, 1949a), and Deane and Seligman (1953) demonstrated that hypox in the rat results in marked atrophy of the ZF and the ZR while glomerulosa remains relatively intact and indeed actually broadens. The retention of the integrity of

the ZG following hypox is significant in that there is a considerable body of evidence which at least suggests the continued secretion of adrenal cortical salt and water retaining fractions following hypox (Swann, 1940; Ingle, 1942). On the other hand, the relationship of the fascicular layer to the elaboration of glycogenic corticoids is further emphasized by Greep and Deane (1949), who demonstrated that injections of B into the intact rat result in alterations in the distribution of sudanophilic material identical with those observed after hypox, while the lipids of glomerulosa remain essentially unaffected. They also observed that 28 days after hypox in the rat, a great depletion of the lipids occurred from the ZF, while the ZG remained uninfluenced. Later, when they administered DOC to the same animals for a period of 1 month, they observed the disappearance of lipid material from the ZG as well. The authors concluded that the ZG in the rat is capable of function independently of the adeno-hypophyseal influence.

Somewhat earlier, Reese and Moon (1938) had noted that following the injection of ACTH there occurred a striking hypertrophy of the Golgi apparatus, particularly in the outer portion of the

fascicular layer. A little later, Carnes, Ragan and Ferrebee (1941), and Sarason (1943) reported that following injection of DOC, there is a disappearance of lipid from the glomerulosa.

However, the view put forward by Greep and Deane (1949) that the ZG in the rat is capable of function independently of adenohypophyseal influence, has been questioned by Selye and Stone (1950), and Feldman (1951), who maintain that the entire rat adrenal cortex is under pituitary control and that following hypox, a decrease in size of the ZG occurs, although the decrease is not as marked as that observed in the other cortical zones.

Later, however, Jones and Roby (1954) showed that male mice that have been hypophysectomized (hypoxed) for as long as 100 days show no abnormalities in their intake and output of Na and K. The hypoxed animal thus can control its water, Na and K metabolism.

Some of the disagreement among the various observers may perhaps be explained by the observation of Lever (1956) that shortly after hypox the glomerular layer actually broadens in width, but subsequently becomes progressively more narrow.

Jones and Wright (1954) suggested that the greater the concentration of circulating ACTH, the smaller the ZG becomes as the ZF and ZR increase in size. In the rat and in the human subject, the prolonged exogenous administration of ACTH may result in a virtual disappearance of the glomerular zone, while the fascicular and reticular layers become more prominent (O'Donnell, Fajans and Weinbaum, 1951; Baker, 1952). In any event, it would appear that although the glomerular zone in the rat is at least histologically responsive to ACTH (Jones and Wright, 1954), its dependence on the adenohypophysis is nevertheless less complete than is that of the other cortical zones, and indeed, this zone is apparently capable of a certain degree of autonomy.

Yates and Urquhart (1962) reported in their review paper that plasma levels of adrenocorticoids fall considerably after hypox, and that the secretion rates of F and B drop to values that are less than 20% of those in intact animals.

Lee and Williams (1952), and Hechter and Lester (1960) reported decreased uptakes of amino acids and sugars by adrenal glands <u>in vivo</u> after hypox of experimental animals, and their return to high levels

following administration of various ACTH preparations. Rogers (1959) described similar results *in vitro*.

Young (1950) reported that atrophic adrenals can be almost completely restored to their normal histological structure by daily homotransplants of the pituitary gland, or, as reported by several groups of workers (Houssay et al., 1933; Collip et al., 1933; Emery and Atwell, 1933; Reese and Moon, 1938), by the use of adrenocorticotropic pituitary extracts.

One further point of interest in this respect is the relationship between the pituitary and the compensatory hypertrophy of the remaining adrenal in the unilateral adrexed animal. In the intact animal, the removal of one adrenal is promptly followed by a compensatory increase in the size of the cortex of the remaining adrenal. This phenomenon does not occur in the hypoxed animal. However, if ACTH is administered to such an animal, the usual hypertrophy of the remaining adrenal will ensue.

Furthermore, certain clinical and experimental hyperpituitary states are associated with hypertrophy of the adrenal cortex. Thus, the adrenals in acromegaly are characterized not only by hyperplasia

of the cortical cells, but frequently actual adenomata of the adrenal cortex are encountered (Cushing and Davidoff, 1927). Also, Wilson (1958) observed that mice bearing tumours of the pituitary gland secreted twice as much corticoids as control animals. Experimentally, the adrenal cortical hypertrophy has been induced in both the intact and the hypoxed animal by treatment with ACTH (Houssay et al., 1933; Collip et al., 1933; Emery and Atwell, 1933; Reese and Moon, 1938).

Moreover, changes in adrenocortical function induce changes in pituitary function. The nerve supply to the anterior pituitary is scanty, and few histologists consider it adequate for the purpose of controlling the rate of secretion of ACTH. Most workers assume that the control is humoral.

The peripheral humoral concept of Sayers (1950) suggests that ACTH secretion by the pituitary varies inversely with the concentration of the circulating CSs. This hypothesis is based on the reasonable assumption that under conditions of stress, there is an increased peripheral utilization of cortical hormone. The drop in concentration stimulates the adenohypophysis to discharge ACTH and

thus brings the blood titre of CSs back towards the initial level. However, this view does not take into consideration the role of the hypothalamus which is critical, as will be discussed later.

3.3.1. ISOLATION AND PURIFICATION OF ACTH

In 1942-1943, the isolation from sheep and pig pituitary glands respectively of two active ACTH protein preparations was announced by Li, Simpson and Evans (1943), and Sayers, White and Long (1943a).

These preparations were both reported to have a molecular weight of approximately 20,000. Li and his associates (1943) working with pig, sheep, and beef pituitaries, have demonstrated that the ACTH is a straight-chain polypeptide with 39 amino acids. The sequence of first 24 amino acids is identical in corticotropin from the three species. Slight species differences exist in the order and arrangement of the remaining residues.

In later experiments, Li (1949); Payne, Raben and Astwood (1950); and Morris and Morris (1950) succeeded in preparing material of considerably

higher biological activity and concluded that the true hormone was probably a polypeptide of much lower weight than was previously supposed. Subsequently, Harris and Li (1954) showed that sheep ACTH is a polypeptide with 39 amino acid residues and with a molecular weight of approximately 4,500. The amino acid sequence of porcine, ovine, bovine and human ACTH has been elucidated by Bell (1954), Levy and Kushinsky (1955), Lee, Lerner and Janusch (1959, 1961), and Li, Dixon and Chung (1961). Lee et al., (1961) and others have pointed out that in the ACTH molecule, the amino acid residues 1 to 24 and 33 to 39 are probably the same in all four species, but that species differences occur in positions 25 to 39.

3.3.2. THE EFFECTS OF ACTH ON THE ADRENAL CORTEX

Some of the effects of the ACTH on adrenal cortex are:

1) augumented oxidative phosphorylation (increased oxygen uptake and radio-phosphorus (P^{32}) incorporation)
2) increased protein synthesis (increased RNA, incorporation of acetate and amino acids into protein) stimulating growth of the adrenal

3) accelerated glycolysis (increased active phosphorylase)
4) altered lipid metabolism (depletion of adrenal cholesterol and lipid, increased formation of at least 12 steroids, with the delta 4-3 ketone grouping, and
5) ascorbic acid depletion

In view of the many effects of ACTH on the adrenal cortex, it has been difficult to decide which effects are primary and which are secondary consequences of ACTH action. The fact that ACTH increases synthesis of active phosphorylase (an effect mediated by increased concentrations of a co-factor, adenosine 3', 5'-phosphate) in the adrenal cortex has great significance because these enzymatic changes might provide a mechanism for increased steroid synthesis. Because of the increased phosphorylase, more glucose-6-phosphate (G-6-P) might be available for the hexose monophosphate (HMP) shunt, a pathway which is particularly important in the adrenal cortex and provides the reduced triphosphate pyridine nucleotide (TPNH) necessary for steroid biogenesis.

In other words, the evidence from a variety of indirect studies seems to favour the hypothesis that ACTH exercises its steroidogenic effect at some rate-determining step in the biosynthetic sequence between cholesterol (Ch) and delta 5-Pregnenolone to Prog (Kass, Hechter, Macchi and Moon, 1954). Various theories have been advanced as to the mechanism whereby ACTH influences adrenal steroidogenesis (Haynes and Berthet, 1957; Haynes, Sutherland and Hall, 1960; Hechter and Halkerston, 1964; McKerns, 1964). However, they all recognize the availability of TPNH as being the principal rate-limiting factor in this process, but how the hormone acts precisely to increase the co-factor availability <u>in vivo</u>, is not known.

One interesting aspect of the effect of ACTH on the adrenal is the fact that calcium (Ca) is required for ACTH action <u>in vitro</u>. The role of Ca in steroid production in response to ACTH is not clear; however, it is known that Ca ions are not involved in the binding of ACTH by the adrenal cortex (Birmingham, Kurlents, Lane, Muhlstock and Traikov, 1960).

The influence of ACTH in the regulation of Aldo secretion has not been as clear-cut as its role in the control of glucocorticoid (GCC)

production. However, in view of all the evidence available at present, it appears that although the influence of the pituitary on Aldo secretion by the adrenal cortex is less marked than its control over the production of GCCs, the demonstration of a definite effect on Aldo production cannot be denied. This effect of ACTH will be discussed in greater detail later.

3.4. HYPOTHALAMIC-HYPOPHYSEAL CONTROL

Superimposed upon the basal secretion of ACTH is the stress response whereby a large and diverse group of stimuli triggers the release of CRF from the median eminence of the hypothalamus, which, in turn, activates the pituitary-adrenocortical axis with a consequent outpouring of CSs into the blood. The early thought that blood levels of CSs influence ACTH release is still a matter of investigation, especially in the area of hyphothalamic involvement.

Thus, Egdahl (1964) reported that in dogs with isolated pituitaries, the high adrenal secretion rate cannot be suppressed by dexamethasone. He concluded that the anterior pituitary is not the site of the CS inhibition of ACTH release.

It should be emphasized, however, that the responsiveness of the pituitary to ACTH-releasing stimuli may be slightly diminished by CSs, even though the adenohypophysis is not the major site of physiological feedback.

The importance of the hypothalamus to the secretion of ACTH is well established (Guillenin, 1968). However, the precise nature of hypothalamic control is not clear. Although a neurohumoral regulation through the hypophyseal portal system is accepted by most investigators, there is some disagreement whether CRF of neural or other origin can activate the pituitary through the general circulation (Hume, 1953; Brodish and Long, 1962; Gagong, 1963), or require a direct passage from hypothalamus to adenohypophysis (Harris, 1955; David, Horvath and Kovacs, 1961; Greer, Kendall and Duyck, 1963; Gagong, 1963).

Matsuda, Duyck and Greer (1964) demonstrated that pituitaries transplanted under the median eminence are capable of inducing cpd B secretion at a level undistinguishable from that of intact control rats. Previous studies had indicated that the median eminence-stalk-pituitary complex is the only part of the forebrain essential to ACTH secretion (Matsuda, 1963).

It is now clear that the CRF activity is present mainly in the median eminence (Matsuda et al., 1964), but perhaps also throughout the whole tuber cinereum and infundibular process of the hypothalamus. Stimulation of the hypothalamus in or close to the median eminence causes ACTH release (Mason, 1958; McHugh and Smith, 1964), a response that is not prevented by pre-treatment with CS. This important observation suggests that the part of the hypothalamus immediately involved in CRF release may not be CS-sensitive. However, introduction of CS directly into the hypothalamus will diminish adrenal cortical function under a variety of experimental conditions (Smelik and Sawyer, 1962; Davidson and Feldman, 1963; Bohus and Endröczi, 1964; Chowers, Feldman and Davidson, 1964). The steroids are effective when placed in or near the median eminence and the anteromedial region. CSs in the pituitary itself do not similarly diminish adrenal cortical function (Smelik and Sawyer, 1962; Bohus and Endröczi, 1964). These results and those obtained after hypothalamic lesions (Brodish, 1963), or dexamethasone injected intravenously in rats with the forebrain removed (Kendall, Matsuda, Duyck and Greer, 1964) or in normal

rats (Kendall, 1962), suggest that the comparator element and the CRF-releasing element lie in the midline of the floor of the third ventricle. The feedback element may be even more diffused than this, however, because CS implants in the reticular formation of the rostral mesencephalon or parts of the forebrain also impair ACTH release (Endrőczi, Lissak and Teheres, 1961; Davidson and Feldman, 1963; Bohus and Endrőczi, 1964). Dexamethasone implants in the septa area interfere with compensatory adrenal hypertrophy (Eisenstein, 1967). Steroid implants in the preoptic nuclei or in parts of the thalamus or neocortex have little, if any, effect on ACTH release (Bohus and Endrőczi, 1964).

At present, it seems wise to assert only that the CS-sensitive element of the adrenal cortical feedback system probably lies in the medial, basal hypothalamus, septum and rostral midbrain in rats and that the final step involved in CRF-release is not CS-sensitive.

It has been proposed that ACTH inhibits its own release by a negative-feedback action on the brain or pituitary (Kitay, Holub and Jailer, 1959).

Experiments of Vernikos-Danellis have shown that in rats bearing ACTH-producing transplanted pituitary tumours, the increase in ACTH content of the normal in situ pituitary that ordinarily follows adrex can be prevented. The author concluded that in the absence of corticoids, an extra-pituitary source of ACTH may be able to inhibit adenohypophyseal ACTH synthesis and release (Vernikos-Danellis, 1965).

Motts and Martini (1965) report that implants of solid ACTH into the median eminence of normal male and female rats depress blood B levels 1 week after implantation, and that control implantations of gonadotrophic hormones are ineffective. These results are cited to indicate the possibility that an ACTH feedback loop exists. Its contributions to control of GCC secretion rates or plasma concentrations, if any, remain to be discovered.

In adrenal insufficiency, a slowing of the electrical discharges in the electroencephalogram (EEG) (Feldman, 1962), which is reversible by F but not by DOC, has been clearly established. The threshold for electrical excitation of the brain is definitely lowered by F (Feldman, Todt and Porter, 1961).

This phenomenon accounts for an increased tendency to epileptic seizures in subjects prone to this disease who have been treated with F. Conversely, DOC diminishes the tendency. Psychiatric disturbances are common both with lack of F and when F is present in excess; a large number (40%) of patients with Cushing's syndrome show some major psychological abnormalities (Cleghorn, 1965). How this effect of the adrenal cortex on the EEG and on personality disturbances are related to the effect of the CSs on hypothalamic-anterior pituitary function, is presently not known.

3.5. THE EFFECTS OF STRESS ON THE ADRENAL CORTEX

A large variety of circumstances call forth the activation of the production of corticoids by the adrenal cortex, (Ingle, 1938; Tepperman, Engel, and Long, 1943; Sayers, 1950), and Selye (1936, 1940) called these conditions "stress". In the absence of the pituitary, stress does not bring about the morphological and histochemical changes that characterize its action on the adrenal cortex (Firror and Grollman, 1933; Selye and Collip, 1936; Sayers, Sayers, Liang and Long, 1945), as it is quite established now

that the anterior lobe of the pituitary gland is the site of formation, storage and release of the ACTH, which activates the adrenal cortex. The anterior pituitary usually has large stores of its hormones, including ACTH. In response to stress, there is a release of ACTH from storage, as shown by a fall in the ACTH content of the pituitary gland immediately after stress (Rochefort, Rosenberger and Saffran, 1959). Under normal circumstances, the adrenal cortex contains only minor amounts of the corticoids, but after stress and accompanying release of ACTH, the adrenal tissue contains higher-than-usual concentrations of corticoids indicating an accelerated synthesis (Holzbauer, 1957). The depleted pituitary stores of ACTH are quickly restored after stress, suggesting that its synthesis too is probably accelerated (Rochefort et al, 1959).

The pituitary, however, is not essential to maintain a minimal secretion of cortical hormones, as shown by the inability of adrexed animals to survive the operation for more than a few days (Britton, 1930; Firror and Grollman, 1933), whereas after hypox, the animal may survive for long periods of time (Horsley, 1886; Smith, 1927; Jones and Roby, 1954). However, the administration of ACE increases

the resistance of hypoxed animals to stress (Baird, Cloney and Albright, 1933; Tyslowitz and Astwood, 1942). Adrexed animals have a diminished resistance to a variety of damaging stimuli (Swingle and Remington, 1944; Sayers, 1950).

When the ruminant is stressed or injected with ACTH, there is no noticeable change in morphology of the adrenal gland. On the other hand, the rat responds with the disappearance of lipid from ZF, and adrenal hypertrophy (Ingle, 1938; Tepperman et al., 1943; Simpson, Evans and Li, 1943a; Sayers, 1950; Fortier, Skelton, Constantinides, Timiras, Herlant and Selye, 1950), while in the human adrenal cortex lipid depletion occurs in a focal manner; that is, whilst some areas of the ZF are rich in lipid, adjacent ones are devoid of it. If the stress continues, all stainable lipid disappears and ZF and ZR become a uniform zone of enzyme-rich but lipid-depleted compact cells.

Symington (1959, 1960) reported that under stress ZF and ZR of human adrenal gland are one and the same zone. ZR is the site of production of CSs (except Aldo), androgens and possible estrogen hormones, and the clear cells of the ZF are a storage

zone for steroid precursors (Ch and Ch-esters). In conditions of stress when endogenous ACTH is liberated, or after its exogenous administration, the steroid precursors in the cells of ZF nearest the ZR are used for steroids synthesis, and in time this results in the morphological, histochemical and hydroxylating enzymatic changes observed in the cortex (Symington, 1962a).

This view is consistent with the observations on the rat by Yoffey (1953, 1955), while contrary to the Zonal Theory (Jones, 1948, 1957) which attributes to the reticular zone the production only of sex hormones.

Selye (1936) put forward the concept of "General Adaptation Syndrome" which postulates that when subjected to stress, an animal, besides undergoing adaptive changes specific to that particular stress, also undertakes a non-specific defense which is mediated by the pituitary-adrenal system. According to Selye, the pituitary-adrenal reaction is believed to be a defense or resistance mechanism common to all types of stress and to consist of four phases: shock, countershock, resistance, exhaustion, terminating in death should the stress be sufficiently severe. Either adrex and/or hypox affects only the resistance stage by

considerably shortening it, but does not influence the shock and counter-shock phases (Selye, 1946).

3.6. DIURNAL VARIATION IN ADRENAL CORTICAL FUNCTION

In man, F production is maximal in mid-morning, relatively stable in the afternoon, and gradually declines throughout the afternoon and evening, with minimal activity in late evening and early morning. This has been shown for plasma levels (Peterson and Wyngaarden, 1956; Migeon, Tyler, Mahoney, Florentin, Castle, Bliss and Samuels, 1956; Perkoff, Eik-Nes, Nugent, Fred, Nimer, Samuels, Rush and Tyler, 1959; Doe, Vennes and Flink, 1960; Ekman, Hakansson, McCarthy, Lehman and Sjogen, 1961; De Moor, Osinski, Deckx and Steeno, 1962; Martin, Mintz and Tamagaki, 1963; Vermeulen and van der Straeten, 1964; Iisalo and Pekkarinen, 1965), and for urinary corticoids (Sandberg, Nelson, Glenn, Tyler and Samuels, 1953; Migeon et al., 1956). This diurnal variation persists under conditions which obliterate the estrus cycle of mice (Halberg and Vissher, 1952) and it develops earlier than the menstrual cycle in human beings (Halberg and Ulstrom, 1952). In the mouse, a rise in eosinophils occurs before that in B, while the peak of mitoses in adrenal cortex occurs later (Halberg, Peterson and Silber, 1959).

Halberg, Albrecht and Bittner (1959), and Halberg (1962) made an extensive study of the circadian rhythm in B level of serum and adrenal in the mouse under standardized conditions with respect to the physical environment and the history of the animal, including their genetic background. On a regimen providing for light from 0600 to 1800 hours, alternating with darkness, B levels in mouse serum and adrenal were highest at about 1600 and lowest at about 0400. The occurrence of peak levels in adrenal and blood CS in this nocturnally-active rodent differs from that recorded for blood and urinary steroid levels in diurnally-active man, but in mice as well as men, spontaneous adrenal activation occurs prior to the onset of daily activity (Halberg, 1959). The adrenal cycle is not a mere direct and immediate reaction to extrinsic stimuli acting upon the gland via ACTH stimulation. In addition to the fluctuating pituitary or central nervous system (CNS) stimulation which influences the state of the adrenal at any one time, periodic changes in the metabolism of the mouse adrenal itself may contribute critically to the gland's intrinsic circadian cycle.

The site of the control of most biological, and in particular, of adrenal rhythms, has been located in the CNS and chiefly in the limbic structures; lesions of the fornix seem to modify phasic adrenal activity (Ganong, 1963). Moreover, in man the diurnal rhythm of the plasma CS level is altered or abolished by CNS disorders (Eik-Nes and Clark, 1958; Hokfelt and Luft, 1959; Perkoff et al., 1959; Krieger, 1961; Krieger and Krieger, 1966), or by administration of depressant drugs, such as morphine (McDonald, Evans, Weise and Patrick, 1959). In the rat, some nervous structures seem to control the diurnal variations in adrenal production and plasma B level. Saba, Saba, Carnicelli and Marescotti (1963) previously reported that the integrity of the median eminence is also essential for maintenance of the night-time rhythm in adrenal cortex secretion and/or plasma steroid level. The effect of median eminence lesions might be related either to the suppression of some autonomous activity of this structure or to the destruction of pathways originating elsewhere; in this regard, the reticular activating system must be considered as a possibility (Anderson et al., 1957; Egdahl, 1961). Furthermore, this last hypothesis was confirmed by Tronchetti, Marescotti and Saba (1967) who observed similar effects as a

consequence of midbrain transection, i.e., after interruption of not only the most sensitive pathways, but also of the reticular activating system. Taylor and Farrell(1962) considered this system as a complex network of autonomous structures which regulate adrenal activity.

Therefore, the diurnal CS concentration and adrenal secretion rhythm originate not in the adrenal or pituitary, but in the CNS which thus regulates plasma levels and adrenal B secretion in some humoral way, probably through cyclic variations in the ACTH secretions. This is also confirmed by the persistance of the phasic behaviour of plasma B levels observed in rats with adrenal autografts (Tronchetti et al., 1967). The same authors conclude that at normal levels of adrenal activity, plasma B levels are quite related to the changes of hormone metabolism. On the contrary, in conditions of adrenal hyperfunction, peripheral plasma B levels are closely dependent on adrenal production.

3.7. ROLE OF THE ADRENAL CORTEX IN METABOLISM

3.7.1. CARBOHYDRATE METABOLISM

As early as 1910, Porges pointed out

the frequency with which hypoglycemic episodes occurred in adrexed dogs. Maranon (1925) demonstrated that patients with Addison's disease were markedly sensitive to minute amounts of insulin. Later, Britton and Silvette (1932) demonstrated the occurrence of hypoglycemic seizures in adrexed guinea pigs, cats, and other species. They further found that the liver and muscle glycogen of the adrexed animals was considerably reduced, and that the ability of these animals to form liver glycogen from injected dextrose or sodium lactate was diminished. Simpson (1932) supplemented the above-mentioned observations by showing that patients with Addison's disease failed to show a rise in the blood sugar level comparable to that of normal individuals following the injection of a standard dose of epinephrine. The question arose, too, as to whether the CHO disturbances observed were not due primarily to removal of the adrenal medulla. Patients with Addison's disease who have atrophy of the cortex, but with relatively intact medulla, nevertheless display the same characteristic disturbances in CHO metabolism as do those patients with extensive and universal destruction of the adrenals due to tuberculosis. Similarly, Zwemer, Smith, Shirley and Sullivan (1930, 1934) found that in demedullated cats no changes in the

blood sugar level occurred as a result of the operative procedure. Boggild (1925) observed similar results in dogs. Long et al., (1940) found that in both normal and adrexed fasting rats and mice, the administration of ACE was followed not only by an increase in liver glycogen and blood glucose, but also by a parallel increase in urinary nitrogen excretion. The same authors also pointed out that in some species overwhelming changes in electrolyte metabolism occur so promptly as to obscure any alterations in CHO metabolism. In others, the animals survive long enough to permit these changes to become manifest.

During the early periods of investigation of the functions of the adrenal cortex, the relationship of the cortex to CHO metabolism was a source of great conflict between those groups who insisted that the CHO disturbances observed in adrexed animals were fundamentally related to the absence of the adrenal cortex, and their opponents who postulated that these disturbances were non-specific in character and rather related to malnutrition so commonly present in the adrexed animals. Today there is no question concerning the fundamental role which the adrenal plays in CHO metabolism, inasmuch as the administration of GCCs can reverse the effects of adrex on CHO metabolism.

3.7.2. ELECTROLYTE METABOLISM

3.7.2.1. Earlier Studies

The role of adrenal cortex in electrolyte and water metabolism was the first of the important adrenal cortical functions clearly recognized when studies of the cortical physiology began to take precise form in the early 1930's.

3.7.2.1.1. Effect on Na

It was in 1927 that Marine and Baumann showed that the Na content of the blood of cats decreased following bilateral adrex. The authors also showed that injections of salt solution prolong appreciably the life of these animals. Britton (1930) found that the symptoms of adrenal insufficiency in dogs were relieved slightly by saline injections, while Harrop, Weinstein, Soffer and Trescher (1933) reported a slight drop in the total base of the adrexed dogs. In 1932, Rogoff stated that the intravenous administration of physiological saline becomes an indispensible adjuvant in the treatment of Addison's disease with extracts representing the hormone of the adrenal cortex.

In 1933, Loeb, Atchley, Benedict and Leland reported the retention of K ion in adrenal

insufficiency even though this ion passes through the kidney with greater facility than either the Cl or Na ions. They also confirmed the earlier reports that the Na concentration of the blood decreases in adrexed cats and dogs, and in patients suffering from Addison's disease. The authors also reported a decrease in the Cl and bicarbonate (HCO_3) concentrations in the blood.

Rubin and Krick (1933), using white rats, showed that the adrenal insufficiency not only leads to loss of Na^+ and $C\bar{l}$ ions, as reported by several investigators, but a great loss of elements such as N, P, K, Ca and Mg also occurs. The authors state:

> "We do not feel certain that the action of adrenal hormone is primarily one of salt regulation, although this is quite probable".

Later, Loeb, Atchley and Stahl (1935) after an extensive study in dogs, concluded that there is a definite relationship between Na metabolism and the active principle of the adrenal cortex. Also, [illegible] the Na concentration of the blood is decreased because of an increased rate of its excretion, and the administration of salt will frequently

alleviate acute adrenal insufficiency. However, salt alone will not maintain life when there is a complete destruction of the adrenal gland.

Several investigators (Harrop and Thorn, 1936; Thorn, Garbutt, Hitchcock and Hartman, 1937; Hartman, Lewis and Toby, 1938) have shown that when cortical extract is injected into the patient with Addison's disease, the normal human subject, the adrexed dog or the intact dog, the excretion of Na is decreased, and that of K increased, and the Na excretion is closely proportioned to the dosage given.

By now, several investigators (Thorn, Engel and Eisenberg, 1938; Thorn, Howard, Emerson and Firror, 1939; Kuhlman, Ragan, Ferrebee, Atchley and Loeb, 1939; Loeb, Atchley, Ferrebee and Ragan, 1939; Kendall, 1940; Wells and Kendall, 1940) had confirmed that the most potent adrenal hormone possessing an action causing renal retention of Na is DOC. It was, at that time, the most potent of all known cortical hormones in maintaining the life of adrexed animals, and in the control of certain phases of electrolyte metabolism. Some investigators, however, pointed out that the absorption of electrolyte from the lumen of

the intestine is distinctly abnormal in adrexed animals (Clark, 1939; Dennis and Wood, 1940; Stein and Wertheimer, 1942), indicating that extra-renal defects may also be contributing to the impaired Na metabolism.

Increases in interacellular hydration which should accompany depletion of extra-cellular Na and Cl by renal wastage, have been repeatedly demonstrated in adrexed animals (Winter and Hartman, 1933; Silvette, 1934; Ponder and Gaunt, 1934; Muntwyler, Mellors and Mantz, 1940).

3.7.2.1.2. Effect on K

Baumann and Kurland (1927), and Hastings and Compere (1931) had earlier reported that the removal of the adrenal glands was followed by a rise in the serum K level, so that comparatively high concentrations were reached at death. Later work showed that not only was the adrexed animal exceedingly sensitive to administered K (Allers, Nilson and Kendall, 1936; Cleghorn and McVicar, 1936; Zwemer and Truszkowski, 1937; Truszkowski and Duszynska, 1940; Winkler, Hoff and Smith, 1941), but that K salts given to the intact animal in quantities sufficient to raise the plasma concentrations to those typical of terminal adrenal insufficiency would reproduce many of the symptoms of

the adrexed animal, and might lead to death (Hastings and Compere, 1931; Zwemer and Sullivan, 1934; Zwemer and Truszkowski, 1936, 1937). The therapeutic value of a diet low in K in the treatment of Addisonians or for the maintenance of adrexed animals has been repeatedly demonstrated (Allers et al., 1936; Allers and Kendall, 1937; Nilson, 1937; Wilder, Kendall, Snell, Kepler, Rynearson and Adams, 1937).

3.7.2.1.3. Effect on H_2O

In most, but not all, animals, the altered electrolyte excretion which follows adrex is associated with a diuresis. This subsides, as symptoms become severe, and terminally an oliguria or anuria ensues (Harrop et al., 1933; Swingle, Pfiffner, Vars and Parkins, 1934; Rubin and Krick, 1934; Sandberg, Perla and Holy, 1937; Gaunts, Potts and Loomis, 1938; Loeb, 1939). Yet despite this water diuresis associated with Na loss, the ability of the kidney to excrete water shows deficiencies. If distilled water is administered by mouth, even in small doses, the diuretic response is far below normal (Rowntree and Snell, 1931; Margitay-Becht and Gomari, 1938; Levin, 1943), and susceptibility to water intoxication is extreme (Rigler, 1935; Swingle, Parkins, Taylor and Hays, 1937; Gaunt, Remington and Schweizer, 1937; Eversole, Gaunt and Kendall, 1942).

Swingle, Remington, Hays and Collins (1941), and Eversole et al., (1942) reported that DOC shows activity in preventing water intoxication in adrexed animals, but whole gland extract or the corticoids are more effective (Eversole et al., 1942).

Swingle and Remington (1944), reviewing the work of numerous investigators since 1930, prepared a concise summary, as follows:-

> "...The desoxycorticosterones, i.e., those lacking a Keto or OH group at Carbon 11 appear to be chiefly concerned with the regulation of electrolyte and fluid balance, acting (1) directly upon the renal tubules to allow them to conserve Na and H_2O and to release K; (2) in a manner less clearly defined, on fluid and perhaps electrolyte partitioning across cell membranes, capillary endothelium and intestinal mucosa; and finally, (3) through their regulation of phases of mineral metabolism, primarily those involving Na and K, to produce secondary effects upon extra- and intra-cellular hydration."

Later, in 1949, Kendall wrote an extensive review of the previous work on electrolyte and water metabolism and stated: that there was ample evidence that the metabolism of Na, K and Cl was in large measure controlled by the hormones of the adrenal cortex. It is also true that the physiologic response

to administration of the hormones of the adrenal cortex is in large measure determined by the amount of Na and Cl present in the body. (Harrison and Darrow, 1938).

Kendall (1949) further stated that the adrenal cortex furnishes a mechanism to the body, which modifies the transfer of Na, K, and Cl through cellular structures. In man, the dog, or the cat in adrenal insufficiency, an increase of the intake of Na is followed by an increase of the output of K. The reverse is also true, but in the intact animal, an increase of the intake of either Na or K causes but a brief increase of the output of the other ion and the normal balance is promptly restored. It would, therefore, seem that the hormones of the adrenal cortex modify the transfer of electrolytes in the kidney and permit this organ to perform work to overcome the direction of the ionic changes which occur in the absence of the adrenal cortex.

The salt-regulating activity of the adrenal cortex controls (a) the acid-base balance of the body through its control of Na and Cl ions; (b) the body's degree of hydration and osmotic pressure through the control of the extracellular fluid and (c) muscle irritability through its control of K ions.

3.7.2.2. Aldosterone

Despite the considerable amount of work conducted up to 1950, some crucial facts relating to the regulation of electrolyte metabolism by the adrenal cortex were still lacking. The discovery of Aldo was a major step in the filling of this gap. Indeed, the discovery of Aldo provides a remarkable example of the value of applying new techniques to older problems. For a good many years it had been recognized that lipoid extracts of adrenal cortex contained life-saving factors for adrexed rats and other animals. A great deal of skillful work had also been done by chemists to extract the compounds responsible for this powerful action. This effort had led to the isolation and chemical recognition of some 28 different steroids, and among this number were included cpds cortisone (E) and F. Many of the rest, however, seem to be physiologically inert. Compounds F and E were recognized as having some life-saving properties, but adrexed animals, especially rats, treated with them nevertheless remained very vulnerable and readily died. It was also known that the steroid DOC would act powerfully to prevent the urinary salt loss and so prolong survival, even in the absence of saline to drink, but

DOC was not among the 28 steroids isolated from the adrenal cortical extract. The amorphous residue from this extract, after known steroids had been removed, was found still to possess the life-prolonging action, but the isolation of the active principle remained an unsolved problem until the application to it of a new technique.

In 1951, Bush suggested to Tait and his colleagues, that they should apply the relatively new technique of paper chromatography to the problem. It was found that when concentrated adrenal extracts were run on the toluene/propylene glycol system, strong Na-retaining activity was present in elutes of the zone corresponding to the position of E on the paper. Careful assay of this activity made it clear that the Na-retaining effect was much greater than could be accounted for by the amounts of E present in this zone. It was apparent, therefore, that some other, as yet unknown, cpd must also be present in that zone and that it possessed a high degree of Na-retaining potency. This early challenging evidence, published in 1952 by Tait and his co-workers, provided a strong stimulus to an intensive effort to isolate and characterize the cpd

responsible. These workers gave it the provisional name "electrocortin". Within a very short time the active substance on the chromatograms was obtained sufficiently pure to be crystallized by Simpson, Tait, Wettstein, Neher, Von Euw, Schindler and Reichstein (1954). That the chemical formula of such a complex and elusive substance could be worked out completely with only a very few milligrams (mgs) available for study, is a remarkable example of the skill of modern chemists. In a further year the perhaps even greater triumph of the total chemical synthesis of this cpd was announced by Schmidlin, Anner, Billeter and Wettstein (1955), the process involving 30 separate stages. Such a chemical synthesis produced a racemic mixture of D- and L-forms in equal proportions. But only the D- form is found in nature and is hormonally active (Cope, 1965). That the laevo-form of Aldo is devoid of activity was shown in a series of elegant experiments by Leutscher, Dowdy, Lew and Callaghan (1962). By 1960, evidence strongly suggested that the renin-angiotensin system (R.A.S.) might be the primary regulatory mechanism for Aldo secretion. During the next few years a large number of reports have provided convincing evidence that the R.A.S., plasma electrolyte concentrations, and ACTH influence the rate of secretion of Aldo.

Apparently, the secretion of the renin is significantly influenced by electrolyte concentration so that, as with F secretion, a feedback mechanism operates although entirely apart from the GCC control system (Yates and Urquhart, 1962).

There are also suggestions that other less important factors are involved in the control of Aldo secretion.

3.7.2.2.1. Secretion Mechanisms for Aldosterone

3.7.2.2.1.1. Role of Renin-Angiotensin

Several lines of evidence indicate that the R.A.S. is a regulator of Aldo secretion. Renin is an enzyme produced by the juxtaglomerular apparatus in the kidney. It interacts with plasma alpha-2 globulin fraction, which contains renin substrate, to form angiotensin-I. The angiotensin-I is converted by an activating enzyme to angiotensin-II which is the active material responsible for elevation of blood pressure.

Valine-5-angiotensin-II has itself been shown to be a strongly stimulatory agent in the dog, the sheep and in man (Laragh, Angers, Kelly and Lieberman, 1960; Davis, 1961; Biron, Koiw, Nowaczynski,

Brouillet and Genest, 1961; Wright, 1962; Blair-West, Coghlan, Denton, Munro, Peterson and Wintour, 1962a). Both the renal extract and synthetic angiotensin-II stimulate secretion of Aldo, accompanied by only a small increase of B and without increase of F, thus demonstrating an essential difference from the effects of ACTH (Carpenter, Davis and Ayres, 1961; Davis, 1962).

It has been shown that angiotensin may exert a more direct effect, for the adrenal tissue of rats which have been pre-treated with angiotensin-II was able to synthesize Aldo at a faster rate than normal, although there was no direct action of the peptide on the adrenal <u>in vitro</u> (Glàz and Krisztina, 1962). On the other hand, beef adrenal slices with Ch added as substrate, synthesized Aldo faster when the peptide was added <u>in vitro</u> (Kaplan and Bartter, 1962). It appears that Aldo secretion by the adrenal does not operate as a direct feedback mechanism automatically reducing its secretion (Blair-West, Coghlan and Denton, 1962).

Hartroft (1963) reviewed evidence for increased secretion of renin by juxtaglomerular cells of the kidney during congestive heart failure, hepatic cirrhosis, nephrosis, thoracic vena cava constriction,

chronic Na depletion, hemorrhage and malignant hypertension. However, there are species differences. Thus, in the rat, there is conflicting evidence as to whether angiotensin is the humoral factor since infusions of renin-angiotensin-II have effect on Aldo secretion in some, but not in other, studies (Marieb and Mulrow, 1964; Mulrow, 1966).

3.7.2.2.1.2. Role of Altered Sodium Intake and Depletion

The observation that a low sodium intake auguments the rate of urinary Aldo excretion in normal human subjects was first reported by Leutscher and Axelrod (1954) and confirmed by others (Hernando-Avendano, 1957; Bartter, Mills, Biglieri and Delea, 1959). Also, it has been clearly established that chronic Na depletion increased Aldo secretion in sheep (Denton, Goding and Wright, 1959, 1960; Blair-West, Coghlan, Denton, Munro, Wintour and Wright, 1962, 1964; Denton, 1964); dogs (Davis, 1961; Brown, Davies, Lever and Robertson, 1964; Binnion, Davis, Brown and Olichney, 1965), and rats (Eisenstein and Strack, 1961; Cohen and Crawford, 1962; Marx and Deane, 1963; Mariele and Mulrow, 1964; Eilers and Peterson, 1964; Cade and Perenich, 1965). It is of

interest that B secretion is only very slightly increased in sheep (Blair-West et al., 1962, 1964), unchanged in dogs (Binnion et al., 1965), and decreased in the rat (Eisenstein and Strack, 1961; Cade and Perenich, 1965).

The mechanism by which Na depletion auguments Aldo secretion has been a subject of discussion and debate. Increasing evidence, however, shows that there is augumented activity of the R.A.S. during Na depletion in both man and dog (Davis, Ayres and Carpenter, 1961; Davis, Hartroft, Titus, Carpenter, Ayres and Spiegel, 1962; Binnion, 1964; Brown, 1964; Brown, Davies, Lever and Robertson, 1964a; Veyrat, de Champlain, Boucher and Genest, 1964; Gross, Brunner and Ziegler, 1965). In sheep and rats, the evidence is less complete and some observers (Eilers and Peterson, 1964; Marieb and Mulrow, 1964; Cade and Perenich, 1965) have interpreted their findings in the rat to indicate that the R.A.S. is not important in the hypersecretion of Aldo secondary to Na depletion.

Excessive levels of mineralocorticoids (MCCs) in the organism disturb both electrolyte balance and blood pressure. Considerable evidence suggests electrolyte disturbances in arterial wall may, in some manner, be etiologically related to hypertension.

Restriction of K reduces the secretion of Aldo whilst loading stimulates it (Liddle, Bartter, Duncan, Barber and Delea, 1955; Laragh and Stoerck, 1955; Bartter, 1956).

3.7.2.2.1.3. Role of Anterior Pituitary

The effects of hypox were studied by Singer and Stack-Dunne (1959) in rats, and by Rauschkolb, Farrell, and Koletsky (1956) in dogs; both groups of workers discovered a decrease in Aldo secretion after hypox. This effect of anterior pituitary ablation was confirmed by others (Davis, Bahn, Yankopoulos, Klinman and Peterson, 1959; Ganong, 1959; Davis, Yankopoulos, Lieberman, Holman and Bahn, 1960; Ross, Van't Hoff, Grabbe and Thorn, 1960; Davis et al., 1961; Blair-West et al., 1964; Eilers and Peterson, 1964), and most observers have found an 80-90% fall in Aldo secretion following acute hypox in the dog. The observations of Eilers and Peterson (1964) in the rats, and Blair-West and associates (1964) in sheep, indicate that species differences may exist since only 25-35% reduction in Aldo secretion was observed by these workers.

Davis et al., (1960) demonstrated that the stressful stimuli provoked by laparotomy stimulate

Aldo production. Mulrow and Ganong (1961) reported that hemorrhage auguments Aldo output in hypoxed dogs.

Holzbauer (1964) observed that a high level of circulating ACTH was responsible for the high Aldo secretion in acutely laparotomized dogs. Much of the early confusion on the effects of hypox on Aldo production was due to failure of observers to recognize that laparotomy increased Aldo secretion. Also, in some of the early studies, the effect of hemorrhage, which is now known to stimulate renin release, was superimposed on the stimulus of laparotomy.

Although, as mentioned earlier, the adrenal cortex is capable of secreting at least Aldo even in the absence of the hypophysis, many workers (Liddle, Duncan and Bartter, 1956; Farrell, Fleming, Rauschkolb, Yatsu, McCally and Anderson, 1958; Bartter et al, 1959; Davis et al., 1960) have demonstrated the potent effect of different corticotropin preparations on Aldo secretion. The response is striking in every mammalian species studied, with the exception of the rat; in the rat, ACTH does increase Aldo output, but the reported response is not as great as in other species (Muller, 1956; Eilers and Peterson, 1964).

There is abundant evidence to show that ACTH exerts an influence on the biosynthesis of Aldo, but the precise action of ACTH in steroidogenesis is unknown. A popular hypothesis has been that of Haynes and Berthet (1957), who suggested that ACTH stimulates the formation of cyclic adenylic acid and the final outcome is the production of TPNH, used for steroids hydroxylation at the 11, 17, 18 and 21 positions.

ACTH is also known to stimulate Aldo secretion, particularly in conditions involving Na deficiency, in which Aldo secretion is enhanced (Ganong, Boryczka, Shackleford, Clark and Converse, 1965).

Csánky, Van der Wal and de Wied (1968) reported that in the rat, as in other species, both the kidney and the pituitary contribute to the maintenance of the basal rate of Aldo production in normal Na balance, and these organs are responsible for the increased rate of Aldo production in dietary Na restriction. ACTH, however, though a potent stimulus for Aldo secretion, appears only to augument the already enhanced production of Aldo in the Na-deprived rat.

3.7.2.2.1.4. Role of Miscellaneous Factors

Insight into the physiological control of Aldo secretion has also been gained from a variety of clinical and experimental disorders. Carpenter et al., (1961) and Davis et al., (1962) cited evidence for hypersecretion of Aldo with acute hemorrhage, chronic thoracic vena cava constriction, chronic Na depletion, congestive heart failure and malignant renal hypertension. The connecting links between hypersecretion and these disorders are still under discussion. Two considerations in these studies, however, are decreased circulating volume and Na depletion, which in man can respectively induce a thirty-fold and ten-fold increase in Aldo secretion.

In cross-circulation experiments the adrenal gland showed appropriate Aldo responses (Yankopoulos, Davis, Klinman and Peterson, 1959) and it appears, therefore, that a humoral mechanism, as proposed by Fleming and Farrell (1956), and not a nervous mechanism, excites Aldo secretion.

Recently, Müller and Weick (1967) presented the evidence that the high Aldo-stimulating activity of rat serum is not due to ACTH, angiotensin-II,

or a monovalent cation, but rather to a still unidentified substance of small molecular size. Later, Müller and Ziegler (1968) assumed that this substance is identical with serotonin and substantiated their assumption with an *in vitro* study by incubating rat adrenal cortex with a measured dose of serotonin and found a significant and dose-dependent increase of Aldo and DOC, but this did not alter the production of B. The authors proposed that serotonin acts mainly on the conversion of Ch to pregnenolone. Müller (1966) earlier reported that the site of action of ACTH, angiotensin-II, K and ammonium ions, was Ch to pregnenolone.

Nevertheless, there is suggestive evidence that the higher centres may mediate a response in Aldo secretion. For example, emotional stimuli in man may increase it (Venning, Dyrenfurth and Beck, 1957), and the sheep is a particularly sensitive animal to salt craving (Denton et al., 1959). In man, posture and activity exerts an important effect, standing with exercise stimulating it, reclining reduces it (Müller, Manning and Riondel, 1958).

A variety of stimuli, e.g., a painful stimulus, undoubtedly induces increase or decrease in the rate of Aldo secretion by activating classical

pathways up the spinal cord, through the brain stem to the region of the pineal. Pharmacological agents may depress the rate of Aldo secretion (Farrell, 1959).

Müller and Ziegler (1968) further stated that since it had little effect on the production of B, it probably acts only on the ZG. This is consistent with the findings that it also stimulates the production of DOC, which like Aldo is mainly produced in the ZG of the rat adrenal cortex (Sheppard et al., 1963).

3.7.2.2.2. Excretion

Aldo and its reduced transformation products are excreted chiefly as conjugates of glucuronic acid. The urinary conjugates are mostly tetrahydroaldersterone. Conjugation takes place primarily in the liver, but some acid-labile conjugates may be formed in the kidney (Siegenthaler, Dowdy and Leutscher, 1962; Leutscher, Hancock, Camargo, Dowdy and Nokes, 1965).

The distribution and clearance of Aldo from plasma are abnormal in congestive heart failure. The altered distribution enhances the rate

and extent of response of plasma Aldo to changing secretion rate. The reduced plasma clearance tends to increase the plasma concentration in the steady state (Leutscher, Cheville, Hancock, Dowdy and Nokes, 1967).

3.8. STEROIDS SECRETED BY THE ADRENAL GLAND

Of roughly 50 steroids isolated from adrenal cortices, only a very few have been shown to be normally secreted into the blood stream. These include corticoids, Aldo and sex steroids (Grant, 1960; Short, 1960). The remainder are intracellular intermediates. The adrenal CSs may be defined as steroids secreted by the adrenal cortex and possessing 21 carbon atoms and three or more oxygen atoms. The corticoids may be further divided into those steroids possessing the characteristic delta 4-3 ketone in ring A, a grouping present in all biologically active corticoids, and those with a reduced ring A structure. The cpds having glucocorticoidal action influence CHO, fat and protein metabolism. Aldo appears to be the major MCC and is essential for normal electrolyte balance. Most corticoids exhibit both kinds of activity to some extent. C-19 steroids having weak androgenic

activity are also secreted by the adrenal cortex (Short, 1943; Gassner, 1951; Bush, 1953; Bloch, Dorfman and Pincus, 1954; Vogt, 1955; Lombardo, 1959). C-18 steroids with estrogenic potency (Hardy, 1958a, 1958b; Engel, 1962), and progesterone (Beall, 1938, 1940; Short, 1960; Grant, 1962) have also been reported. The daily production of these hormones is approximately equivalent to that of F but their physiological role has not been elucidated. However, their concentration in the circulation is so small in comparison with the contributions of other endocrine glands that these substances are not classified as primary adrenal cortical hormones. Abnormalities of biosynthesis, however, can occur in which they may become major products and thus cause serious hormonal imbalances.

Out of a long list of CSs discovered so far, only a few show predominantly glucocorticoidal or mineralo-corticoidal activity. The GCCs include corticosterone, 11-dehydro-corticosterone (A), 17 a-OH-corticosterone, cortisol, 11-dehydro-cortisol, 11-deoxycortisol (S), 11-deoxy-corticosterone, and the most potent mineralo-corticoid: aldosterone, which exists in three forms: Aldo, hemiacetal (C_{11} - C_{18} oxygen bridge) and hemiketal (C_{18} - C_{20} oxygen bridge), (Reichstein and Schoppee, 1943; Simpson et al., 1954).

Until the isolation of Aldo by Simpson et al., (1954) the MCC activity, that is, of excretion of K ion and reabsorption of Na ion by the distal renal tubule, has been associated with DOC. Prog, pregnenolone and 17-OH-pregnenolone have also been isolated.

Apart from these active steroids, the cortex also produce C-19 steroids such as delta 4-androstenedione (ASD) and its 11 β-OH and 11-keto derivatives, and dehydroepiandrosterone (DHEA) which have been isolated.

The synthesis of estrogens (C-18) by the adrenal is much less clear than that for the androgens.

3.9. SPECIES DIFFERENCES IN ADRENAL CORTICAL FUNCTION

Throughout their history there seems to have been little change in the hormonal mechanism that maintain the vertebrate body though changes have occurred in the adrenals which secrete controlling steroids, and in the organs of conservation, for example the kidney and the widely differing extrarenal target organs. Clearly, further work in the field of comparative endocrinology will illuminate problems of form and function of the adrenal cortex.

3.9.1. MAMMALS

There are intriguing species differences in the production of adrenal corticoids. The physiological significance of the variations between different animal species whether they produce F or B as their major CS have not been found attributable to the environment (Bush, 1953). Thus Bush, (1953) made an elaborate study on the adrenal cortical function in a variety of species. He showed that the composition of adrenocortical secretion is subject to considerable species differences which appear to be genetically determined and related neither to the mode of life nor to dietary habits. Compound B or F, either alone or in combination, are the chief secretory products, and ranged from 74-100% of the total a, β-unsaturated, 3-keto-steroid in the adrenal venous blood. The relative proportions of F and B vary in different species (Bush, 1953; Yates and Urquhart, 1962; Bern and Nandi, 1964; Gottfield, 1964).

3.9.1.1. Man

In the human, cortisol is the major CS (Bush and Sandberg, 1953; Romanoff, Rodriguez, Seelye, Parent and Pincus, 1953; Hudson and Lombardo, 1955; Morris and Williams, 1955; Heard, Bligh, Cann,

Jellinck, O'Donnell, Rao and Webb, 1956; Tamm, Beckmann and Voigt, 1959; Short, 1960), while B is present in one-tenth of the amount (Peterson, 1960). Human fetal adrenals contain 19-hydroxylase, but the activity of this enzyme in adult human adrenal tissue could not be detected (Griffiths, 1963).

Salhanick (1957) and Mahesh and Herrmann (1963), using pathological human adrenal tissue, observed the <u>in vitro</u> production of various estrogens.

3.9.1.2. <u>Cattle</u>

The major steroid secreted by sheep is F (Bush and Ferguson, 1953; McDonald and Reich, 1959), while the ox secretes about equal amounts of B and F (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Shenker and Pincus, 1951; Schindler and Knigge, 1959a). Hayano and Dorfman (1955), and Levy and Kushinsky (1955) demonstrated the <u>in vitro</u> 19-hydroxylation of DOC using beef adrenal preparations, while Mattox (1955) isolated 19-OH-DOC from beef adrenal glands. Beall (1940) extracted estrone from beef adrenal glands.

3.9.1.3. Mouse and Rat

In the mouse (Southcott, Bendy, Newsom and Darrach, 1956; Cohen, Bloch, and Celozzi, 1957; Wilson, Borris and Bahn, 1958; Halberg et al., 1959; Bloch, Cohen and Furth, 1960; Triller and Birmingham, 1965a, 1965b), and rat (Bush, 1953; Hofmann and Davison, 1954; Elliott and Schally, 1955; Rosenman, St. George, Freed and Smith, 1955; Vogt, 1955; Holzbauer, 1957; Birmingham and Kurlents, 1959), the major corticoid is B.

Varon and Touchstone (1964) showed the biosynthesis of F in adrenal glands of immature mice, and reported that the 17 a-hydroxylase activity diminishes with increasing age. When testosterone was administered to the young mice, it altered the steroidogenesis towards B instead of F, thus supporting the 17 a-hydroxylase activity.

Later, Huseby and Dominguez (1964), using Balb/c female mice, showed that castration led to adrenal hyperplasia as well as production of androgenic steroids. In vitro, the adrenal glands converted pregnenolone to ASD and testosterone, and with Prog precursor they obtained 17 a-OH Prog thus confirming 17 a-hydroxylase in mouse adrenal gland.

Bush (1953) in spite of his elaborate study on adrenal cortical function and species differences, could not detect any 17-hydroxylated steroids in rat, and suggested that the adrenal cortex of the rat lacks 17-hydroxylase activity. No F was found in the plasma of Balb/c mice by Halberg et al., (1959). Several other workers (Hofmann, 1956; Raman, Ertel and Ungar, 1964; Ertel and Ungar, 1964; Triller and Birmingham, 1965a, 1965b) reported the absence of F in incubation studies of mouse adrenals. Recently, Nandi, Bern, Biglieri and Pieprzyk (1967) reported extremely small amount of F in young male Balb/c mice, but the authors interpreted that the presence or absence of F in the secretions by mouse adrenal may depend in part on (1) conditions of the experiment, and (2) also appears to vary with the strain. They also observed that the identification of F is complicated by the fact that this substance can readily be confused with various other polar steroids, particularly with 18-OH B. This cpd has been reported in several instances (Raman et al., 1964; Ertel and Ungar, 1964; Triller and Birmingham, 1965a, 1965b). The matter regarding the presence or absence of 17-hydroxylase activity in mouse and rat is still a controversial one, though much of the above-mentioned evidence supports the idea of

no 17-hydroxylase activity in these species.

In other studies, Lucis, Carballeira, Idler and Venning (1964) reported the 19-hydroxylase activity in rat adrenal. Ryan, Ferro and Beyler (1964) observed 17-ketosteroids (17-KS) in the urine of castrated rats, their level increasing after the administration of ACTH. It is difficult to visualize the endogenous formation of 17-KS in the absence of 17 a-hydroxylase in the adrenal cortex.

3.9.1.4. Rabbit

Rabbits are mainly B producers (Bush, 1953; Kass, Hechter, Macchi and Moon, 1954; Vogt, 1955; Reif and Longwell, 1958), but prolonged stimulation by ACTH injection induces the secretion of F (Kass et al., 1954).

3.9.1.5. Mongolian Gerbil

Oliver and Péron (1964) showed the presence of 19-hydroxylase in the adrenal glands of the mongolian gerbil. From adrenal venous blood of these animals, two major steroids, F and 19-OH, 11-deoxycortisol were isolated in equal amounts; Aldo

and an unidentified, less polar than E steroid, were also isolated (Oliver and Péron, 1964).

3.9.1.6. Golden Hamster

Schindler and Knigge (1959a, 1959b) reported that golden hamsters secrete mainly F.

3.9.1.7. Ferret

The ferret secretes roughly equal amounts of both F and B (Hechter et al., 1951; Schindler and Knigge, 1959a).

Several authors (Birmingham and Ward, 1961; Peron, 1961; Cortes, Péron and Dorfman, 1963) have reported the innate differences in the qualitative nature of major end products other than the chief steroids B and F, by showing the abundant secretion of 18-OH-DOC in rat, but not in the mouse. Similarly, 19-OH-DOC appears as a major component in the hamster (Griffiths, 1963).

3.9.1.8. Miscellaneous

The major corticoid secreted by the monkey (Bush, 1953; Holzbauer, 1957; Lanman and

Silverman, 1957), dog (Reich, Nelson, Zaffaroni, 1950; Bush, 1953a; Holzbauer, 1957), hog (Dobriner, Katzenellebogen and Schneider, 1954; Heard et al., 1956) and guinea pig (Bush, 1951; Heard et al, 1956) is F.

3.9.2. NON-MAMMALIAN VERTEBRATES

Jones et al., (1962) in the first systematic investigation of the nature of the adreno-cortical secretions throughout the non-mammalian vertebrates analysed either adrenal venous effluent or, where anatomical difficulties precluded this, peripheral blood of representatives of the various classes. It was found that either B or F, or both constitute the major proportion of the total CSs content in species of birds, reptiles, amphibians, lung-fish, bony-fish, dog-fish, skates and rays (Jones and Philips, 1957). The analysis of adrenal venous blood from a marsupial showed to contain B and a major unidentified component (Jones et al., 1962).

The lower vertebrates show a cortical secretory pattern similar to that demonstrated for man and the common laboratory (lab) animals. In

mammals and throughout the vertebrates the relative proportions of B and F may vary considerably; cpd E is sometimes present. However, in non-mammalian vertebrates, Aldo was initially detected only in the capon (Philips and Jones, 1957). The authors also found a low concentration of Aldo (0.12 mg/100 ml) in a pooled plasma of spawning and spent fish. Later, Carstensen, Burgers and Li (1961) found that Aldo was the major CS in the frog (Rana Catesbeiana) being present 4-times the concentration of B. Several other workers have already reported that Aldo, B and F are the predominant CSs throughout the Gnathostomata and that their biosynthetic pathways are similar to those operating in eutherian mammals.

Sander, Lamoreux and Lanthier (1963) have also reported that the adrenals of the domestic duck, an aquatic bird, had higher 18-hydroxylating activity than the adrenals of chickens, a result which would tend to associate with Aldo production with an aquatic existence. Also, 18-OH-B appears as a major component in ducks and chickens (Sander et al., 1963). While Aldo is present in fish (Philips, Holmes and Bondy, 1959), there is no evidence of a high concentration of Aldo relative to GCCs in these animals. Moreover, there is insufficient information to assess its probable

importance in maintaining the internal milieu of the animal during migration from salt to fresh water or from water to the land.

The striking finding that comparative studies of adrenal steroid biosynthesis have brought out is that all of the steroid hydroxylases seem to be present in the lowest vertebrates, the elasmobranchs, and that, so far, quantitative differences among species and families cannot be related to any evolutionary or environmental factor.

4. BIOSYNTHESIS OF THE ADRENAL STEROIDS

Research into the biogenesis of the adrenal steroids is concerned with the nature and amounts of the steroids secreted by the adrenal cortex and how these substances are produced in the adrenal cells. Beginning with the early experiments of Brown-Séquard (1856), attempts were made to prepare extracts of the adrenal cortex which would prolong the life of adrexed animals. None was successful, however, because it was assumed that the cpds in question would be water-soluble. Later, the discovery that sex hormones were steroid in nature, led to the experiments

with lipid extracts, and in 1929-1930, three groups of workers - Rogoff and Stewart (1929); Hartman and Brownell (1930); and Swingle and Pfiffner (1929), all reported the preparation of lipid-soluble fractions from adrenal tissue which would maintain the life of adrexed animals.

4.1. GLUCOCORTICOIDS

Adrenocortical hormones were first isolated from the adrenal gland in 1936 (Mason, Myers and Kendall, 1936a, 1936b; Reichstein, 1936; Wintersteiner and Pfiffner, 1936). The gross structures, though not their details, were elucidated during 1937 and 1938. A partial synthesis was completed in 1946; the first total synthesis in 1951 (Fieser and Fieser, 1959). The first important clinical paper on the effect of an adrenocorticoid was published by Hench, Kendall, Slocumb and Polley (1949), describing the effect of E in rheumatoid arthritis.

As early as 1918, Laignel-Lavastine (1918) observed that human adrenal glands removed at autopsy from individuals who had died of severe infections or other stresses, contained less Ch than those of persons who had died from less stressful causes.

Between 1937 and 1939, Kendall and co-workers, and Reichstein and his associates, isolated a large number of steroids from the extracts of bovine adrenal gland, among which were physiologically active substances later shown to be secreted by the adrenal cortex. Their structural resemblance to that of Ch directed attention to this cpd as a common precursor. This idea was reinforced in 1944 when Sayers, Sayers, White, Fry, Long and Lewis (1943, 1944a, 1944b) reported that 3 to 6 hours after the injection of pituitary adrenocorticotropic extracts into rats, there occurred a fall in the concentration of Ch in the adrenal glands. The Ch content of the adrenal returned to normal in about 24 hours. This decrease occurred entirely in the Ch ester fraction, while the free Ch remained unaffected. Similar results were observed in hypoxed rats, but the Ch content of a variety of other tissues was not affected by these injections. The continued injection of ACTH over a 3-day period increased the adrenal Ch content above that of the normal controls.

The adrenal cortex is also unusually rich in ascorbic acid (vit C), and following the injection of ACTH there occurred a sharp drop in ascorbic acid content of the gland. This decrease became evident

within 20 minutes after the injection, and reached a peak within an hour. As with Ch, exposure of normal animals to various stresses resulted in a marked decrease in adrenal ascorbic acid, while similar changes did not occur in the hypoxed animals.

Studies on the nature of the chemical and structural changes occurring in the adrenal cortex in response to the direct action of ACTH, revealed that the usual sequence of events is an initial, very rapid enhancement of the rate of CS synthesis and secretion, followed by an almost as rapid decrease in the concentration of Ch, stainable lipid and ascorbic acid. Finally the signs of anabolic processes leading to increase in adrenal size and weight are manifested.

The initial steroidogenic effect of the hormone can become evident within seconds after incubation <u>in vitro</u> is begun, or within a few minutes after administration <u>in vivo</u>. Inversely, removal of the pituitary gland from the dog is followed by a prompt decline of the major steroids, B and F.

Sayers et al., (1943, 1944a, 1944b) also found that exposure of normal animals to various traumatizing procedures (scalds, cold, hemorrhage)

resulted in a similar decrease in adrenal Ch, but identical procedures did not alter the adrenal Ch content in the hypoxed animal.

When the stress is slow in onset and prolonged, the change noted is an increase in size of the adrenal rather than any decrease in the adrenal content of Ch or ascorbic acid. If the stress is continuous and severe, there is complete adrenal cortical depletion of Ch and ascorbic acid. Similarly, if ACTH is administered over a prolonged period of time, the adrenal hypertrophies, but its storage of Ch and ascorbic acid remains low. When recovery ensues following a severe stress, the adrenal content of Ch and ascorbic acid is depressed, but returns to normal after several days. During this period the adrenal may hypertrophy markedly (Soffer, Dorfman and Gabrilove, 1961a).

Long (1947) emphasized the excellent correlation which exists between the decline in adrenal Ch and ascorbic acid, and such manifestions of adrenal cortical activity as the decrease in liver glycogen in fasting animals, and the fall in the number of circulating lymphocytes and eosinophils.

Symington, Currie, Curran and Davidson (1955), and Currie and Symington (1955) studied alterations in the lipid content of ZF. They maintain that under the influence of ACTH, compact cells, such as are seen in the ZR, fill the ZF from within outward. They further maintain that the lipid is a reflection of the storage of precursors for adrenal steroid metabolism.

Further information on the biosynthesis of the CSs was provided by the following studies: Bloch (1945) fed deuterium - labelled Ch to a pregnant woman and demonstrated that the pregnanediol excreted in the urine contained the isotope. In 1949 Pincus, Hechter and co-workers (Hechter, Jacobsen, Jeanloz, Levy, Marshall, Pincus, and Schenker, 1949; Hechter, Zaffaroni, Jacobsen, Levy Jeanloz, Schenker and Pincus, 1951; Hechter, Solomon, Zaffaroni and Pincus, 1953; Hechter and Pincus, 1954) used ^{14}C label to study the synthesis of the adrenal steroids. They perfused bovine adrenal glands with blood containing ^{14}C-acetate, and found that a large number of radioactive steroids could be isolated from perfused blood. The same was true when ^{14}C-Ch was used. Thus, they obtained evidence of a sequence of reactions leading to the

formation of the most active natural GCC, cortisol.

Long (1947) and several other workers have demonstrated that labelled Ch, when incubated with adrenal cortical tissue, yields labelled Prog and which in turn is an intermediate in the biosynthesis of CSs. Similar conversions were shown by Haines (1952) using minced adrenal tissue.

Caspi, Rosenfeld and Dorfman (1956), Caspi, Ungar and Dorfman (1957), and Caspi, Dorfman, Khan, Rosenfeld and Schmid (1962) degraded radioactive S and B synthesized by perfusing bovine adrenal glands with either acetate-1-^{14}C, or acetate-2-^{14}C. The results indicate that the individual carbon atoms of these structures originate from the same acetate carbons as the equivalent carbons of Ch.

The sequences of reactions involved in the biosynthesis of adrenocortical steroids are still not fully known. Present evidence indicates that the adrenal steroids are normally synthesized from acetyl coenzyme A (Co A) via cholesterol, i.e., Acetate → Acetyl Co A and Acetoacetyl Co A → Mevalonic Acid → Squalene → Lanosterol → Zymosterol → Cholesterol → Pregnenolone → Progesterone → CORTICOSTEROIDS.

This sequence of reactions which is generally accepted today, is based on the work of Pincus and his colleagues with the perfused ox adrenal gland (Hechter et al., 1951, 1953). The work of others has resulted in modification of the original scheme, and additions to it, but without any drastic alteration. There is reason to believe that the same or very similar sequences occur in man (Pincus and Romanoff, 1955; Lombardo, Roitman and Hudson, 1956; Lombardo and Hudson, 1959). Perhaps the most controversial question is whether Ch is an obligatory intermediate in adrenocortical steroid biosynthesis. Evidence in favour of a pathway not involving Ch is based mainly on the comparison of specific activities of Ch and CSs formed by adrenal preparations when ^{14}C-acetate is used as a precursor. It has, for instance, been observed that whereas ^{14}C is incorporated into CSs, little or no ^{14}C is found in the Ch isolated (Heard et al., 1956). It is possible, however, that metabolic pools of Ch exist (Loud and Bucher, 1958) and that ^{14}C is in fact incorporated into the Ch of a small pool involved in CSs biosynthesis. The ^{14}C-Ch might be rapidly transformed into labelled steroid before it could equilibrate with other pools of Ch.

It would appear that the matter may have been settled by the reports of Chaikoff and his associates (Morris and Chaikoff, 1959; Werbin, Chaikoff and Jones, 1959; Werbin and Chaikoff, 1961), who fed isotopic Ch to rats and guinea pigs until the specific activity of adrenal Ch no longer increased. This required several weeks, pointing to a slow rate of exchange on the part of a significant portion of the adrenal Ch. When equilibrium was achieved, it was found that F isolated from the urine of the guinea pigs, and B isolated from the blood and adrenals of the rats, had the same specific activity as the adrenal free Ch. Moreover, in the rats the specific activity of the adrenal free Ch was very close to that of the blood Ch, pointing either to a small amount of Ch synthesis in the adrenal cells or to a rapid exchange with the Ch of the blood. In guinea pigs the adrenal synthesis of Ch appeared to be about 40% of the free Ch present.

Krum, Morris and Bennett (1964) working with dogs, showed that Ch was an intermediate in adrenal steroids biosynthesis, by observing cpd F in the adrenal venous blood with the same specific activity as the adrenal free Ch. Earlier, Hechter et al., (1953) have shown in experiments involving the perfusion

of beef adrenal glands, the greater incorporation of acetate carbon into F than into the total free or esterified Ch. This also led to the thought of two distinct Ch pools. Thus, according to the work of Chaikoff et al., (1959, 1961), and Krum et al., (1964), under normal conditions Ch is an intermediate in the synthesis of the CSs and the anomalies found by Hechter et al., (1953) would appear to be due to the fact that only a small portion of the adrenal-free Ch is in the metabolic pool from which the adrenal steroids are synthesized. The metabolic pool may be mitochondrial-free Ch (Ichii, Kobayashi and Matsuba, 1965).

While the evidence thus supports the concept that Ch is normally an intermediate on the path from acetate to the adrenal steroids, the introduction of triparanol (MER 29) as an inhibitor of Ch synthesis (Blohm and MacKenzie, 1959; Avigan, Steinberg, Vroman, Thompson and Mosettig, 1960) demonstrated that Ch is not an obligatory intermediate. Treatment of human beings with MER 29 results in a fall of the plasma cortisol level initially, but later the near-normal level is restored; associated with this is an increase in blood concentration of desmosterol

(Steinberg, Avigan and Feigelson, 1961), a precursor of Ch with a double bound between carbons 24 and 25.

Goodman, Avigan and Wilson (1962) showed that when ^{14}C-desmosterol is incubated with rat adrenal homogenates, it is quantitatively comparable to Ch as a precursor of CSs. It would appear that either Ch or a very closely related structure is required as a steroid precursor. In the normal adrenal gland, however, such cpds are present only in minute amounts, and Ch, therefore, is probably the normal precursor. The pathway from Ch to Prog via pregnenolone appears to be common to all steroid-secreting glands.

The study of adrenal steroid biosynthesis was helped greatly by the development of a commercially feasible synthesis of Prog-4-^{14}C, by Fujimoto and Prager (1953). Utilizing adrenal homogenates Prog itself can be hydroxylated at several positions (C_{11}, C_{17}, C_{18}, and C_{21}) depending on the species origin of the homogenate.

4.2. ALDOSTERONE

Although there has been intensive investigation of the biosynthesis of Aldo since its

isolation in 1953 by Simpson and Tait, this problem is still a matter of controversy owing to conflicting results.

Wettstein, Kahnt and Neher (1955) reported that the yield of Aldo from beef adrenal homogenate incubations was increased in the presence of DOC, and decreased with cpd B or Prog. Later, they isolated 18-OH-DOC and also demonstrated the conversion of labelled DOC to radioactive Aldo (Kahnt, Neher and Wettstein, 1955). They postulated that B was not an intermediate in such conversion.

Ayres, Pearlman, Tait and Tait (1958), contrary to the above results, showed the conversion of Prog, DOC, and B to Aldo by incubating with capsules stripped from ox adrenals, and postulated that B was the immediate precursor to Aldo. Stachenko and Giroud (1962) reached the same conclusion using a similar preparation.

Rosemberg, Rosenfeld, Ungar and Dorfman (1956) tested Prog, DOC and B as precursors and found only Prog increased Aldo content in the effluent of the perfused calf adrenals. Thus, the most likely mechanism of biosynthesis of the 18-aldehydic

group of Aldo would be preliminary 18-hydroxylation followed by dehydrogenation.

Travis and Farrell (1958), using Ayres et al.'s technique (1958), incubated radioactive Prog, DOC, and B, and claimed not only that Prog gave higher yields of Aldo than did DOC or B, but the specific activity of Aldo relative to that of labelled precursors was higher in Prog incubations than in those with DOC or B. Thus, they suggested an alternate pathway not necessarily involving B.

For the biosynthesis of Aldo, the 18-hydroxylation followed by dehydrogenation to 18-aldehydic group in the steroid structure has been earlier reported by Wettstein et al., (1955).

Birmingham and Ward (1961), and Péron (1961), reported 18-OH-DOC as the major 18-OH-steroid of the rat adrenal gland, while Raman et al., (1964) observed 18-OH-B and Aldo as the major metabolites of Prog-4-^{14}C incubation with an <u>in vitro</u> adrenal gland preparation of the mouse. Raman et al., (1964) demonstrated the conversion of Prog to 18-OH-B and Aldo, and also reported the presence of 18-OH-steroids in adrenal tissue as well as in the adrenal vein blood.

Pasqualini (1964) found that where low concentrations were used, 18-OH-B was converted to Aldo in good yield by both normal and tumourous tissue of the human adrenal gland.

Nicolis and Ulick (1965) pointed out that 18-OH-steroids are present mainly in 18-20 hemiketal form in solution, and this hemiketal form may be more resistant to enzymatic processes.

In view of this evidence, 18-hydroxylation seems to be the most probably process in view of the currently available knowledge of steroid biosynthesis.

Although there are many studies on the regulation of Aldo secretion, only a few attempt to define the site in the biosynthetic pathway that is stimulated by Na depletion (Kaplan and Bartter, 1962; Davis, Burwell, Kelly, Casper and Bartter, 1966; Bledsoe, Island and Liddle, 1966; Müller, 1966). The biosynthesis of Aldo occurs in the ZG of the adrenal gland and little, if any, takes place in the ZF-ZR zones (Stachenko and Giroud, 1964). The only step that is unique in the biosynthesis is conversion of B to Aldo, which occurs in the mitochondrial fraction

(Psychoyos, Tallan and Greengard, 1966; Raman, Sharma and Dorfman, 1966; Marusic and Mulrow, 1967). The steps involved are presumably B $\longrightarrow$ 18-OH-B $\longrightarrow$ Aldo. Only the production of Aldo is specific for the ZG. Some 18-OH-B is synthesized in the ZF and ZR as well, but the quantity appears to vary with different species (Marusic and Mulrow, 1967).

Marusic and Mulrow (1967), reviewing the Na depletion effect and Aldo secretion, put forward the following: Na depletion may stimulate Aldo synthesis by

1) Stimulating the early steps in the pathway as mentioned above, thus delivering more B to the mitochondrial site for conversion to Aldo;

2) stimulating the last steps, conversion of B to Aldo;

3) producing hypertrophy or hyperplasia of the ZG cells, and therefore increasing the number of biosynthetic units, and

4) stimulating by some combination of these three mechanisms.

Presumably, Na depletion stimulates release of a humoral factor which must alter the activity of a specific mitochondrial enzyme either through stimulating synthesis of a new enzyme or increasing enzyme activity.

As cited before, there is conflicting evidence, in the rat, as to whether angiotensin is the humoral factor, since infusions of angiotensin have little effect on Aldo secretion (Marieb and Mulrow, 1965; Mulrow, 1966). Since Na depletion stimulates the last steps, these studies suggest that a mechanism other than angiotensin is responsible for the increased conversion of B to Aldo in the rat (Marusic and Mulrow, 1967).

4.3. ANDROGENS

C_{19} steroids have been isolated from extracts of adrenal glands and also they appear in the adrenal venous blood in higher concentration than in the general circulation. Steroids like ASD and its 11-β-OH and 11-keto derivates, and DHEA have been isolated.

Bloch, Dorfman and Pincus (1957) isolated ASD and its 11-β-OH derivative from beef adrenal glands perfused with ^{14}C-acetate or ^{14}C-Ch.

Rao and Heard (1957) showed the conversion of ^{14}C-Prog to ASD. This indicated that the metabolic pathway already established by Slaunwhite

and Samuels (1956) for the synthesis of ASD in the testis via Prog and 17-OH-Prog probably applied to the adrenal also. The sequence has been repeatedly confirmed. A similar pathway seems to occur in the ovaries as well (Sweat, Berliner, Bryson, Nabois, Haskell and Holstrom, 1960; Axelrod and Goldzieher, 1962).

The major route of formation of DHEA in the adrenal appears to be by way of 17-OH-pregnenolone which is then acted on by 17-desmolase. Neher and Wettstein (1960) found 17-OH-pregnenolone in extracts of adrenal glands, and Kahnt, Neher, Schmid and Wettstein (1961) were able to show that either pregnenolone or 17-OH-pregnenolone would be converted to DHEA when incubated with adrenal breis. Cohn and Mulrow (1962) showed that DHEA readily undergoes conversion to ASD.

Thus a number of routes of more or less importance exist for the metabolism of DHEA, other than its direct secretion into the adrenal venous blood.

4.4. ESTROGENS

In spite of the biological evidence that, at least under certain circumstances, the adrenals of some species can secrete estrogenic substances (Mancinini and Tarantino, 1957), the chemical evidence of either their biosynthesis or their secretion is not nearly so clear-cut as that for the androgens.

Meyer (1955a, 1955b) reported that 19-OH-ASD was a product of the incubation of ASD or DHEA with homogenates of bovine adrenals. Meyer, Hayano, Lindberg, Gut and Rodgers (1955), and Meyer (1957) later showed the conversion of this cpd to estrogens by bovine adrenal preparations. However, Engel and Dimoline (1963) incubated human adrenal slices with 19-OH-ASD but could not detect any estrone or estradiol.

Vinson and Jones (1964) reported the formation of 16-keto-estrone and 17-epiestriol when ^{14}C-Prog was incubated with finely minced mouse adrenals. Their identification was based on their phenolic character, and it looks like the first good chemical evidence of the formation of phenolic C_{18} steroids by the adrenals of any species.

5. METABOLISM OF ADRENAL CORTICAL HORMONES

5.1. GLUCOCORTICOIDS

The cpd F or B which is continuously produced in the adrenal is carried in the blood to the tissues which take up the steroids but, except for the liver, kidney, muscle and possibly other tissues, do not modify them. Subsequently, they are released and their main fate is to undergo a variety of oxidative and reductive metabolic changes, mostly of the latter type. The bulk of the catabolic changes takes place in the liver but this is not the exclusive tissue for such changes. The finding of efficient 20a- and 20β- reduction of 3β, 17 a-dihydroxy - delta 5 - pregnen - 20-one to the extent of 20% by muscle strip points to this tissue as an important site of steroid catabolism (Thomas, Forchielle and Dorfman, 1960). The metabolic products are excreted in the urine and feces with the steroid ring structure still intact. In humans, the bulk of the metabolites are excreted in the urine with negligible amounts appearing in the feces.

Cpds B and A differ only in the state of oxidation of C-11 and are interchangeable in metabolism.

The primary product produced in the gland is B which is in part oxidized to A, and both cpds are secreted into the blood.

Of cpds F and E, the former appears to be the primary biosynthetic product and the two cpds are interchangeable in metabolism by virtue of oxidation - reduction at C-11. Both steroids are probably secreted into the blood. Under normal steady state conditions, the reaction is slightly in favor of the oxidized cpd E (Bush and Willoughby, 1957; Romanoff, Rodriguez, Seelye, Parent and Pincus, 1958; Holness and Greenaway, 1959). The half-life of F in man has been determined by various groups and the results of three representative studies are in good agreement: Done, Ely, Olsen and Kelley (1955) reported a value of 90 $\pm$ 7.4. min.; Brown, Willardson, Samuels, and Tyler (1954) found 111 $\pm$ 5.3; and Peterson and Wyngaarden (1956), a value of 115 min.

Cortisol metabolism is altered during pregnancy and near term half-life is prolonged 100%. This has been established by using ^{14}C labelled hormone. The increased plasma content suggests a slower rate of metabolism (Migeon, Bertrand, Wall, Stempfel and Prystowsky, 1957). Sandberg and Slaunwhite (1959),

Daughaday, Holloszy and Mariz (1961), Seal and Doe (1966), and Daughaday (1967) have shown that estrogens (and pregnancy) induce a high level of CBG in blood and as a result of this increase most of the extra plasma F is 95% bound instead of normal 75%.

The principal C_{21} metabolites in the urine are tetrahydrocortisone and tetrahydrocortisol and such transformations have been shown to take place in the liver (Burstein, Savard and Dorfman, 1953; Miller and Axelrod, 1954; Peterson and Wyngaarden, 1955, 1956). As such, it is the principal determinant of the secretion rate of F.

The enzymes and co-factors involved in the removal of side chain are unknown. The substrates, however, are almost exclusively the 5β-reduced-tetrahydro cpds. This follows from the fact that the urinary metabolites are almost exclusively of the 5β-series.

The major portions of the metabolites of F or B are excreted as conjugates of glucuronic acid while small amounts of conjugates with sulphuric acid and possibly small amounts of mixed conjugates are normally produced (Pasqualini and Jayle, 1961; Tamm, Volkwein and Voigt, 1964). In the newborn infant,

sulphurylation of B by adrenal tissue is a prominent reaction. There is no evidence that the cpds conjugated with glucuronic acid participate in further biochemical transformation. Again, the liver appears to be the prime site for the union with glucuronic acid.

Reduction at C-20 may be the major step in the metabolic transformation of B and its 11-keto analogue (Engel, Carter and Fielding, 1955). Cleavage of the side chain has not been reported for B. Hydroxylation of B at C-18 is known to occur in adrenal tissues but could not be demonstrated in peripheral tissues. Dehydroxylation of C-21 of B has been demonstrated by the isolation of 11-keto-5β-pregnane-3a,20a-diol (Baulieu and Jayle, 1957), a reaction analogous to that observed with Aldo (Engel et al., 1955).

5.2. ALDOSTERONE

A very small portion of the hormone is excreted unchanged. A rather larger amount (about 6%) is conjugated in such a way that it is released in the free state by hydrolysis at pH 1. About 40% of the

hormone undergoes reduction of ring A and probably conversion of the 18-aldehyde to a hydroxyl group (Ulick and Lieberman, 1957). Neher and Wettstein (1960) reported that the carbonyl structure at C-18 remains unaltered, and that 18-OH metabolites of C_{21} steroids isolated from the urine, are probably not derived from Aldo, but appear to be metabolites of 18-OH-B, a likely precursor of Aldo.

Aldo, and its reduced transformation products, are excreted chiefly as conjugates of glucuronic acid. The urinary conjugates solvolyzed by acid are mostly of Aldo; the predominating tetrahydroaldosterones are extractable after β-glucuronidase hydrolysis. Conjugation takes place primarily in the liver but some acid-labile conjugates may be formed in the kidney (Sigenthaler, Dowdy and Leutscher, 1962; Leutscher et al., 1965).

5.3. ANDROGENS

Studies employing ^{14}C-labelled testosterone have confirmed and extended the initial findings of Dorfman, Cook and Hamilton (1939), and Callow (1939) who used unlabelled hormone, that androsterone and etiocholanolone are the principle

metabolites. With labelled testosterone it has been possible to show that in the human only trace amounts of this cpd or its metabolites are present in the feces, while by far the greatest amount is contained in the urine (Fukushima, Dobriner and Gallagher, 1954). In other species such as dogs, mice and rats, however, a significant portion of the hormone appears in the bile or feces (Barry, Eidinoff, Dobriner and Gallagher, 1952; Ashmore, Elliott, Doisy and Doisy, 1953; Paschkis, Cantarow, Rakoff, Hansen and Walkling, 1954).

6. DISEASES OF ADRENAL CORTEX IN MAN

6.1. ADDISON'S DISEASE (ADRENOCORTICAL INSUFFICIENCY)

Addison was the first to describe this chronic primary adrenocortical insufficiency and its fatal outcome, as early as 1855. This deficiency does not become clinically manifest until nine-tenths of the adrenocortical tissue has been made unresponsive as a consequence of tissue destruction or involution. Guttman (1930), in an analysis of 566 cases of Addison's Disease between the years 1900 and 1930, noted the

following causes of pathological changes in the adrenals:

bilateral tuberculosis:	68%
destructive atrophy:	19%
amyloid disease:	2%
neoplasms:	1%

A variety of conditions such as fatty degeneration, pressure atrophy, venous thrombosis, arterial emboli, syphilitic infection and hemochromatosis caused fewer than 10%. Carcinoma of the bronchus frequently metastasizes to the adrenals. By 1948, tuberculosis having become much less frequent, Friedman (1948) found that the primary atrophy was the cause of 60% of the pathological changes in the adrenals and tuberculosis was the cause of 40%. Addison's Disease is relatively rare, but when it occurs, it is most common between the ages of 20 and 50 years. The disease is rare in infants. The onset of Addison's Disease is generally insidious, although occasionally it develops with a crisis associated with or following an acute infection, traumatic injury, operation or termination of pregnancy. Addison's description of the disease is still the best:-

> "The leading and characteristic features of the morbid state to which I would direct attention are anemia, general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach and a peculiar change in the colour of the skin".

Death commonly occurs during crisis; in rare instances patients drift slowly into stupor, coma or shock-like state, and death.

The cardinal signs and symptoms of Addison's Disease are as follows:

(1) weakness and easy fatigability
(2) abnormal pigmentation
(3) weight loss and dehydration
(4) hypotension and small heart size
(5) anorexia, nausea, vomiting and diarrhea
(6) dizziness and syncopal attacks
(7) hypoglycemic manifestations
(8) nervousness and mental symptoms

Changes in gonadal function and in secondary sex characteristics are exceedingly rare. Hypoglycemic episodes occur in the clinical course of Addison's Disease. The signs and symptoms most frequently associated with hypoglycemia are hunger, headache, weakness, sweating, trembling, emotional instability and negativism. In more severe cases there may be blurring of vision, diplopia, apprehension, disorientation, irregular and shallow breathing and unconsciousness, with or without convulsions.

Patients with Addison's Disease complain frequently of muscle cramps - due to the reduction in blood flow and deficiency in salt and

extra-cellular fluid. The growth of body hair is decreased in both sexes but more strikingly so in females who may show a complete absence of axillary hair.

Therapy consists of a regular administration of cortisol or cortisone, usually supplemented by a salt-retaining hormone.

6.2. CUSHING'S SYNDROME

Adrenocortical cells normally capable of secreting GCCs, MCCs, estrogens and androgens, may under pathologic conditions, secrete an excess of any one of these. In hyperplasia of the adrenal cortex, and in the presence of an adenoma or adenocarcinoma, a variety of syndromes may appear, depending on the excess production of one or the other corticosteroids.

Harvey Cushing (1932) first described the syndrome of preponderent GCC excess in 1932, relating a basophilic pituitary adenoma with bilateral adrenocortical hyperplasia and suggesting that excess activity of the pituitary gland was the cause of the disease. Cushing's Syndrome, with primary pathology in the pituitary or adrenals, is a relatively rare condition

and occurs far more frequently in females than in males. The peak incidence is between the ages of 30 and 40 and particularly following pregnancy, but the disease has been known to occur in persons younger than 1 year, and older than 70 years (Forsham and Melmon, 1968).

Cushing's Syndrome is due to the excessive production of cortisol. About 60% of adults show bilateral adrenocortical hyperplasia, 30% have tumours and 10% show no overt pathology. Of these, one-half are benign adenomas, one-half adenocarcinomas. They are bilateral in less than 2%. Bilaterial hyperplasia is usually associated with widening of the adrenal cortex, particularly marked in the ZF; frequently hyperplastic nodules are seen also. A basophilic adenoma of the anterior pituitary may be present. Nelson and Meaking (1960), and subsequently by Salassa, Kearns, Kernohan, Sprague and MacCarthy (1959), and others, reported chromophobe tumours of the anterior pituitary which often lead to enlargement and partial destruction of the sella turcica, and which may occasionally give rise to Cushing's Syndrome associated with bilateral adrenocortical hyperplasia.

The systematic changes found in Cushing's Syndrome are marked and progressive atherosclerosis in the larger blood vessels. The kidneys

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manifest nephrosclerosis and sometimes calcinosis. The ovaries are often small and sclerotic.

Adenocarcinoma of the bronchus, pancreas and thymus has been described in association with Cushing's Syndrome, and elevated ACTH content has been demonstrated in the former.

Depending on the secretion of excess F, androgens, Aldo or estrogen, a varying number of symptoms and signs may be present. Cushing's Syndrome and adrenogenital syndrome represent extreme clinical variants; a mixed picture is not uncommon. Centripetal fat distribution without necessarily any marked gain in weight is common. Redistribution also leads to fat pads on the back of the neck (buffalo hump), and in the cheeks (moon face and fish mouth). Polycythemia, which in turn leads to a red face. Poorly healing wounds, frequently infected, are usually present. As wasting of muscle tissue progresses, marked weakness occurs. Backache is common and severe. Arteriosclerosis develops rapidly and is associated with hypertension.

The onset of Cushing's Syndrome is usually insidious, while the onset is more rapid in

pregnancy and after delivery. As the disease progresses, the patient becomes weaker and bed-ridden because of the loss of muscle strength and multiple-vertebrae fractures. There is an increased susceptibility to infection. Also, the patients show the following symptoms: osteoporosis, abnormal glucose tolerance test and diabetes mellitus. In over 90% of the patients, there is total absence of diurnal variation. There is a definite elevation of plasma corticoids.

In Cushing's Syndrome there is an inordinately steep rise, some eight-fold to ten-fold, of urinary free F, presumably because of the weaker binding of larger quantities of circulating hormone and consequent rise in glomerular filtration rate.

Adrex is the therapy of the choice in the rapid progression of Cushing's Syndrome.

6.3. ADRENOGENITAL SYNDROME

The adrenogenital syndrome due to congenital adrenal hyperplasia, long recognized as a clinical disorder of man, has been defined in terms of precise defects at several points in the pathway to F. The disorder has for a number of years been regarded as

one constantly associated with virilization, rapid somatic maturation associated with very large adrenal glands. These features, it is now known, are not constant in all types; the one common denominator in all forms is a defect along the course of F biosynthesis. Hechter and Pincus (1954) have provided evidence for the several biochemical reactions from Ch to F and the steroidal peculiarities in the adrenogenital syndrome have been translated on the basis of their fundamental experiments. In one form, the commonest type, due to 21-hydroxylase deficiency, a direct absence or disturbance of this enzymatic substance has been demonstrated by Bongiovanni (1958).

6.3.1. 21-HYDROXYLASE DEFICIENCY

This form of the disorder is accompanied by a marked elevation of urinary 17-KS and by the preponderance among the C_{21} steroids, of C-21 methyl substances. In particular, pregnanetriol may be easily measured and its abundance is of great diagnostic significance. The disorder is present from birth, and the females almost always present some ambiguity of the external genitalia from earliest life. Within the first few years of life there is rapid growth and virilization

in both sexes. The female does not usually achieve normal feminization because the large quantities of adrenal androgens suppress the pituitary gonadotrophins. The abnormalities may even be detectable in amniotic fluid (Jeffcoate, Fleinger, Russell, Davis and Wade, 1965). Treatment with small replacement doses of F or its congeners leads to an amelioration of both the chemical peculiarities and the clinical virilization.

In certain individuals with this disorder there is a marked tendency to salt and water loss with an Addisonian-like crisis leading rapidly to death unless it is quickly recognized and treated. The basic biochemical observations suggest that this represents a more severe form of the deficiency of 21-hydroxylase (Bongiovanni, 1967).

6.3.2. 11-HYDROXYLASE DEFICIENCY

It has been known for some years that hypertension may occur in the rare case of adrenogenital syndrome with its usual features as described above. Eberlein and Bongiovanni (1956) have shown that this form of the disease is due to a disturbance of 11-hydroxylase. The major steroidal product is 11-deoxycortisol and the urine contains large amounts of the

reduced tetrahydro derivative as well as significant quantities of tetrahydro-DOC. These two products would be expected in the absence of 11-hydroxylation since all other hydroxylations are normal. The virilizing features are virtually the same as with the 21-hydroxylase deficiency and the urinary 17-KS are extremely elevated. This form also responds well to replacement doses with F and both the hypertension and the virilization are controlled (Prunty, 1967).

6.3.3. 3β-HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

Bongiovanni (1962) described a severe form of the adrenogenital syndrome due to the lack of 3β-hydroxysteroid dehydrogenase. One peculiarity of thie form of the disease is the very high mortality rate despite the administration of large doses of therapeutic steroids (CSs administration reduce the excessive excretion of 17-KS). Later, Goldman, Bongiovanni, Yakovac and Prader (1964) demonstrated by histochemical techniques that the designated enzyme is indeed missing.

6.3.4. 17-HYDROXYLASE DEFICIENCY

Most recently Biglieri (1966) has described a single case with a deficiency of 17-hydroxylase.

This occurred in an adult woman with normal sexual structures who had never matured sexually and who excreted no 17-KS, and no estrogens whatsoever. The major steroidal product was cpd B, but there was also an elevation of secretion of DOC. She had symptoms of weakness and frequent upper respiratory infections, and she also had hypertension.

6.3.5. THE C-20 BLOCK SYNDROME

The C-20 block with lipoid hyperplasia of the adrenals with generalized steroid hormone insufficiency syndrome was first described by Siebenmann and Prader (1955). An enzymatic defect seems to preclude the formation of steroid hormones from Ch and leads to large lipid-filled adrenals. A genotype male has female external genitalia and is, in fact, a male pseudohermaphrodite. In this rare syndrome, patients die from adrenal insufficiency in early infancy; no survivals have been reported. However, with earlier recognition and adequate substitution therapy, they might survive in the future.

6.3.6. 18-HYDROXYLASE DEFICIENCY

Ulick, Gautier, Vetter, Markello, Yaffe and Lowe (1964) have described a defect in C-18 hydroxylation with decrease in Aldo secretion.

Bongiovanni (1967) concluded that the adrenogenital syndrome could now be attributed to a defect of several enzymes, and the 21-hydroxylase deficiency is the most common form of the disorder with several grades of severity.

CHAPTER II

THE LYMPHATIC TISSUE

1. NORMAL LYMPHATIC TISSUE

1.1. INTRODUCTION

The lymphatic system, in its gradual development from fish to mammals, shows a steady increase in complexity and organization, an increase which is an expression of its fundamental importance to mammals. Yet in spite of the great advance in medical science made during recent years, there are still serious gaps in our knowledge of the ultimate functions of lymphatics, lymphocytes and the organized collection of lymphocytes known as "lymphoid or lymphatic tissue". It is well established now that if lymphocytes and lymphatic tissue suffer extensive destruction, death occurs, and that if lymphatics are blocked, disasterous changes appear in the regions involved; but the real reasons behind either of these gross results still elude us.

1.2. ANATOMY AND HISTOLOGY

1.2.1. MAMMALS

Wherever it is found in mammals, lymphoid tissue consists essentially of a mass of free cells — the vast majority of which are lymphocytes of various sizes — together with a supporting framework consisting of reticulum cells, and of fibrous, elastic and sometimes muscular elements.

In mammals, the lymphatic tissue forms distinctly outlined organs, the lymph nodes, which are arranged along the course of the lymph vessels. It is present in small amounts in the bone marrow and in large amounts in the spleen, where it may undergo specific modifications depending on the peculiar type of blood circulation in the organ. In addition, lymphatic tissue is scattered in the mucous membranes of the alimentary canal and the respiratory passages, in the conjunctiva and elsewhere. The thymus, also an important lymphoid organ, differs from the rest of lymphatic tissue in that it has, intermixed with the lymphocytes, a large epithelial component, and is currently believed to be an important gland of internal secretion (Bloom and Fawcett, 1968).

Two microscopic constituents can be distinguished in the lymphatic tissue: a sponge-like framework, or stroma, and free cells in the meshes of the stroma. These constituents are present in different proportions in various parts of the lymphatic tissue, so that we may distinguish "loose lymphatic tissue", consisting predominantly of stroma, and forming the sinus or pathways for the flow of lymph through the lymph nodes; "dense lymphatic tissue", in which the free cells predominate; and "nodular lymphatic tissue", especially dense accumulation of free cells within the loose or dense lymphatic tissue. Under various physiological and pathological conditions, each of these types of tissue may turn into either of the other two types.

In the lymphatic tissue, several groups of lymphocytes can be distinguished. The "small lymphocytes" form the vast majority of the cells; the "medium-sized lymphocytes", scattered everywhere among the small lymphocytes, are present in a much smaller number; "large lymphocytes" or "macro-lymphocytes", are scattered singly among the other lymphocytes. These three types are connected with one another by an uninterrupted series of transitional forms. In the small ones, mitoses are extremely rare under normal conditions,

the main, immediate source of the lymphocytes of the blood being the medium-sized lymphocyte.

Plasma cells are common in the lymphatic tissue, especially in the medullary cords of the lymph nodes; their number is subject to marked variation, particularly under pathological conditions. In some animals (rat, mouse) plasma cells are unusually numerous (Bloom and Fawcett, 1968).

1.2.1.1. The Thymus

The thymus is a lymphoepithelial organ that produces lymphocytes and, very probably, a hormone. In man and many mammals, the thymus is situated in the anterior mediastinum in close connection with the pericardium and the great veins at the base of the heart. The organ presents marked variations in its structure depending on the age and the condition of the organism as a whole.

In relation to the BW, the thymus is largest during embryonic life and childhood up to the period of puberty; after this, it begins to involute, a process that proceeds gradually and continuously throughout life under normal conditions. This change

in its structure is spoken of as "age involution". During the course of some infections and wasting diseases, the normal slow involution may be greatly accelerated. This is called "accidental involution". Unexplained is the enormous overgrowth of this gland in some cases of "exophthalmic goiter" and in "mysathenia gravis".

The thymus is divided into a number of macroscopic lobules, separated from one another by connective tissue, and are divided into a darkly-staining, peripheral (cortical) area and a lighter-staining, inner (medullary) portion. The difference between cortex and medulla is due to the proportion of lymphocyte to reticular cells in each. The cortex consists mainly of densely packed small lymphocytes and a few reticular cells. The medulla, on the other hand, has large number of reticular cells and relatively few lymphocytes. There is no sharp line of demarcation between the two zones. Thus, the principal cells of the thymus are lymphocytes and epithelial reticular cells.

The lymphoid cells of the cortex exhibit intense mitotic activity and this activity is concentrated in the peripheral regions of the cortex

and along the epithelial sheaths surrounding radial vessels in the cortex.

1.2.1.2. The Spleen

The spleen, one of the blood-cell forming and destroying organs, contains a large amount of lymphatic tissue and plays important roles in metabolism and defense mechanisms of the body. But unlike those collections of lymphatic tissue that are interposed in the lymph stream, the spleen is in the bloodstream. Its purple colour is due to its great content of the blood. There is a peculiar type of blood vessel that allows the circulating blood to come into contact with the macrophages of this organ, so that the spleen acts in many respects as a filter for the blood; this property becomes greatly accentuated in some immune reactions.

The spleen, much like the lymph nodes, has a collagenous framework within which is suspended a reticular framework. As in the lymph nodes, the collagenous framework consists of a 'capsule' thickened at the hilus of the organ, where it is attached to folds of the peritoneum and where arteries enter and veins leave the viscus. Branching and anastomosing

continuations of the capsule, called 'trabeculae', penetrate the organ and form part of its framework.

The reticular framework fills the spaces between the capsule, hilus and trabeculae, and forms, together with the cell present, the splenic tissue. This is composed of typical lymphatic tissue (white pulp) and an atypical lymphatic tissue (red pulp).

The structure of the spleen and the relations between the red and white pulp depend on the distribution of blood vessels and change markedly in certain infections, intoxications, and disturbances in blood-cell formation (anemia, leukemia).

1.2.1.3. The Lymph Nodes

A lymph node is a large accumulation of lymphatic tissue organized as a definite lymphatic organ. Such nodes are always located along the course of lymphatic vessels, whose contents pass through the nodes on their way to the thoracic or right lymphatic ducts. Lymph nodes are scattered in large numbers, usually in groups, throughout the prevertebral region, in the mesentry, and in the loose connective tissue of the axilla and groin.

In general, lymph nodes are distributed not where lymph originates (as do the lymphatic nodules), but rather along the course of the main tributaries that flow into the thoracic and the right lymphatic ducts.

The lymph nodes are flat, well-defined bodies varying from 1 to 25 mm. in diameter. Their form is rounded or kidney-shaped, and their surface is somewhat rough. Usually, there is a slight indentation, the 'hilus' on one side of the node, where blood vessels enter the node at many places over its convex surface; they leave it only at the hilus. The sectioned surface of a lymph node shows the organ divided into an outer cortical and an inner medullary part. The difference in appearance between the cortex and medulla consists mainly in differences in arrangement of the lymphatic tissue in the two zones.

1.2.1.4. <u>The Bone Marrow</u>

The bone marrow comprises of 4 - 5% of the BW. The myeloid tissue, like the lymphatic, consists of the sponge-like framework or stroma, which is intimately connected with the blood vessels, and the free cells in the meshes of the stroma. The bone marrow may be divided into 'red' (active hemopoietic)

and 'yellow' (inactive, fatty) marrow. In the embryo and newborn, only red marrow is found. With progressing age, the red marrow is partially converted to yellow marrow, so that in the normal adult, approximately equal amounts of red and yellow marrow are found. No sharp line can be drawn between the two types of bone marrow. The amount of myeloid tissue increases at the expense of the fat when there is an increased rate of formation of myeloid cells. After prolonged starvation or in some wasting diseases, the amount of myeloid tissue decreases and the fat acquires a peculiar gelatinous appearance, a condition known as serous fat atrophy.

The myeloid tissue has a variety of functions, the most important of which is the production of myeloid elements for the blood. It is an important site for storage of iron, which is readily available for synthesis of hemoglobin; the heterophilic leukocytes produced in it are actively phagocytic for some bacteria and so are fixed macrophages. Little is known about the large amount of fat stored in it. Nodules of lymphatic tissue are found normally in the marrow. In some vertebrates, the chicken for example,

these nodules are present in large numbers. In man, the amount of lymphatic tissue in the marrow varies with age.

1.2.2. LOWER VERTEBRATES

Lymphoid tissue occurs throughout the vertebrate series. In fish, amphibia and reptiles, it is closely associated with tissues giving rise to myelocytes and granulocytes, and together with these cells, it constitutes the so-called lymphomyeloid organs.

In the lower vertebrates, although lymphocytes are plentiful, their regeneration is not localized in special lymphatic organs but occurs in many places in the connective tissue. In fact, lymph nodes are usually absent. The most important difference in comparison with the mammals is that the lymphatic tissue is not sharply separated from the myeloid tissue.

Drzewina in 1905 made an extensive survey of lymphoid tissue in fish and in amphibia, and found that in these creatures it consists essentially of a cellular reticulum with both granular and

non-granular leukocytes in its interstices. Lympho-myeloid masses may be found in one or more of a whole series of organs — kidney, liver, heart, genital glands, spleen, skull and alimentary canal. The association of lymphoid tissue with the alimentary canal is observed almost at the commencement of vertebrate evolution, and has persisted ever since. It is well-marked in cyclostomes — e.g., lamprey in which the spleen has not yet evolved — being situated immediately under the mucous membrane, where it forms the so-called spiral valve, the main blood-forming organ in these animals. In fish, generally — apart from the cyclostomes — a new lymphoid organ appears, the spleen, and this organ persists throughout all the subsequent phases of vertebrate evolution, though its functions undergo some modification. The bone marrow first makes its appearance in amphibia. Conditions in reptiles are essentially the same as in amphibia, except that in the crocodile there may be a rudimentary accumulation of lymphoid tissue in the mesentery. It is not, however, until we reach birds and mammals that the lymphomyeloid tissues begin to show a really marked separation into their component elements (Yoffey and Courtice, 1956).

1.2.3. REGENERATIVE CAPACITY OF THE LYMPHATIC SYSTEM

1.2.3.1. Lymphatic Vessels

Regeneration of injured lymphatic vessels begins in the lymphatic capillaries and proceeds by vascular budding. In some cases, however, for reasons not known, regeneration of the lymphatic vessels does not take place.

1.2.3.2. Lymph Nodes

The tissue of the lymph nodes responds to local injury at first by the rounding up of reticular cells and their transformation into macrophages, which multiply by mitosis. The lymphocytes, which at first are unchanged, then begin to multiply and hypertrophy into exudate mononuclear cells. But this attempt at regeneration is limited, and healing is usually brought about by the development of ordinary scar tissue.

After excision in young rabbits, lymph nodes may regenerate from local cells. With advancing age, such regeneration ability decreases markedly.

1.2.3.3. Lymphatic Nodules

These are the dense accumulations of lymphocytes embedded in a relatively scanty cellular and fibrous reticulum, and are usually the expression of some stage of lymphocytopoietic activity focused at a small area in the lymphatic tissue. These nodules appear and disappear, or pass through a series of cyclic changes during which an intense new formation of lymphocytes proceeds through proliferation of pre-existing lymphocytes and to a lesser extent through transformation of the primitive reticular cells.

1.3. PHYSIOLOGY

A primary function of the lymphatic tissue is to defend the body against disease-inducing agents that gain entrance to it. One very important role it performs in this respect is to provide lymphocytes, macrophages and plasma cells which, in response to disease-bearing agents (antigens), develop into cells that function immunologically. The distribution of lymphatic tissue in the body provides a line of defense underneath epithelial membranes against all those antigens which penetrate such membranes. For that

purpose, little depots of lymphatic tissue called lymphatic nodules, are scattered about in the loose connective tissue beneath the epithelial membranes. Also, antigens that gain entrance to lymphatic capillaries anywhere in the body enter the lymph, and thence into larger tubes, the lymphatics, which empty into the convex surfaces of lymph nodes. Here the lymph comes into contact with reticulo-endothelial cells, and cells of the lymphocyte series, which act as precursor cells for the formation of cells that function immunologically. Furthermore, antigens that gain entrance to the blood either by the direct invasion of the venules in infected tissue anywhere in the body, or via the lymph stream, come in contact with the spleen.

Although all the lymphatic tissue described above is advantageously located so as to be encountered quickly by any disease organisms that gain entrance to the body, there is one large depot of lymphatic tissue that is not; this is the thymus gland. This gland produces lymphocytes, probably some of those for delivery into the blood and lymph and inexplicably so far exists in an environment which protects it from, rather than exposes it to, antigens.

Moreover, not only do the antigens and lymphocytes contact each other by the movement of

the antigens to the lymphocytes, but the lymphocytes also migrate to where the antigens are. This is true not only of a relatively small number of newly formed lymphocytes from the thymus but also of those from lymph nodes which migrate into the sinuses and are carried away by the lymph stream into the lymphatics and the thoracic duct, and thence into the blood where the antigens circulate. In addition, numbers of small lymphocytes from the blood migrate directly into the lymphatic tissue through the endothelium of its venous capillaries.

In some pathological conditions, the lymphatic tissue may also be involved in extramedullary myelopoiesis; that is, it can also become a source of granulocytes.

From the foregoing it is obvious that the distribution of much of the lymphatic tissue in the body is such that disease agents that gain entrance to the body or spread in the body soon encounter lymphatic tissue and so set into motion the process of antibody formation. The fact that the function of the lymphatic tissue is to combat invaders in the shape of disease organisms is emphasized by the fact that if animals are born and raised in a germ-free

environment, their lymphatic tissue is poorly developed.

1.3.1. THE FUNCTION OF THE THYMUS

1.3.1.1. Introduction

As mentioned earlier, the thymus produces lymphocytes, a few plasma cells and myelocytes. In rat and opossum embryoes it is the first organ to produce large numbers of small and medium-sized lymphocytes.

The thymus has also been found to play a definite role in immune body formation. Although the organ does not produce antibodies to circulating antigens, it does form specific antibodies to antigens injected into it. If the thymus is injured, it will also produce specific antibodies to circulating antigen.

It has been known for several decades that removal of the thymus from young rats, mice, and rabbits causes a depletion of lymphocytes in the lymphatic tissue, lymph and blood. It has also been known that the younger the animal, the more extensive were the effects of removal of the thymus. Miller, in 1961, and Martinez, Dalmasso and Good (1962) found that

removal of the thymus from newborn mice resulted, after a few months, in a wasting, gradually fatal disease with underdevelopment of the lymphocyte-producing tissues and a decreased immunological competence. Their ability to reject skin grafts and to produce antibodies was decreased.

In comparison to the lymphatic tissue of animals raised in a normal environment, the lymphatic tissue of animals raised in an aseptic environment is not so well developed and shows no change after removal of the thymus.

The change from a large organ during embryonic development, infancy and childhood, into a gradually disappearing organ with the development of sexual maturity, led many authors to ascribe an endocrine function to the thymus. The clear evidence in favour of an internal secretion by the thymic epithelium comes from experiments with grafts of thymus in a capsule made with minute pores, which permit the passage of substances in solution but are impenetrable to cells. In these grafts lymphocytes die, but the epithelium does not. Such grafts prevent the development of changes, characteristic of removal of the thymus, and this is taken as evidence of an internal secretion (Bloom and Fawcett, 1968).

Further evidence of a possible thymic hormone has been given by Klein, Goldstein and White (1965). They found that injection of thymic tissue extracts into mice stimulated the production of lymphocytes, as measured by an increased incorporation of labelled precursors into DNA and total protein of the peripheral lymph nodes, as well as a significant increase in their weight.

The age involution of the thymus may also find some explanation in the following findings. Repeated injection of horse gonadotropic hormone causes atrophy of the thymus, but this does not occur in castrated rats. On the other hand, castration causes hyperplasia of the involuted gland in the rat (Rieke, 1966; Bloom and Fawcett, 1968).

1.3.1.2. Lymphocyte Migration from the Thymus

Since the thymic population of small lymphocytes is renewed at such a rapid rate while the size of the organ remains constant, it has been tacitly assumed by many that these cells must migrate in large numbers, presumably to other lymphoid organs. Many investigators have demonstrated that suspensions

of thymic lymphoid cells, when injected intravenously, will seed in the spleen and lymph nodes (Fichtelius, 1960), but it is difficult to assess from such experiments that lymphocytes would leave the thymus in normal circumstances.

Metcalf and Nakamura (1962) working with adult 6-months old C3H and AKR mice, reported that the thymus probably produces between 40 to 65% of all new lymphocytes. They made such calculations from the weights of the various lymphoid organs and the mitotic indices of the lymphoid cells in these organs. By utilizing ^{3}H-thymidine in autoradiographic studies, they showed that the rate of replacement of the small lymphocyte population in the thymus far exceeds that in lymph node, blood or bone marrow. Since the weight of the thymus remains relatively constant over long intervals, it must be kept so by a massive movement of lymphocytes from this organ to the rest of the body — primarily to the non-thymic lymphoid organs (Metcalf, 1964).

The question of whether or not lymphoid cells leave the thymus has been recently dealt with by Nossal (1964), and Murray and Woods (1964).

They labelled the guinea pig thymus by direct intra-organ injections of ^{3}H-thymidine. In both investigations, in the days following labelling heavily labelled lymphoid cells (presumably thymic in origin) were observed in the spleen and lymph nodes, particularly in newborn animals. However, in the better controlled work of Nossal, the number of thymus "migrants" was surprisingly small in relation to the production rate of thymic cells. Thus, their experiments indicate that some thymic cells do leave the organ and seed in peripheral lymphoid organs, but that possibly the majority of the lymphoid cells produced in the thymus may remain in this organ. Later, Matsuyama, Wiadrowski and Metcalf (1966) further investigated this question in C57Bl mice with multiple thymus grafts. Their calculations from the relative weights of thymus tissue and host non-thymic lymphoid tissues indicated that fewer than 0.5% of cells produced in the thymus grafts or host thymus could have seeded in the host lymphoid tissues. Observations on the liver, kidney and gut of these thymus-grafted animals also failed to reveal any excessive accumulation of labelled lymphoid cells in these organs.

From the above considerations it seems likely that relatively few cells produced in the thymus ever leave the organ, the vast majority being destroyed on a non-random basis at the end of 3 to 4 days. Thus, it is clear from the above evidence that such limited cell migration as does occur is necessarily of little importance to the body, and that the average life span of non-thymic lymphocytes is much longer than that of thymic lymphocytes (possibly 30-100 days versus 3 days). Furthermore, thymic and lymph node lymphocytes differ not only in size but also in heterogeneity of cell size. The failure of thymectomy (thymy) to have any effect for several weeks on lymph node size or cellularity also argues against a continuous mass influx of lymphocytes into the lymph nodes from the thymus (Metcalf, 1966).

Several groups of investigators (Harris and Ford, 1964; Leuchars, Cross and Davies, 1964; Miller, 1965) have contradicted the conclusion that few of the cells produced in the thymus leave the organ. They have shown in chromosome marker experiments that thymus-grafted, thymectomized mice contain a high proportion of mitotic cells of graft origin in host lymph nodes. This is certainly indisputable

evidence for a substantial migration of thymus graft cells into the host. However, these observations have not established the time at which this cell migration from the graft occurred. Further, it is now well known that substantial necrosis of thymus tissue occurs immediately after grafting and this is accompanied by a widespread breakdown of the architectural framework of this tissue. During the immediate post-grafting period it is possible that many cells may escape from the disrupted graft tissue, lodge in host organs and subsequently proliferate freely — particularly if the host is in a lymphocyte-depleted state. However, such cell migration may no longer occur once the architecture of the thymus graft is restored (Metcalf, 1966).

Ford (1966), reviewing an extensive article on the "traffic of lymphoid cells", concluded that the evidence for a natural movement from the thymus is not decisive, since it rests largely on grafting experiments, though it seems unlikely that so specific a property could be entirely an artifact. There may also be a movement directly from myeloid tissue into the lymph nodes without the intervention of the thymus.

In summary, there is evidence that some lymphocytes produced in the thymus migrate from this organ to other lymphoid organs. However, the vast majority (99%) of cells produced in the thymus in the normal animal appear to die locally in the thymus at the end of their intrathymic life span of 3 to 4 days (Metcalf, 1966).

1.3.1.2.1. Stress, Lymphocyte Depletion in the Thymus, and Fluctuation of Blood Lymphocytes

Selye found that a large variety of noxious agents and stressful conditions produced a marked atrophy of the lymphoid tissues and a hyperplasia of the adrenal cortex (Selye, 1936, 1936B). The thymic atrophy, is believed to be due to the liberation of adrenal cortical hormones; it does not occur if the adrenal cortex is removed. Purified ACTH causes a striking reduction in the weight and size of the thymus in rats. This is presumably due to stimulation of the adrenal cortex which results in an increased production of CSs.

The lymphocytes are among the most sensitive cells in the body to steroids, toxins such as mustard gas, and ionizing radiations. Unexpectedly,

reticular cells, which are more primitive than lymphocytes, are remarkably radio-resistant.

It is frequently stated in the literature that cpd E leads to weight loss in the thymus by causing lymphocytes to emigrate from the thymus. This thesis seems also to be advanced to cover stressful situations during infections and is used in support of the idea that in such situations the thymus exports large number of antigenically competent cells to the lymph nodes to be used for defense purposes. In order to check the validity of such publications, Matsuyama and Metcalf (1966) carried out their studies on young C57Bl mice with multiple thymus-grafts. The thymus graft tissue was labelled with ^{3}H-thymidine. The authors came to the conclusion that the vast majority of thymic lymphocytes do not leave the organ after the administration of E, and since thymus tissue weight does decrease sharply, there must be massive local death of cells to account for this weight loss. The percentage of labelled lymphocytes in the blood at no time rose above control levels of 10% in C57Bl mice. Such <u>in situ</u> death of thymic lymphocytes following E administration is, of course, well known and has been documented repeatedly by Dougherty and his associates (Dougherty and

White, 1947; Dougherty, 1952; Dougherty, Berliner and Berliner, 1960; Dougherty, Berliner, Schneebeli and Berliner, 1964).

In essence then, all E administration or stress appears to do is to accelerate the normal process of intra-thymic destruction of small lymphocytes. It seems unlikely therefore that there is any substantial excess migration of potentially useful lymphoid cells from the thymus to locally reacting lymphoid organs.

If most thymic lymphocytes do not leave the thymus, and yet whilst in the thymus exhibit little morphological evidence of any functional activity, while there remains an intense lymphopoiesis in the thymus, one of the major unsolved problems of thymus physiology arises.

The weight and cellular activity of lymphoid tissues and particularly the levels of blood lymphocytes in the mouse are extremely labile, fluctuating widely in response to influences which often appear trivial. In the mouse, minor procedures like earmarking, tail-pricking to obtain blood or sham injection, lead to lymphopenia. This lymphopenic response is maximal after 30-60 min. and passes off

after 3-6 hours. It is not related to adrenal-mediated stress responses, and occurs in adrexed animals (Metcalf, 1966). Absolute blood lymphocyte levels in apparently normal mice fluctuate from one period of the year to another. The basis for these fluctuations is very poorly understood, but population density, temperature and the level of endemic infections and infestations in the mouse colony are all known to be involved. The blood lymphocyte levels of larger lab animals seem much less prone to fluctuation.

1.3.1.3. Effect of Thymectomy

1.3.1.3.1. Guinea Pigs

As early as 1904, Paton and Goodall showed that blood lymphocytes decreased to 30% following thymy in neonatal guinea pigs. Gyllensten (1953) reported that subtotal thymy in guinea pigs resulted in a slight hyperplasia of the lymphoid organs. However, Reinhardt and Yoffey (1956) observed a fall in lymphocyte count in the thoracic duct output following thymy in adult guinea pigs. Later, investigators showed a remarkable decree of unanimity in their observations on the effects of thymy in various species.

1.3.1.3.2. Rabbits

Nakamoto (1957) reported that thymy in young adult rabbits was followed by development of a lymphopenia and a decrease in the lymphocytes in the efferent lymph from the popliteal lymph nodes. He also noted atrophy of lymph nodes in such animals.

1.3.1.3.3. Rats

Thymy in adult rats led to a fall in thoracic duct output but not a measurable fall in lymph node weights or mitotic activity (Bierring, 1960). Schooley and Kelly (1964) demonstrated that thymy at 6 days of age in rats caused lymph node weight loss, followed by lymphopenia in 2 months' time.

In a later study, Bierring (1963) observed that neither adult thymy nor did thymy combined with partial lymph node resection in rats have any effect on bone marrow lymphocytes.

1.3.1.3.4. Mice

Metcalf (1959, 1960) reported that thymy in four-weeks old C57Bl mice was followed by the slow development of a moderate degree of lymphopenia

(lymphocyte levels falling to 30-50% below control levels) and a corresponding degree of weight loss in the spleen and subcutaneous lymph nodes. These changes occurred gradually in two to three months after the operation. The population of primitive lymphocytes did not appear to be depleted by thymy. Similar findings on the effect of adult thymy on the spleen and lymph nodes in AKR mice were confirmed in detail by Nakakuki and Nishizuka (1964).

Doak and Cross (1963) reported that thymy in adult mice was followed by the development of lymphopenia and that the degree of lymphopenia was accentuated by whole body irradiation. Bone marrow injections did not improve the lymphopenic state, but did allow survival of the mice when the dose of irradiation used was lethal (850 r), by providing erthyropoietic and myelopoietic cells for the animals.

Miller, in 1961, while observing the effects of thymy in neonatal (less than 1 day old) mice on lymphoid leukemia (LLk) development, noted that thymy at this age produced dramatic changes in blood lymphocyte levels and lymph node and spleen morphology (Miller, 1961, 1962). Simultaneously, Archer and Pierce (1961), Archer, Papermaster and Good (1962), Good,

Dalmasso, Martinez, Archer, Pierce and Papermaster (1962) working on the effect of thymy on immune responses also performed thymectomies on newborn rabbits and mice, and apart from observing changes in immunological responsiveness in such animals, also observed changes in lymphoid tissues similar to those noted by Miller (1961, 1962). Subsequently, Waksman, Arnason and Jankovic (1962) made a detailed study in rats of the effects of neonatal thymy on the lymphoid organs. The changes described by this group have now been confirmed repeatedly by others in rats and mice, and also by studies in hamsters, and a reasonably clear picture of the effects of neonatal thymy has now emerged.

Following neonatal thymy in mice, as after thymy in adult life, little change is observed in the lymphoid organs for several weeks. These organs increase in size at a normal rate and contain dense follicular aggregates of small lymphocytes and active germinal centres. However, blood lymphocyte level do show an immediate response to thymy. In newborn mice, lymphocyte levels in the blood are relatively low and normally increase rapidly in the first 10 days of life. In neonatal thymectomized mice, lymphocyte levels tend

to remain at the initial low levels, showing little increase with increasing age of the animal. Miller (1964a) reported that the small lymphocytes of the blood are more severely affected by neonatal thymectomy than the large lymphocytes, whilst polymorphonuclear (PMN) and monocyte levels remain normal. After several weeks (the length of the latent period varying from one strain of mouse to another, and from one species to another) the lymphoid organs of neonatally thymectomized animals begin to lose weight. Plasma cell and reticulum cell populations in the lymph nodes are not reduced by thymy and in many studies, some evidence for an actual increase in the number of these cells has been obtained (Metcalf, 1966).

Thus, the changes in lymphoid organs caused by adult and neonatal thymy are essentially similar qualitatively, the difference being that neonatal thymy leads to a more rapid development of lymphoid organ atrophy and to a more severe degree of depletion of lymphocytes in these organs than does adult thymy.

1.3.1.4. Secretion of the Thymus Gland

Evidence has accumulated that the

thymus secretes a hormone. The crippling effects of early thymy upon development of the lymphoid system and of immunological competence can be reversed by a thymic graft, even when that graft is enclosed in a diffusion chamber which precludes any exchange of cells between graft and host (Levey, Trainin and Law, 1963; Law, Trainin, Levey and Barth, 1964; Osoba and Miller, 1964). There are several reports that saline extracts of thymus will induce proliferation of lymphocytes. Metcalf (1956) recorded increased number of circulating lymphocytes in newborn mice and thymectomized adults injected with a heat-labile extract of mouse thymus; Gregoire and Duchateau (1956) described hyperplasia of lymphoid tissues in adult rats treated with extracts from rabbit and pig thymus; Camblin and Bridges (1964) used extracts of rat or rabbit thymus to restore circulating levels of lymphocytes to normal in adult rats rendered lymphopenic by irradiation, and Klein et al., (1965) described increased rates of incorporation of radioactive precursors into DNA and protein in lymph nodes of mice injected with thymic extracts from mice, rats and calves. Accelerated synthesis of DNA was observed within an hour of injection — an indication that the stimulus to proliferation may act at this level. Extracts of other lymphoid organs and foreign

proteins, used as controls in these studies, were ineffective. Thus, the thymus appears to contain some heat-labile, water-soluble material that rapidly induces proliferation of lymphocytes in other lymphoid organs.

There are indications that this material is associated with the epithelial cells of the thymic medulla. Metcalf (1956) reported that extracts of medulla were more effective than extracts of cortex. Osoba and Miller (1964) stated that within diffusion chambers thymic lymphocytes soon degenerated, leaving a gland which, although consisting almost entirely of epithelial cells, was still capable of overcoming the effects of thymy. Gregoire and Duchateau (1956) obtained their extracts from thymuses in which an almost purely epithelial structure had been produced by destroying the lymphocytes with irradiation.

With advancing age, the epithelial cells (large epithelial cysts) increase in the number and degenerate to form Hassal's corpuscles (Arnesen, 1958), presumably bringing secretory activity to a close.

Clark (1966) summing up the available data, stated that thymic secretion begins before birth

with the formation of small basophilic granules, waxes during the first week after birth by shifting to the secretion of mucoid droplets, and wanes slowly, from two weeks after birth, by storing the secretory product in epithelial cysts. Metcalf (1956) has earlier reported that the lymphocytosis-stimulating-factor (LSF) was undetectable in the thymus of mice 2 days old, but had reached high levels by 7 days after birth. Clark (1966) further stated that the rate of release of thymic secretion product is variable and subject to control. The adrenal cortex may be one controlling factor. Injections of ACTH, E or F, induce an acute thymic involution during which the number of PAS-positive inclusions and epithelial cysts increase (Baker, Ingle and Li, 1951; Arnesen and Kierulf, 1961; Ito and Hoshino, 1962). Furthermore, the involution produced by several toxic drugs can be prevented by adrex (Selye, 1936).

All through this discussion it has been assumed that the role of the thymic hormone is to stimulate proliferation of lymphocytes, but the exact mechanism of its action remains obscure. It is also implied that the thymic hormone, by stimulating lymphocytic mitosis, interferes with the proliferative

response to antigens (Ito and Hoshino, 1962). That this may be true within the relatively secluded environment of the thymus has already been suggested (Clark, 1964), but many other possibilities need to be considered in trying to understand the role of the thymus in the complex responses of the lymphoid system to antigens.

The question also arises whether there is but one thymic humoral factor regulating thymic lymphopoiesis, lymphoid and plasma cell antigen initiated proliferation and influencing leukemogenesis, or whether there are a multiplicity of thymic factors, as for example in the case of the diverse secretions of the pituitary, adrenal, and salivary glands. Although future work may indicate the existance of multiple factors, at present it is still possible to hold to the simple concept that there is but one humoral factor and that the different phenomena influenced by the humoral factor reflect qualitative differences in the target cells responding to stimulation.

1.3.2. FUNCTIONS OF THE SPLEEN

The spleen is closely related to the lymphatic and hemal nodes and the bone marrow, and is

an important hemopoietic organ. Lymphocytes are produced in it mainly in the white pulp and in particular, in its nodules. From the white pulp they migrate into the red pulp where some of them perhaps become monocytes. Lymphocytes and monocytes actively enter the venous sinuses through the reticular wall.

In some pathological conditions, especially in myeloid leukemia (MLk), the red pulp of of the spleen undergoes myeloid metaplasia. In this case a large number of erthyroblasts and megakaryocytes appear in the tissue, so that the red pulp acquires a structure suggesting that of red bone marrow.

After the removal of the spleen the number of lymphocytes in the blood increases (lymphocytosis); this is explained by an excessive compensation on the part of lymph nodes. There is also an increase in the number of eosinophilic leukocytes. Both of these phenomena soon disappear.

The spleen also acts as a store for red blood cells. From time to time large numbers of them are retained in the red pulp and then given up to the bloodstream as they are needed in the circulation.

The destruction of erthyrocytes occurs in the spleen, with a varying intensity in different species, for they are phagocytosed by the macrophages in the splenic cords and sometimes by those lining the sinuses.

Closely connected with erthyrocyte destruction by the spleen is its function in iron metabolism. The iron-containing component of hemoglobin is freed from the disintegrating erthyrocytes and stored in the reticular cells of the spleen. This accumulated iron is reutilized in the formation of hemoglobin.

The spleen is thought by some to regulate the formation and destruction of erthyrocytes by the production of a hormone which decreases the erthyropoietic capacity of the bone marrow. Others think that a hormone is produced by the spleen which inhibits the formation of leukocytes in the other hemopoietic organs. In short, the spleen possesses a combination of phagocytic, cytopoietic and antibody forming activities (Taliaferro, 1956).

1.3.3. FUNCTION OF LYMPH NODES

The lymph nodes are the most active structures for the formation of the lymphocytes, except perhaps for the thymus. The stimuli for lymphocyte production are probably brought to the lymph nodes by both lymphatic and arterial vessels. Although great numbers of lymphocytes are produced in certain infections and the lymphatic leukemias (LLks), the actual stimuli for lymphocytopoiesis in these conditions, as well as in physiological states, are unknown. As the lymph nodes are composed essentially of lymphocytes and phagocytes, it is obvious that their main functions depend on these cells.

Because of the phagocytic activity of the reticular cells, particularly in the sinuses, the nodes serve as the filters in which various particles arising locally or brought with the lymph from other regions of the body, are taken up and often destroyed. Phagogenic bacteria brought to the lymph nodes are frequently ingested and sometimes destroyed by the macrophages. Just like all the other tissues and organs containing many macrophages and lymphoid cells, the lymph nodes elaborate antibodies.

2. THE LEUKEMIAS

2.1. LEUKEMIA IN MAN

2.1.1. HISTORY

Leukemia (Lk), since its recognition as a distinctive disease, has a history of little more than 100 years and it is therefore a comparative newcomer among the major known scourages of humanity. The rising incidence of Lk and its possible relationship to background or occupational exposure to ionizing radiations in this atomic age have brought the disease increasingly before the public eye. As an example of cellular proliferation, closely akin to the generality of neoplastic processes, Lk has been of special interest to innumerable workers in the field of cancer research since the blood and hemopoietic tissues can be so easily and repeatedly sampled.

Towards the middle of the nineteenth century the time became ripe for the recognition of this disease. Cases of splenic enlargement accompanied by pallor, purpura, lymphatic glandular swelling, and other signs commonly found in Lks had been described often since the earliest medical records.

Richard Bright, Physician Extraordinary to the Queen, in a paper entitled "Observations on abdominal tumours and intumescence; illustrated by cases of disease of the spleen", makes clear the position of advanced medical opinion on this subject at that time (1838). "With regard to the functions of the spleen", he wrote, "we have every reason to believe that it affords important assistance in preparing the blood, but whether chiefly as accessory to the process of digestion, or as having within itself the power of acting beneficially on the blood needs to be inquired. It is an established fact, that it is provided with a structure which affords it peculiar elasticity so that it can accommodate itself to great changes in the volume of the blood it contains". Among a large number of structural alterations of the spleen categorized by Bright, was a condition described as "fleshy hardness with enlargement". Bright noted that "in this state, the spleen often attains a prodigious size, filling up the whole left side of the abdomen. It produces very little constitutional irritation, and chiefly injures by its bulk, and its tendency to favour serous effusion. In young children, this form of disease is still more frequent than in adults, and with them it is more fatal.

Such children seldom live above a year, or two or three; and fall victims to emaciation and often to mesenteric disease". Although Bright included many case reports in this instructive article and one may reasonably interpret several of them as examples of some form of Lk, no reference appears to blood examination, either macroscopic or microscopic, during life or post-mortem. Yet how near Bright and, no doubt, many of his contemporaries were to the concept of splenic involvement in a generalized blood disease, may be seen from his penetrating final remarks in this paper: "It is probable that the spleen is greatly influenced by the derangement of many of the other organs of the body; for we cannot doubt, that whatever acts decidedly on the circulating system, must, in some degree, influence the spleen; which obviously, from its structure and appearance, receives large quantities of blood, as subsidiary to the process of sanguification or circulation".

While clinicians were so close to the idea of a disorder of "sanguification" involving the spleen, pathologists and microscopists had been surprisingly slow to exploit and extend the microscopic studies of blood and the recognition of "white globules" initiated by William Hewson in 1774.

It seems likely that the first accurate description of a case of Lk was given in 1827 by Velpeau. His patient, a 63-year old florist and seller of lemonade, "who had abandoned himself to the use of spirituous liquor and of women without, however, becoming syphilitic", fell ill in 1825 with a pronounced swelling of the abdomen, fever and weakness, and symptoms caused by urinary stones. He died soon after admission to the hospital and was at autopsy found to have an enormous liver and spleen, the latter weighing 10 lbs. The blood was thick, "resembling in consistency and colour the yeast of red wine...one might have asked if it were not rather laudable pus, mixed with blackish colouring matter, than blood." It was, in fact, the peculiar character of the blood, as seen on post-mortem, which first attracted the attention of all the early observers of Lk.

Almost two decades later, in 1845, Lk was first described almost simultaneously by two brilliant young men - Bennett, in Scotland, and Virchow, in Germany - who, after applying their great gifts to a meticulous exploration of its features in the living and the dead, engaged at once in an almost venomous wrangle over the honour of having been the first to

identify this fatal disease. Their independent publications within one month of each other, of two cases of same new disease, was less remarkable than the fact that each observation came from the pen of a man who was to become a leader in his own field; Bennett, in physiology, and Virchow, in pathology. In each of the first two cases, it was the post-mortem appearance of the blood which first gave the hint that an unusual condition was present. In Virchow's patient, the blood vessels contained a "yellowish-white almost greenish mass". The relation between red and colourless corpuscles was reverse of the normal, so that Virchow coined the term "white blood" (weisses blut) to describe the condition. The spleen weighed 7 lbs. 12 oz. in Bennett's case, and measured nearly a foot in length in Virchow's patient. While the findings were similar, the two authors interpreted them in a different fashion: Bennett as "suppuration of the blood"; Virchow, much more cautiously, as probably not "pyemic". Interestingly enough, David Craigie, who observed his case several years before those of Bennett and Virchow, published his case report of a patient with enlarged spleen and liver weighing 115½ and 90 oz. respectively, and also found "globules of purulent matter" in the blood, in the same edition of Edinburgh Medical and

Surgical Journal of October 1, 1845, as did Bennett, but did not realize its significance.

Neither Craigie nor Bennett regarded the disease they described as primary disturbances of leukopoiesis. Craigie thought that the disorder to be due to chronic inflammation of the spleen and argued that the structure of the spleen was such that pus formed there would not accumulate as an abscess but pass directly into the blood stream. Bennett also thought the colourless corpuscles were pus cells rather than white blood cells and concluded that the disease was a suppuration of the blood.

A few months later (August, 1846), having reconsidered not only his own case but also those published by Bennett (1845), Craigie (1845), and Fuller (1846), Virchow took a much more definite attitude against the 'pyemic theory of Lk', pointing out that there was no evidence of local suppuration which could have spread to the blood, that the pus corpuscles were identical with the colourless bodies normally occurring in the blood, and that in Lk (still called "white blood") there was merely an increase in the normal number of these latter cells. Such an increase was also shown by Fuller (1846). Later, in

1847, Virchow introduced the term "leukemia", but this name did not receive unqualified acceptance. Bennett, while reading his paper to the Societe de Biologie in Paris, in 1851, described and discussed 4 cases which he studied personally and a further 8 gathered from the literature, objected to the term "leukemia" as a misnomer, since the blood in this disease was not white. He proposed "leucocythemia" (meaning white cell blood) as a more satisfactory descriptive term. He did, however, accept Virchow's interpretation of the nature of the disease and withdrew his former concept of blood suppuration. We must agree with Gowers, who discussed this problem of terminology in 1879, that "leucocythemia" would really be a more apt and accurate name, particularly since the white blood corpuscles came to be generally known as "leucocytes" after the first appearance of this word in 1855, in Littré and Robin's Dictionnaire de Médecine (Nysten), but perhaps because it is shorter, "leukemia" seems now firmly established.

After introducing the term Lk, Virchow published a series of brilliant studies on the nature of the disease and which he summarized in 1856, in a paper of great interest and importance. He started by

asserting that the colourless corpuscles are always present in normal blood and are increased after digestion, in pregnancy, and in most inflammatory conditions. Such an increase is not by itself a disease and must be distinguished from Lk, which is a definite pathologic state characterized not only by an increase in colourless cells, but also by a decrease in the number of red corpuscles, and dependent on changes in certain organs. He also introduced the terms splenic and lymphatic leukemias (LLks). This paper of Virchow contains in a rudimentary form many of the views on the pathology of Lk which are still held today. This is all the more remarkable as there was extremely little knowledge at that time of the sites and mechanisms of hematopoiesis, and the functions and fate of the blood cells.

The general view on the origin of the red blood cell was still that put forward by Hewson in the eighteenth century - that is, that red cells are formed from the colourless corpuscles in the blood itself. Thus, Bennett suggested that the red cell was the "liberated nucleus of the colourless cell". Colourless corpuscles were thought to be formed in the "lymphatic glands", including the spleen, thymus,

thyroid, suprarenals and pineal body, whence they entered the blood. Virchow, himself, like many others, had at first accepted this prevailing concept and had explained Lk as a retardation in this process, with the production of increased number of white and of decreased quantities of red cells. By 1856, however, he had abandoned this view and now regarded the white corpuscles as "simple, non-specific cells" which are not transformed into red corpuscles once they have left the sites at which they themselves are produced; they are rather "a sort of superfluous excess". The transformation of lymph corpuscles into red cells does take place in the spleen and lymph nodes, but once they have reached the blood stream, "their specific metamorphosis into coloured corpuscles becomes impossible". They circulate for a brief while and then perish.

This first phase of research on Lk may be summarized by saying that within 12 years of its recognition, the two chief varieties of chronic leukemia (CLk), as well as the acute form (Friedrich, 1857) had been described, and the main clinical and pathological features tabulated. Owing to the exceedingly crude hematologic methods then available, it was possible to make only the most superficial examination of the

leukocytes themselves, and though it was realized that there was more than one variety of these cells, they could not be characterized morphologically or traced to the sites of their formation. It had, however, been acknowledged even by those who, like Bennett, had originally regarded Lk as the result of a special kind of inflammation, that the changes in the blood were not caused by an admixture of pus, but probably by a proliferation of those white corpuscles which are a normal constituent of blood. The primary changes in the disease were now sought in the lymphatic organs rather than in the blood itself.

Among the many paper, now far too numerous to list, which appeared during these years, we may particularly note Biermer's first record of Lk in childhood (1861), and Bryant's early unsuccessful attempt to halt the progress of the disease by removing the spleen (1866). In these early years Lk was chiefly discovered in association with gross splenomegaly, although Virchow (1847) had already differentiated two forms of the disease, splenic and lymphatic, with emphasis on the involvement of lymph node, particularly in the latter form. Further major advance had to wait the discovery of specific staining methods for blood cells.

A few years later, Neumann (1870, 1872, 1878) demonstrated that the bone marrow was an important site for the formation of blood corpuscles in health and disease. His reports of extensive changes in the bone marrow in Lk led to difficulties in classification and nomenclature, since he now separated a third category of the disease, "myelogenous leukemia" (MLk), from the recognized splenic and lymphatic forms. Neumann (1878) also showed that the immediate precursors of the red cells were nucleated red cells which he found regularly in the marrow, and sometimes also in the leukemic blood. If a transformation of white to red cells did occur, he reasoned Lk could be caused either by an over-production of the former, or by a failure of their transformation to the latter; but if red cells were formed independently of the white ones, then there must also be a disturbance in their production in the marrow in order to account for their diminution in leukemic blood.

Gowers (1879), in a masterly monograph on Lk, took this argument a step further by pointing out that anemia in Lk might theoretically be caused either by a diminished formation of red cells, or by their excessive destruction. He believed that

the increase in the number of white cells which occurred in splenic Lk was only a symptom accompanying the primary changes in the blood-forming organs, and that it need not be present before the diagnosis of Lk could be established.

By this time the introduction of differential staining methods, developed and expanded by Paul Ehrlich from 1877 onwards, and described in his monograph in 1891, greatly facilitated fresh advance of knowledge about Lk as in so many hematological fields. Qualitative study of the blood films from cases of Lk, with differentiation of the various types of cells present, now came to replace the former rough assessments, and it was soon apparent that Virchow's splenic Lk was essentially identical with the MLk of Neumann. At this stage two major sub-divisions of Lk were thought to exist; the "splenomyelogenous" form in which large numbers of PMN and mononuclear cells containing specific granules were present in the blood, and the "lymphatic" form in which many non-granular, mononuclear lymphocytic cells were found.

An increasing number of cases next came to attention in which the cells of the peripheral

blood included differing proportions of both granulocytes and large atypical non-granular, mononuclear cells, and these were first regarded as "mixed leukemias". What we now know to be the acute myeloblastic termination of MLk was also observed and taken to be a transformation from MLk to LLk. The confusion thus brought about began to be clarified when Otto Naegeli, in 1900, recognized and described the myeloblast.

The existance of such a non-granular, mononuclear precursor of the granulocyte series of cells provided an explanation for the mixed leukemias and the transformation anomalies and the idea of different phases of Lk with varying proportions of mature and immature cells was established. The recognition of the myeloblast contributed also to the understanding of acute leukemia (ALk) states. Earlier, in 1857, Friedrich had noted the occurrence of a new form of Lk with a rapid, acute course, and Gowers (1879) made reference in his review of "splenic leucocythemia" to "many cases on record in which the symptoms lasted 6 months only" and some with an even shorter course. He stated, however, that "the most acute cases on record, in which the disease runs its course in a few weeks,

are usually attended with great and rapid enlargement of the lymphatic glands and spleen", and since this picture is hardly typical of ALk, as we now know it, the cases may have been examples of acute terminal exacerbation of previously undetected "chronic granulocytic leukemia" (CGLk).

The distinction of acute from CLk came about gradually; Ebstein, in 1889, described the clinical picture of ALk on the basis of 16 cases already recorded in literature, and Fraenkel assumed that the atypical mononuclear cells he found were early lymphocytes, and he and most of his contemporaries believed that all ALks were lymphocytic, but he nevertheless concluded that these "lymphocytes" were young forms capable of transforming into polynuclear cells. Ehrlich (1905), writing on "History of the Blood, Normal and Pathologic", treats these views of Fraenkel as "plainly contradictory to the facts". Ehrlich believed all non-granular mononuclear cells of the blood to be either monocytes or lymphocytes and incapable of becoming anything else. Ehrlich remarked that it was "very difficult to conceive of conditions which would prevent the natural maturing" of the blood elements.

Leukemias recognized before 1913 were all regarded as examples of disease of the lymphocytic or granulocytic series of cells, and the occurrence of a leukemic proliferation of monocytes had not been reported, but in that year Reschad and Schilling-Torgau (1913) described a new form of Lk involving splenocytes or monocytes. The cytological study made by these authors does not appear to have convinced many hematologists of the existance of "monocytic leukemia" as a separate entity, for in the next 15 years only 6 further cases were reported (Clough, 1932), but from about 1930 onwards, increasing numbers of reports evidenced the spreading acceptance of the concept. With the definition of a monocytic variety of the disease, the major landmarks in the panorama of Lk had been identified. Controversy over borderline states, inter-relationships and innumerable details continued, and indeed is still maintained today, but a general concept of Lk had emerged that has not required radical modification in the last four decades.

During the late nineteenth and early twentieth centuries, hematologists conjured with a host of new terms like pseudoleukemia, leukosarcoma, chloroma, lymphosarcoma, myelosis, myeloma and various combinations.

Many of these were ill-defined at the time of their first appearance, and definitions had to be altered in the light of accumulating clinical or pathologic experience, generally under the pressure of attacks by rival schools of physicians. Recent developments in phase and electronic microscopy and studies of chromosomal details by simple methods have served to bring about a renaissance in the cytologic field. There can be no question that looking at a cell still has considerable importance, although it must also be realized that highly similar cells morphologically may be quite different physiologically. This is being brought out by radioautographic studies using ^{3}H-thymidine and by the response on the part of apparently identical lymphocytes to antigenic stimulation.

2.1.2. GENERAL CONCEPT

Although the term Lk seems at first glance to be readily understood, its exact definition becomes rather difficult. Leukemia (white blood) implies a condition of the blood characterized by a greatly increased leukocyte count. Although this is frequently the case, it is by no means a constant phenomenon. Furthermore, it is not in reality the

blood that is fundamentally abnormal in Lk, but rather the tissues — the several tissues that produce the blood cells and that are proliferating in an unduly rapid or otherwise abnormal fashion, as well as the many tissues in which the proliferating and exfoliated cells may settle and often accumulate. Indeed, the blood may be said to be of secondary importance in Lk. In a measure, it may be considered only as a "traffic stream" through which cells pass from their site of origin in the blood-forming organs to tissues all over the body, and from these tissues to their site of eventual destruction. Thus, with a disturbance of one of these blood-forming organs, characterized by rapid proliferation and exfoliation of cells, the blood stream usually shows considerable number of leukocytes on their way to various tissues. On the other hand, should exfoliation and death of the cells be simultaneously very rapid, the blood itself might carry relatively few cells. Also, if the outlying tissues were particularly "avid" for the leukocytes just produced in the proliferating tissues, the number of blood leukocytes might be normal, whereas those in tissues might be greatly increased. In many instances, it seems that the processes of maturation and

exfoliation of a given white-cell-forming tissue are relatively slow, although by inspection, the tissue seems to be proliferating intensely: this, too, may result in a relatively normal or even low leukocyte count.

Also, in the presence of a high leukocyte count, one cannot be certain, furthermore, that a given leukocytic tissue is proliferating to an unusual degree; the high white cell count may be due to delay in the removal of cells from the blood, i.e., they may be accumulating there. Thus, a high leukocyte count may be due to an excessive degree of proliferation with parallel exfoliation or it may be "accumulative". This in turn may be due, at least in some instances, to an increase in the survival time of certain leukocytes.

These various possibilities, some of them theoretical, are to emphasize that Lk is not a "blood" disease <u>per se</u>, but actually an abnormal proliferation of one of the leukocytic tissues, in which the blood cells may or may not become affected.

Thus, Lk may be said to represent an abnormal form of proliferation of one of the white

cell forming tissues, i.e., the bone marrow, the lymphoid tissues, the reticulo-endothelial system, or the system of plasmocytes. The abnormal means a new kind of white cell growth, in fact, the presence of a new "race" of leukocytes. Fortunately, not all leukocytic proliferations are leukemic; in fact, benign proliferations of the white cells represent one of the commonest forms of normal bodily reactions. Thus, PMN leukocytosis occurs with trauma, exercise or even with psychologic stress or excitement. The leukocytosis of many pyogenic bacterial infections (streptococci, staphylococci, etc.), in which there is a great increase in the PMN cells of the blood, represents fundamentally a proliferation of the bone marrow granulocytes in response to a specific bacterial organism. This has invaded the body and lodged in a certain area such as the lung (pneumococcus pneumonia), or the meninges (meningitis), or the appendix (appendicitis). By obscure, though probably humoral mechanisms, the local infection evokes unusual activity, i.e., proliferation of one of the white cell forming tissues, in this case, the bone marrow granulocytes. This proliferation is a meaningful one, i.e., a useful or essential response to combat an invader, in this case a

coccal bacterial organism. Once the invading organisms have been overwhelmed and there is no longer any need for excess numbers of granulocytes, the bone marrow reverts to its normal growth pattern, and the blood leukocytosis gives way to normal blood count.

A similar mechanism, but involving a different blood-forming system, is that found in the response to certain viral agents as in rubella, poliomyelitis, and perhaps in infectious mononucleosis. It is conceivable that the generalized proliferative reaction which takes place in these disorders is actually immunologic in nature, i.e., that immunocytes of the lymphoid variety are proliferating with the eventual development of an immune response (Dameshek and Gunz, 1964).

The PMN responses of diverse origin, the lymphoid proliferative disorders known as infectious mononucleosis, the reticulo-endothelial proliferation of tuberculosis, these are generalized proliferations of one of the white cell forming tissues, but they may be considered as self-limited, i.e., reactive. An agent appears, there is (ordinarily) a useful response of one or other leukocytic tissue in response

to the agent, the agent is suppressed, and eventually the reaction of proliferation subsides. Leukemia is not like this. What, then, is leukemia?

Leukemia may be said to be an abnormal, generalized self-perpetuating proliferation of one of the white cell forming tissues, in response to a virus (in mice at least), and apparently without purposeful or utilitarian value to the body.

This abnormal growth process may be said to have an innate hardiness which gives it an ecologic advantage; thus it becomes a successful population. The principles of population dynamics - ecology - have been applied by Burnet (1959), Gorman and Chandler (1963), and others to various types of cellular proliferative activities: immunologic, lymphoid, etc. They may be said to have particular relevance in the case of Lk, where the principle of "competitive exclusion", as stated by Hardin (1960) can be invoked so well. Biological competition is most acute between the most similar populations (i.e., between the white cells which are almost, but not quite, similar). The most successful population tends to

displace all others from a given ecologic niche, although a stable co-existance of two or more populations within a niche can occur. The reasons for superiority of one population over another are often extremely subtle and are often not clear even after extensive study of a competitive situation (Gorman and Chandler, 1963).

Thus, Lk may be defined as a neoplastic disorder of the hemopoietic cells and may arise either from red or white cell precursors in the bone marrow or from lymphocyte precursors in the lymphoid tissues. Many of the newly formed neoplastic cells do not mature. These precursor cells proliferate rapidly and may be found not only in large numbers in the marrow or lymph nodes, but also in the blood and in a variety of organs such as liver and spleen, and eventually leading to anemia, thrombocytopenia and death.

The definition of Lk has its limitations. Thus, there is no hint as to the cause of the proliferation; why should one of the leukocytic tissues suddenly develop this "obsessive" outburst? This "self-perpetuating" mechanism still lacks the

explanation and has a tendency to recur even after reversal by a therapeutic agent. Its "relentless" character terminates only with death of the individual.

2.1.3. CLASSIFICATION

2.1.3.1. Myeloid and Lymphoid Leukemias

Very broad sub-divisions of Lk may be indicated in terms of the site of origin of the affected cells. Thus, myelogenous or myeloid leukemias (MLks) are those involving leukocytes normally produced from the bone marrow, while lymphogenous or lymphoid leukemias (LLks) involve leukocytes formed chiefly in lymphoid tissues. MLks would therefore include acute myeloblastic types and all forms of subacute and CGLk, while LLks would include acute lymphoblastic and subacute and chronic lymphocytic leukemias (CLLks). This is clear and consistent as an initial approach, but the placing of monocytic and monoblastic leukemias presents a difficulty, the myelomonocytic form of Naegeli should perhaps be regarded as myelogenous, and the Schilling form as lymphogenous or histogenous, but any precise allocation would certainly not find general agreement among hematologists. Despite all

this, the terms myeloid and lymphoid, or myelogenous or lymphogenous, are commonly employed and serve a useful purpose when a more specific designation is unnecessary or impossible (Hayhoe, 1960).

2.1.3.2. Acute and Chronic Leukemias

In classifying Lks, reference may be made to the clinical acuteness of the disease, the number of leukocytes circulating in the peripheral blood and the presence of abnormal forms, the identity of the predominating cells and their stage of maturity, and the site of origin of the proliferating leukocytes. As stated in the previous section, Lk may be considered as a generalized neoplastic proliferation of one of the leukocytic tissues. Since four lines of white cells may be distinguished, i.e., the granulocytes, the lymphocytes, the reticulum cells (histiocytes, monocytes) and the plasma cells, four main types of Lk may be discriminated: granulocytic; lymphocytic; monocytic; and plasmocytic. Something may be said for the possible inclusion of a fifth type, i.e., "mast cell leukemia". Like other types of neoplastic proliferation, Lk is subject to considerable variation in growth rate, varying from the very rapid to the very slow, and the

growth pattern in the very slow leukemic proliferations may differ only slightly from the normal pattern. A general distinction can be made on clinical grounds between acute and chronic leukemias.

In ALk, the onset is commonly rapid and the course short and severe, with dramatic symptoms and physical signs of fever, anemia, hemorrhage, tissue infiltrations, buccal ulceration, secondary infection of respiratory tract, and the like; if the disease is untreated, death usually occurs within 3 months of the onset.

In CLks, on the other hand, the date of onset is often uncertain, so insidiously do symptoms commence; the condition progresses relatively slowly and may remain mild for long periods with few manifestations of disease other than painless splenomegaly, or lymphatic glandular enlargement. Patients with CLks nearly always survive more than a year from the time of first symptoms, commonly from 3 to 5 years, and occasionally for very much longer.

The survival differences between acute and chronic forms of the disease have been used to provide arbitrary limits; cases of duration less

than 3 months (Sturgis, 1955), or 6 months (Custer, 1949) being regarded as acute, and those of more than a year's duration being called chronic, while cases of intermediate survival, between 3 or 6 months and 1 year, are described as "subacute". Whether this term, subacute, should be reserved for the relatively slow acute cases is open to discussion. There is general tendency, however, to designate Lk as either acute or chronic, realizing that many variations may occur in the two groupings. Nevertheless, clinical distinctions between acute and chronic forms of Lk are braodly paralleled by cytological distinctions and clearly reflect important subdivisions of the disease.

2.1.3.3. Subacute and Aleukemic Leukemias

In both acute and chronic Lks, the peripheral blood typically contains far more than normal numbers of circulating leukocytes with many immature cells of a kind not normally released from sites of leukopoiesis. This is the classical Lk picture. Such a peripheral blood picture does not, however, always appear and instead shows two major

variants. Both states present during life the general clinical picture and pathological findings in the bone marrow of Lk, and post-mortem examination reveals the characteristic proliferative and infiltrative nature of the disease. In the first condition the leukocyte level in the blood is not raised above the normal upper limit of about 12,000 cells per cu.mm., and may indeed be decreased below normal levels, but some of the leukocytes present are immature cells. The name "subleukemic leukemia" is commonly and appropriately given to this variant. The second variant resembles the first in having a total leukocyte count within the normal range, below it, or only moderately elevated, but there are no immature cells present and diagnosis from the study of the peripheral blood alone would be impossible. The somewhat paradoxical name "aleukemic leukemia" applies to this condition.

Subleukemic and aleukemic forms are more often encountered in the acute than in the chronic Lks and they usually become fully leukemic at a later stage in the progress of the disease. It is generally true that predominance of more mature cells is characteristic of Lks with clinically chronic symptomatology

and course, while more primitive, less differentiated cells occur in clinically acute Lks.

2.1.3.4. Preleukemic Leukemia

There are, furthermore, some cases of Lk in which, although the course is "chronic" (i.e., lasting a few to several years), the blood and bone marrow show primitive leukocytes in greater or less degree, with apparently little tendency to differentiate. These cases are "acute" in the histopathologic sense, but "chronic" from the clinical or temporal standpoint. Certain cases of the so-called preleukemic state fall into this category. In the "preleukemic status" there is generally a slow course with a relatively small proportion of leukemic cells in the bone marrow. The chief feature is usually anemia with or without leukopenia and thrombocytopenia. Although an unequivocal evidence of Lk may be lacking, primitive leukocytes, usually myeloblasts, may be seen in small numbers in the blood, in association with similar or somewhat higher concentrations of the same cells in the bone marrow. The spleen may be normal or slightly enlarged. There may be a relatively static course of two or several years. The term preleukemic status is probably

misleading since this is actually Lk, albeit the abnormal proliferation is spotty or of relatively slight degree and is apparently coexisting simultaneously with the normal cellular tissues. In other words, groups of abnormally growing, i.e., neoplastic leukocytes, may be present amongst a much larger group of normally growing leukocytes. It seems apparent that not all primitive cell (so-called acute) Lks overwhelm the host immediately. There may be some forms in which a small nest of abnormal leukocytic tissue proliferates, differentiates but little and eventually dies off. There may be others in which a relatively small group or "clone of primitive cells" continues to develop, retain a "foothold" in a small way, but does not become completely generalized. In such cases, the proliferating groups of cells may remain static for several years, eventually increasing in scope, either slowly, or rapidly.

From the theoretical standpoint, it should be possible to observe acute and chronic granulocytic, lymphocytic, monocytic and plasmocytic forms of Lk. However, chronic monocytic leukemia is rare, perhaps non-existant, and although there are variations in the type of cellular differentiations in multiple

myeloma, acute plasmocytic Lk has not as yet been described.

2.1.3.5. Varieties of Granulocytic Leukemia

The granulocytic leukemias (GLks) merit special attention in view of their rather marked diversity. "Chronic granulocytic leukemia", a relatively slow proliferative disorder of the bone marrow granulocytes, appears to be related to the more generalized myeloproliferative disorders, i.e., polycythemia vera and myelosclerosis-meyloid metaplasia. "Acute granulocytic leukemia (AGLk) may be (1) relatively pure or myeloblastic, showing an almost uniform proliferation of myeloblasts with a variable degree of maturation towards the myelocyte; (2) myelomonocytic, the blood showing a well-defined monocytosis, with the marrow showing, rather paradoxically, a marked degree of reticulo-endothelial proliferation; (3)or, a mixed erthyromyeloblastic proliferation, also called Di Guglielmo syndrome. In this form, there appears to be a simultaneous proliferation not only of myeloblastic cells, but erthyroblastic cells as well. This condition may begin with what appears to be a pure red cell proliferation (erthyremic myelosis), but gives way, as the

case progresses, to mixed erthroblast-myeloblast proliferation and eventually to an almost complete leukemic status.

These different forms of GLks — and there are others — indicate that Lk may not be as "pure" as it is customarily pictured. Thus, although it is the usual tendency to think of Lk as purely a disease of one of the white cell systems, there are actually a number of cases in which simultaneous proliferations of both white cells and red cells and, indeed, of megakaryocytes as well, may occur. Others may show pancytopenia, not only in the blood but in the marrow, thus resembling aplastic anemia rather closely; here the entire cellular content of the marrow may have been "insulted" by an unknown agent with a resultant simultaneous cessation in the growth of red cells, white cells and megakaryocytes. The bone marrow in such cases is "hypocellular", but myeloblasts are usually found in small clusters. Later, these cases usually develop outspoken Lk. Finally, reticulum cell proliferations may show a mixture of monocytic cells with plasmocytosis or lymphocytosis of varying degree and types (Dameshek and Gunz, 1964).

2.1.3.6. Leukemia and Leukosarcoma

In Lk there is a generalized proliferation of the white cell tissues but it should be recognized that localized forms of leukocytic proliferations also occur. These conform more closely to the usual conception of neoplastic disease than do the generalized proliferations. Localized forms, although they may occur in the course of the leukemic state, notably in myelomatosis and CLLk, are ordinarily seen as single or multiple tumours originating in various parts of the body, characteristically in lymph nodes. Since these tumours are leukocytic neoplasms of a non-epithelial type, i.e., sarcomas rather than carcinomas, they are designated as "leukosarcomas". As pointed out earlier, the dividing line between lymphosarcomatosis and Lk is often difficult, if not impossible, to define. Thus, certain cases of obvious CLLk may present large sarcomatous masses; conversely, what at one time appears to be an example of localized lymphosarcoma almost invariably becomes, in the course of time, a generalized proliferation of lymphoid tissue, i.e., an example of "LLks". Reticulum cell sarcoma, a primary neoplasm of reticulum cells, is at times associated with a leukemic picture in which the

predominant cell is the histiomonocyte. More often, however, particularly in the relatively chronic forms of reticulosis, the reticulo-endothelial cells of the bone marrow, lymph nodes, liver, spleen, etc., are extensively involved in a generalized proliferation, and the blood picture is relatively normal except for some degree of monocytosis.

Perhaps it would be more logical to classify leukocytic neoplasms, both of the leukemic and leukosarcomatous varieties, under the heading of myeloproliferative, lymphoproliferative, reticulo-proliferative and plasmoproliferative disorders. This would allow for a more flexible interpretation of a given case and might tend to develop more fundamental thinking regarding the biology of the leukocyte growth process. Thus, one could readily understand that the lymphocytic proliferation might at first be localized, initially perhaps in a single clone, then in a mass, finally becoming a generalized or leukemic process.

Certainly, Lk is by no means a single disease; it brings together under one roof a number of essentially different proliferative processes. Surely, there can be nothing more different than Lk of the

acute granulocytic and chronic lymphocytic varieties; to be sure, they both have high white cell counts and may both lead eventually to the death of the patient, but they seem to be etiologically, physiologically and in every other way, entirely different diseases. They are classified together because they represent neoplastic proliferations of white blood cell forming tissues.

2.1.3.7. Paraleukemic States

Under this convenient general heading may be grouped a number of conditions not specifically leukemic in nature, but bearing a close relationship to Lk. These include other proliferative disorders of hemopoietic cells, such as polycythemia vera and erthyremic myelosis, megakaryocytic myelosis and myelofibrosis, which exhibit a close parallelism to the leukocytic disorder of Lk and may develop into partially or even frankly leukemic states. Certain conditions of medullary aplasia must also be included here, since leukemias sometimes undergo an aplastic change and apparently pure aplasias sometimes become unequivocally leukemic.

2.2. LEUKEMIA IN SPECIES OTHER THAN MAN

2.2.1. BIRDS

Cases of lymphogenous Lk have been described in turkeys (Reinhardt, 1925; Cohrs, 1927; Becker, 1928; Jármai, 1929), geese, ducks, and pigeons (Lund, 1926; Haupt, 1928), swans (Geurden, 1934), storks and parrots (Fox, 1923), canaries (Satterlee, 1906; Haupt, 1928), and vultures (Payer, 1935). These cases permit the supposition that Lk may be found in most kinds of birds, but no more detailed investigation of its specific incidence has been made. The knowledge of bird Lks is dominated preponderantly by those of domestic fowls, which have been studied by many workers and used for innumerable experiments.

Fowl Lks have been known very much longer than Lks in other sorts of birds. The first reported case, one of "lymphosarcomatosis", was published by Roloff in 1868. Caparini (1896) described 3 cases of enlarged fowl livers and diagnosed these as leukemic, while a year later, Moore (1897) reported an "infectious leukemia" in fowls. Many cases of fowl Lk were reported during the early 20th century

(Butterfield, 1905; Koch and Rabinowitsch, 1907; Kon, 1907; Warthin, 1907; and others), but the research into fowl Lk was directed in 1908-1909 by Ellermann and Bang, whose discovery that the disease can be transmitted to healthy fowls by intravenous or intraperitoneal inoculation with blood or emulsions of organs from diseases birds, and also by cell-free filtrates, set up a landmark in Lk research. In the course of many years Ellermann collected a large number of cases of fowl Lk occurring spontaneously, and he demonstrated that several types appear with varying frequency. In 1918 he classified these as (1) lymphogenous or 'lymphatic leucosis', (2) myelogenous or 'myeloic leucosis', and (3) anemic forms, or 'leukanemias', which he later altered to "erthyroleukemia" or 'erthyroleucosis'. Ellermann's classification has held good in principle.

The studies of this worker were the impulse which quickened investigations into Lk in many different parts of the world, and there is now a considerable amount of material illustrating the occurrence of these diseases. Several investigators (Ellermann, 1922; Lund, 1926; Mathews and Walkey, 1929; Schaff, 1936) have reported that the lymphogenous

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leukemia is by far the most frequent form of spontaneously occurring disease in fowls, while MLk is of rather infrequent occurrence. Also erthyroleukemia occurs somewhat rarely, but has been observed by most workers on fowl Lk. This last form seems to be peculiar to birds. It has been compared with various conditions in mammals and human beings, pernicious anemia (Ellermann, 1921; McGowar, 1926); erthyroblastosis congenita (Furth, 1931; Engelbreth-Holm, 1933), but none of these comparisons have been justified. The reason for including this disease among the fowl Lks is solely that it is so closely connected with MLk, of whose nature there is no doubt, that the two manifestations are not infrequently found mixed in the same bird.

2.2.2. MAMMALS

2.2.2.1. Introduction

Just as Lk is known in many kinds of birds and may presumably occur in all of them, so Lk conditions have also been found in various kinds of mammals.

Leisering (1858) published a case of LLk in a horse only a few years after Virchow (1845) had

defined Lk as an independent disease in man. Cases of LLk in monkeys were described by Massaglia (1923) and Fox (1923). Siedamgrotzky (1871), Lellmann (1896) and Allen (1901) described cases in cats, Fox (1923), in sea-lions, Finzi (1913), Klügel (1919), Habersang (1924) and others in horses, Nortmann (1938) in buffaloes, Zschocke (1914), Salomon (1932) and Czymoch (1937, 1938), in deer (elk, roe-deer, and fallow deer), Lund (1926, 1927) and Kitt (1931) in sheep, Avérous (1896), and Krause (1921) in goats, Jacob (1908) in elephants, and Fox (1923), in opossums. All the cases seem to have been lymphogenous type, although MLk is also said to have occurred in horses (Jármai, 1934).

2.2.2.2. Domestic Animals

More detailed information is available about Lk in dogs, pigs, cattle and, in particular, rodents, among which mice are of special interest, inasmuch as the study of Lks in mice has contributed considerably to the understanding of the nature of these diseases. Also, in rodents, true Lk has been recorded with certainty in rats (Bullock and Rohdenburg, 1917; Bullock and Curtis, 1930; Wilens and Sproul, 1936), and in guinea pigs (Snijders, 1926).

Since the last decade, because of the differences between human and animal Lks, and because the cells which appear in the blood of animals with the disease are so variable in type and numbers, the term Lk tends not to be used in veterinary pathology. Instead, the term 'leukosis' is used to cover a wide range of disorders characterized by leukemic infiltration in various organs. Indeed, the term leukosis is comparable to the condition of aleukemic Lk in man. In many respects, leukosis in animals may also be compared with lymphosarcoma in man.

Lymphatic leukosis, in cattle, is a sporadic disease which may appear as enzootic disease in some areas. It affects cattle in which it is invariably fatal. The animal develops fever and enlargement of superficial and visceral lymph nodes. The diseased animal is anemic and the blood count often shows the presence of excessive numbers of lymphocytes and lymphoblasts, so that it could reasonably be called LLk. Bovine Lk, with leukemic cells in the blood, is sometimes seen in the fetus, but this is practically always associated with the disease in the dam. In human Lk, on the other hand, transmission from mother to fetus has never been conclusively shown to occur,

nor, for that matter, has Lk ever occurred in man following the transfusion of leukemic blood (Gresham and Jennings, 1962).

Leukemia in the horse is a rare disease and most closely resembles LLk in man. In the pig, however, the disease is usually of a form best classified as a lymphosarcoma and in complete contrast to the equine condition this disease is probably the commonest porcine neoplasm. The closest human counterpart of this disease is the condition called Hodgkin's disease, which is a rare disorder in man, and affects young adults chiefly.

2.2.2.3. Mice

The first description of Lk in mice was given by Eberth (1878). The mice concerned were, like the rats, lab animals — generally albinos. Further cases in lab mice were reported by Tyzzer (1907-1908), Haaland (1911), von Gierke (1914), Levaditi (1914) and others, but it was not until the 1930's that any real advance in the study of the disease was made. Cases were not available for investigation in large numbers until pure inbred strains of mice were employed.

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There is now general agreement that the great majority, if not all, of mouse Lks are genuine malignant tumours (Dameshek and Gunz, 1964). Thus,

(1) mouse Lk cells fulfil many of the morphologic and cytologic requirements of malignancy; they show an increased cell and nuclear size, increased proliferative and infiltrative activity, form tumours and can be transplanted (Lewis, 1935).

(2) Mouse Lk can be induced in high or low Lk strains by means of irradiation with x-rays (Furth and Furth, 1936; Henshaw, 1944; Kaplan, 1947), nuclear particles (Upton, Furth and Christenberry, 1954), by the application of carcinogens (Rask-Nielsen, 1949) or of estrogenic hormones (Gardner, Dougherty and Williams, 1944). It thus resembles many neoplasms which can be established by the same means.

(3) Mouse Lk can be temporarily inhibited by many of the agents (ionizing radiations, alkylating agents and certain steroidal hormones) which are successful with definite neoplasms.

In the mouse, Lk occurs spontaneously in almost all forms which occur in man, with the possible exception of CLLk (Furth, Ferris and Reznikoff, 1935). The lymphocytic forms are far more common than

the granulocytic, and most of them are subacute rather than acute (Kaplan, 1954). Dunham and Stewart (1953), in a valuable survey of transplantable and transmissable animal tumours, listed 37 examples of lymphomas and Lks known to be established in mice at that time. Again the majority of these were lymphocytic, 29 being included under the heading "lymphosarcoma or LLk" or both, in most instances. The remaining 8 tumours were composed of 4 MLks, 2 reticulum-cell sarcomas, a reticulum-cell Lk, and a plasma-cell Lk.

The general picture of Lk in the mouse is a predominantly lymphocytic disease, and LLk and lymphosarcoma are peculiarly closely related and often almost indistinguishable, particularly as far as transplantable tumours are concerned. A considerable proportion of experimental work in this field in the past has involved lymphosarcoma rather than Lk, with invasion of blood and bone marrow usually a minor feature (Kirschbaum and Strong, 1939; Kaplan, 1947), and even when blood and bone marrow involvement has been conspicuous, massive local tumour formation at the site of inoculation, or in lymph nodes, with areas of necrosis and marked histological invasiveness recall

the morphology of lymphosarcoma in man rather than that of either acute or CLLk. Nevertheless, Lk is now generally accepted as an appropriate name for these transplantable mouse tumours, although workers have usually been careful to emphasize the differences between the experimental mouse Lks and the disease in man, and to point out that "leukemic grafts and the animals bearing them are not exact analogues of either the growth or the hosts in spontaneous disease" (Hauschka and Furth, 1957).

Susceptibility to Lk in the mouse is unquestionably strongly dependent upon genetic constitution. Long continued pedigree inbreeding has produced strains of mice of uniformly high or low Lk incidence, corresponding to the uniformity of their inbred, genetic make-up. In the much studied high-leukemic AKR strain, for example, the disease develops invariably at about 6 months to a year, and the great majority of AKR mice that escape an earlier death from pneumonia and survive to this age, die from Lk. Similarly, the C58 strain has a leukemic incidence of 90%, while even the 10% failing to develop the disease yield, when mated, offspring with the same high Lk rate as offspring from leukemic C58 parents. Crossbreeding experiments, using

high-leukemic C58 males and low-leukemic females of the STOLI strain, and backcrossing the first hybrid generation males with STOLI females, produced families of the second generation having wide differences in Lk incidence, ranging from 0 - 43% (MacDowell and Richter, 1935). Such variations are most suggestive of the reassortment of more than one gene influencing susceptibility to Lk, since, if only a single gene were involved, the second hybrid generation might be expected to show a bimodal pattern of incidence, according to whether the gene had or had not been transmitted, rather than the continuous variation in fact found (Law, 1954). Moreover, the pattern of Lk incidence in those families exhibited the diversity to be expected as a result of gene redistribution, rather than the uniform moderate incidence which would probably result from breeding with a low-leukemic strain if the high C58 incidence were originally due to a non-genetic transmissable pathogen, such as a cytoplasmic virus (Law, 1957). This is not, of course, to say that genetic factors are alone responsible for the development of Lk in high-incidence strains of mice, but merely that genetic constitution influences susceptibility to the disease.

By means of strict inbreeding it is possible to obtain many different strains of mice differing in the frequency and the type of Lk. By brother and sister mating, lines of animals can be established which can then be carried through numerous generations. Evidence that such lines are genetically nearly "pure" can be demonstrated by grafting blood cells or tissue fragments from spontaneously occurring cases of Lk into unaffected members of the line. Grafts thus transferred will grow in the host in precisely the same way as in the donor animal and can thence be transmitted to further animals of the same strain. The one prerequisite for grafts to take is genetic compatibility, although transfers will, occasionally, be successful in a certain proportion of hybrids obtained by crossing the donor with an unrelated strain. It is, however, possible to modify foreign, unrelated strains by methods such as x-irradiation so that they will then accept transplants (Furth, Seibold and Rathbone, 1933). Lks so established in a foreign strain remain grafts which cannot be transmitted to normal untreated animals of the host strain; they can only be transmitted back to the donor strain with which they are genetically compatible.

The possibility of obtaining pure lines of Lk-bearing mice and of transplanting spontaneously-occurring Lk to other compatible animals makes mouse Lk a valuable tool for studying the properties of leukemic cells and the etiology of the disease.

2.3. RELATIONSHIP BETWEEN HUMAN AND MURINE LEUKEMIAS

If the neoplastic nature of mouse Lk is conceded, there still remains the question how far it can be equated with human disease. Cytologically, the similarity between leukemic cells in the two species is rather striking, although such resemblances are seen chiefly with the ALks of childhood. The clinical course in acute human cases and that seen in those with a slower rate of progress, are repeated in mice, and the pathological picture is amazingly uniform in both. The same forms of Lk are found — lymphatic and myelogenous most frequently, but monocytic Lk and atypical forms, e.g. cases characterized by megakaryocytic elements, are more rarely seen in mice than in man. Also, there is the same aggressive tumour-like growth and in both men and mice, the invasion of the blood stream by leukemic cells is often a late phenomenon.

Another strong point in favour of the essential similarity between the two species is the fact that many therapeutic agents are equally effective in man and mouse. Examples are x-rays, arsenic, benzol, urethane, nitrogen mustards and their analogues, steroids, and folic acid antagonists (Burchenal, 1954). Many of the cpds now used for the treatment of human Lk were first shown to be effective in mice and then used in clinical work. Mouse Lks, like human ones, differ greatly in the sensitivity which different types show to different agents; again they become resistant to treatment just as do their human counterparts.

While there thus exists a close co-relation in the therapeutic responses of human and rodent Lks, it must be recognized that this is almost entirely confined to the more acute types which occur especially in childhood and young adults. It is much more difficult, on the other hand, to see a likeness between mouse Lk and the chronic human Lks. During most of their course, these are characterized by the presence of cells which are for long periods quite normal in appearance and do not as a rule respond to the same therapeutic agents as the acute varieties.

In spite of such a well-known phenomena as the terminal transformation of CGLk into an acute (myeloblastic) form, it may well be questioned whether CLks are really of precisely the same nature as the acute forms, and hence as the mouse Lks. On the other hand, the mouse may possibly be better equated with the infant or the child, in which the CLks, particularly of lymphocytic variety, are unusual.

Leukemia in mice behaves nearly always as an autonomous neoplasm. The fundamental neoplastic change appears to be in the cell itself which for various reasons has acquired malignant properties, so much so that the disease can be transmitted by implanting a single leukemic cell into a normal host (Furth and Kahn, 1937). Once the malignant transformation has taken place, these cells appear in no way amenable to restraint or control by any physiologic action. While some of the more acute human Lks appear to be similarly unrestrained, others would seem still to be subject to some control. Such types show cells which, morphologically at any rate, can be distinguished only with difficulty or not at all from normal cells. They may be apparently dormant for a long period in a pre-leukemic phase (Block, Jacobson and Bethard, 1953;

Gunz and Hough, 1956), and they are subject to remissions occurring either spontaneously or as a result of treatment. Cases of this type are often GLks (Dameshek and Gunz, 1964).

In summary, it would seem that Lk in animals, especially in mice, is the same disease as Lk in man. The peculiar features presented by the disease in some species, especially birds, are more reasonably explained as being due to the special characteristics of these species than to the possibility that fowl Lk, for example, is a disease of an entirely different nature from Lk in other animals or in man. Further evidence for this will be presented later, c.f. section 2.4.2.1.).

With the solution of the problem of human Lk as the final goal, the study of the nature of the disease and of its various features in animals must therefore be regarded as abundantly justified, inasmuch as the results attained are likely at the same time to elucidate the corresponding features of Lk in man.

2.4. THE ETIOLOGY OF LEUKEMIA

2.4.1. GENETICS

It is quite established now that

genetic factors influence the development of spontaneous and induced mouse Lk. However, leukemogenic agents also include ionizing radiations, chemical carcinogens, and estrogenic hormone. Certain influences (e.g., administered E) may modify the growth of leukemic cells, although the initiation of the disease is not inhibited. Other factors such as caloric restriction, administered androgen, thymy, may actually inhibit leukemogenesis (Furth and Furth, 1936; Mider and Morton, 1939; Gardner et al., 1944; Murphy, 1944; Saxton, 1944; Furth and Boon, 1945; MacDowell, 1945; Kaplan, 1950; Kaplan, Brown and Marder, 1951; Tannenbaum and Silverstone, 1953; Woolley and Peters, 1953; Kirschbaum, 1957).

More and more investigators, conscious of the species variations and inhibited by the unavailability of the human experimental subject, have sought the answers to basic questions regarding the etiology and mechanisms of disease production for man by studying close parallels in animal systems. Nowhere is this more evident than in the field of experimental oncology.

It has been possible, by means of inbreeding, to obtain various strains of mice which

differ in the frequency with which they develop Lk, and in the type of disease which occurs in them. The predisposition of Lk in these strains has been thought to be hereditary in character, although this statement has had to be modified in the light of recent work on viruses. It has also been shown that mice that have a low rate of spontaneous Lk are highly susceptible to the development of the disease following the exhibition of various extraneous agents like x-rays (Kirschbaum and Mixer, 1947), and that thus not only the occurrence of spontaneous but also that of induced Lk may be under genetic control. Considerable work has been done on the complexities of the hereditary factors which are operative in mouse Lk (Kirschbaum, 1951). This problem has been discussed earlier (see section 2.2.2.3.).

In man, it is much more difficult than in mice to prove that heredity is concerned in the etiology of Lk. Whereas mice can be bred in genetically almost pure lines, such experiments would be impossible in the case of human material. Lk is, therefore, found in a genetically quite heterogeneous population, and the relative variety of the disease makes the analysis of hereditary factors difficult.

2.4.1.1. Familial Leukemia

From the earliest days, instances have been reported in which more than one case of Lk occurred in the same family. Videbaek, in 1947, reviewed the previous literature and accepted 26 as authentic cases of "familial leukemia" up to that year. He himself reported a further 17 cases of familial leukemia, and a considerable number have been added since then (e.g., Riel, 1948; Gross and Matte, 1948; Barbier, Guillermet and Morel, 1949; Lentz, 1949; Partman and Robinson, 1951; Bieber, 1951; Debré, Bernard and Buhot, 1951; Anderson, 1951; Ward, Galinsky and Newton, 1952; Johnson and Peters, 1957; Bowie, 1958; Herrell, Ruff and Bayrd, 1958; Videbaek, 1958). Summing up, Dameshek and Gunz (1964) stated that well over 100 authentic cases of familial leukemia have been reported, while there must be much larger numbers which have failed to reach the literature.

That familial leukemia occurs is therefore not to be disputed. It is, however, equally certain that such cases are to be expected as the result of the operation of chance alone, and the only manner in which heredity can be indicated as an etiologic factor in human Lk is by means of the demonstration

that the incidence in the families of patients with Lk is significantly higher than that in a control material. Videbeck (1947) found among 209 patients with Lk, 17 (8.1%) with a family history of other cases of Lk. Among 200 controls there was only one case with a similar history, an incidence of 0.5%. The author concluded that familial Lk was significantly increased among his patients, and that Lk was therefore under, at least partially, hereditary control. Several investigators (Busk, 1948; Garer, 1949; Engelbreth-Holm, 1954; Steinberg, 1957, 1960), however, have challenged Videbaek's data as well as conclusions, and in particular, the selection of his patients and the controls. Busk (1948) considered Videbaek's findings as an unusual predominance of Lk and cancer in the families of Lk patients and regarded the data statistically invalid. Gunz (1961), on the other hand, has produced results comparable to those of Videbaek. Razis, Diamond and Craver (1959) stated that the discrepancy lies in the fact that the leukemic patients or their close relatives tend to be better informed about other similar cases in the family than the control group or the patients with other diseases. Because of these considerations, and because of several other

largely negative investigations (Amiotti, 1953; Schoenbauer, 1953; Kaliampetsos, 1954; Guasch, 1954; Morganti and Cresseri, 1954; Steinberg, 1957, 1960; Razis et al., 1959), it must be considered that a general familial etiology of Lk has not been shown.

The great majority of cases of familial Lk have, for instance, been reported in patients with CLLk or lymphosarcoma (Jelke, 1940; Razis et al., 1959). Although more than one type of Lk may occur in the same family, multiple cases of CLLk are commonest, CGLk is rather rare, while ALk occupies an intermediate position. There is, furthermore, some evidence that cancer, though not unduly common in the families of Lk patients as a class, might be slightly increased in incidence in those of patients with CLLk or lymphosarcoma (Morganti and Cresseri, 1954; Gunz, 1961).

The presence of a hereditary factor in Lk is certainly most suggestive in those instances in which multiple cases have occurred in close relatives. Thus, 3 brothers with CLLk have been reported by Reilly, Rapaport, Karr, Mills and Carpenter (1952); 2 sisters with CLLk and a third with CGLk by Hornbaker (1942); 3 siblings and 1 paternal cousin with LLk by

Boggian (1938); 4, and possibly 6 cases in two generations by Weiss (1927), and Decastello (1939); 4 of 12 siblings with ALk by Johnson and Peters (1957), and most remarkable of all, by Anderson (1951), LLk in 5 of 8 siblings. To this must be added a family observed where the father, the father's identical twin brother, and 1 son all developed CLLk at approximately the same age (Gunz and Dameshek, 1956). It should be noted, however, that the great majority of cases were chronic or acute Lks.

2.4.1.1.1. Leukemia in Twins

It might be expected that an investigation of twins would be able to throw some light on the heredity of Lk as it has done on that of other diseases. Thus, Lk in identical twins was first reported by Dameshek in 1929 (Dameshek and Gunz, 1957), and later a total of 14 pairs of identical and 3 pairs of non-identical twins has been reported with Lk in both siblings (Guasch, 1954). The maximum interval between the onset of the disease in the first and the second twin was 18 months; among the identical twins, 8 pairs had the same type of Lk, all either chronic lymphocytic or acute, and 6 had dissimilar types. Among the 3 pairs

of non-identical twins, 2 had the same type of Lk. There are, however, other reports in the literature (e.g., by Willi, 1936; Kellett, 1937; Hitzig and Rampini, 1959; Osgood, 1960) on Lk in only one of a pair of twins, and Steinberg (195-) mentions no less than 9 sets of twins, 3 identical and 6 non-identical, in all of whom only one twin contracted the disease. One may conclude that 'concordance' (when both members of the pair are affected) does not necessarily point to heredity as a determining factor, for external factors might also be of great importance when 2 patients share a common environment intimately, as is the case with most twins.

2.4.1.2. Leukemia in Mongols

Perhaps the strongest evidence of possible association of genetic factors with the occurrence of Lk is the greatly increased incidence of Lk in children showing the stigmata of mongolian idiocy and indeed in mongols of all ages (Wald, Barges, Li, Turner and Harnois, 1961). It has been shown (Krivit and Good, 1956, 1957; Merrit and Harris, 1956; Sutow and Welsh, 1958; Stewart, Webb and Hewitt, 1958) that Lk is up to 20 times commoner in this group than in

the general population, and also that among the very rare children with 'congenital' Lk are some mongols (Bernhard, Gore and Kilby, 1951). This association could occur either because mongolism predisposes to Lk or because both conditions have a common etiologic background. There is now evidence that both explanations may be correct (Dameshek and Gunz, 1964). It has already been reported that the chromosome complement of mongolian idiots is abnormal (Lejeune, Gautier and Turpin, 1959; Jacobs, Court-Brown, Baikie and Strong, 1959).

An alternate theory explaining the increased incidence of Lk in mongols is that both conditions are caused by the same external factor, called the 'agent', and it has to initiate Lk at perhaps the 6th to 9th week of gestation, and the disease would then have to lie dormant for period up to 6 or 9 years before producing any clinical symptoms (Dameshek and Gunz, 1964). X-rays have been considered as a possible causative factor, but there have been few, if any, cases reported in which intrauterine irradiation had been given to a leukemic mongol (Stewart el al., 1958).

2.4.2. VIRUSES

Time and time again, especially during the period around the turn of the century, all sorts of bacteria, fungi, and the like were said to cause cancer, largely, often solely, on the strength of their presence in the tumours. None of these theories was long-lived, however, for it is one thing to find bacteria in tumours and quite another to demonstrate that they caused the tumours. As a result, there was a period in which some researchers frowned on suggestions that viruses might play a role. The science of virology has come a long way since those days.

Viruses lie at the threshold of life. They are parasites that cannot multiply in any environment except living tissue. They are capable of doing damage out of all proportion to their submicroscopic size. That they are indeed on the borderline between the living and the non-living was proved by Dr. Wendell Stanley who won a Nobel prize for his classical experiment with a virus that causes the so-called mosaic disease in tobacco plants. These virus particles were isolated as crystals, which seemed to have no more life than there is in a lump of coal, but when these same crystals were rubbed into the leaves of a tobacco plant, the

virus sprang to life again. Though small and less complex than a plant or an animal cell, viruses have one essential thing in common with cells, in that the core of every virus particle contains nucleic acid (DNA or RNA).

In spite of the many advances made in recent years in knowledge of what viruses are and what they do, the task of discovering how they cause disease is difficult, for viruses do their damage inside cells. Some viruses, instead of killing cells, may do just the opposite: cause them to proliferate. Also, some viruses that kill cells under some circumstances may cause proliferation under others. This could explain why viruses might cause cancer.

Do viruses cause human cancer? The only answer that can be given at present is that they are responsible for such a variety of cancers in animals, that it would be surprising if they did not cause some types in man, for the basic phenomena of life do not differ very much from one species to another.

Since the turn of the century, support for viruses as specific etiologic agents of Lk, as well

as of neoplasms in general, has had a rather precarious course. In 1908 Ellermann and Bang proved that leukosis of domestic fowls could be transmitted by means of the inoculation of cell-free extracts and thus propagated through several generations of birds. These observations were confirmed by the discovery of Peyton Rous in 1911 that a chicken sarcoma could also be induced by what was presumed at that time to be a virus. In fact, Lks and lymphomas occur commonly in domestic fowl. There is some similarity between the Lk-lymphoma complex in fowl and the same spectrum of diseases in man, which lends significance to the question of whether there are just a few fowl leukosis viruses, each of which can produce a wide range of disorders, or a large number whose individual range of effects is limited.

Whereas the leukosis of fowls was thus clearly demonstrated as virus-induced, attempts to transmit mammaliam Lk by means of cell-free extracts remained unsuccessful for many years, until Gross showed, in 1951, that transmission is possible provided newborn animals are used as hosts. This discovery has since been repeatedly confirmed and has led to a great upsurge of interest in this field. The experiments of

Stewart (1960), and Stewart, Eddy and Stanton (1960) showing that a single virus can produce some 20 different tumours in mice was especially effective in this regard. However, the evidence purporting to show that viruses may be involved in human Lk is so far tenuous. Developments in this field of research are extremely rapid at this moment, and there are many findings whose significance cannot as yet be precisely evaluated.

2.4.2.1. The Gross Virus

The early experiments of Gross were carried out with two inbred strains of mice, Ak and C3H. The former of these has a very high incidence of spontaneous Lk, about 85% of all animals succumbing in middle age to a rapidly growing LLk which shows its most pronounced lesions in the thymus. Like many other murine Lks, it has the characteristics of a lymphosarcoma whose cells eventually invade the blood stream. The C3H strain has a spontaneous Lk rate of 0.5%. Gross succeeded in transmitting Lk from Ak donors to C3H recipients. In his early work he used newborn (less than 1ϕ hours old) C3H mice and inoculated them with suspensions of leukemic Ak cells. Tumours appeared at the site of inoculation within 2 or 3 weeks, and were usually followed within another week or two

by a generalized Lk. These tumours could be transplanted back to normal Ak but not to adult C3H mice; they were therefore merely grafts, the very young C3H mice serving as a medium for the growth of the implanted Ak leukemic cells.

The next step in Gross's experiments was the inoculation of cell-free extracts into newborn C3H mice. The extracts were prepared from leukemic Ak organs and were either centrifuged at very high speeds (105,000 g) or passed through selas porcelain of Berkfeld filters. No immediate effect of these inoculations appeared, but after an interval averaging 10 months, 28% of the C3H mice developed Lk. In contrast to the transplants mentioned above, the leukemic tumours produced in this fashion could be transplanted to further C3H mice. It therefore appeared that the Lk in these animals had been newly induced and not merely grafted. Besides transmitting Lk through filtrates of plasma and organs of leukemic animals, it was also possible to do so by using filtered saline extracts of ground-up apparently normal embryoes of the high Lk strains Ak and C58.

The author concluded that mouse Lk is caused by a filterable, thermolabile agent which is

transmitted in certain families of mice from one generation to another directly through the embryoes (Gross, 1951, 1953, 1955, 1957).

According to Gross's concept, the mouse agent might remain latent for long periods in any one individual, often even throughout its whole lifetime. During this period the host would remain in perfect health. To make the agent active, "triggering" would be necessary. This could be provided by the activity of either intrinsic (hormonal, metabolic) or extrinsic (ionizing radiations, chemical carcinogens) stimuli; as a result the agent would "change into a formidable pathogen, causing rapid multiplication of cells harboring it and killing its carrier. In low-leukemic strains of mice, however, activation of the agent would not occur. The host would remain in good health, even though they would carry the agent and transmit it to their offspring" (Gross, 1958, 1959a, 1960).

In spite of some initial contradictory reports, which were traced back to differences in the strain of mice used and experimental techniques, Gross's results were later amply confirmed, although

in some cases with somewhat different interpretations (Dmochowski, 1959). Confirmation came from Stewart (1953), Furth, Buffett, Banasiewicz-Rodriguez and Upton (1956), Woolley and Small (1956) and Woolley (1958).

2.4.2.2. Leukemogenic Viruses Other Than The Gross Virus

Besides the mouse Lk agent described by Gross, at least four others have been discovered in the past few years.

(1) An agent obtained by Graffi (1957, 1958) from a variety of spontaneous mouse tumours (not Lk), especially the Ehrlich carcinoma. This produces an unusual form of myeloid ("chloro") Lk which can be transmitted by cell-free filtrates to several strains of mice, as well as to rats (Graffi and Gimmy, 1958). Virus-like particles have been found by electron microscopy in the organs of infected mice (Graffi, Heine, Helincke, Bierwolf and Randt, 1960).

(2) An agent similarly derived from the Ehrlich carcinoma of Swiss mice by Friend (1957) and causing in the same strain a Lk, interpreted as being of reticulum cell origin with an erthyroblastosis (Metcalf, Furth and Buffett, 1959).

(3) An agent causing a rapidly progressing lymphosarcoma in Swiss mice and derived from a spontaneous Lk in the same strain (Schwartz, Schoolman, Szanto and Spurrier, 1957; Schwartz and Schoolman, 1959).

(4) An agent derived from mouse sarcoma 37 and causing LLk in a wide variety of inbred as well as in Swiss mice (Moloney, 1960) and in rats (Moloney, 1960a). This agent has been propogated serially in vitro (Manaker, Strother, Miller and Piczak, 1960).

These agents appear to be distinct from the Gross agent and from each other. They not only produce distinctive macroscopic and microscopic lesions in strains of animals most of which will not support the growth of the Gross agent, but they can also be transmitted with ease to adult animals.

While certain murine Lk viruses have been shown to be capable of infecting and causing Lk in rats (Gross, 1961a; Maloney, 1962), attempts to cross other species barriers have thus far been unsuccessful. In contrast, strains of Rous sarcoma virus (chicken sarcoma virus) have been shown to be capable of inducing neoplasms in mice, rats, hamsters, and

guinea pigs (Ahlström, Bergman, Forsby and Jonsson, 1963; Munroe and Sutham, 1964; Rabotti, Raine and Sellers, 1965), rabbits (Zilber, 1961), dogs (Rabotti, 1966), and even monkeys (Munroe, Shipke, Erlandandson and Windle, 1964). The versatility of the prototype of the RNA tumour virus group (Rouse sarcoma virus) has done much to reduce the controversy concerning the applicability of data acquired from studies of virus induced chicken neoplasms for animals and man.

Several investigators (Hanafusa, Hanafusa and Rubin, 1963; Harvey, 1964; Moloney, 1966; Huebuer, Hartley, Rowe, Love and Capps, 1968) have demonstrated the defectiveness of the sarcomatous viruses (Rous, Moloney, etc.,), and with the discovery of similarly defective mouse Lk viruses, the parallels between the avian leukosis group and the mouse Lk group of viruses appear to be on the brink of convergence. The availability of fibrosarcoma-inducing viruses related to the mouse Lk virus group invites speculation that species barriers may soon fall and that antigenic relatives of the mouse Lk viruses may now be capable of travelling the experimental route to other mammals as well as fowls. Up to now, these two groups of

viruses, avian and murine, have not revealed any antigenic relationship to each other. It is intriguing to speculate on the possibility that the defectiveness of Rous sarcoma and murine fibrosarcoma viruses may now make it possible for helper viruses of alternate species to assist in viral replication, and, by so doing, concomitantly introduce their own antigenic imprint. The implication of these findings and theoretical projections have obvious ramifications for the possible rescue of as yet unisolated Lk-associated viruses of domestic animals and man.

Virus-like particles resembling RNA tumour viruses have now been observed in Lks of cats (Jarrett, Crawford, Martin and Davie, 1964), dog (Chapman, Bopp, Brightwell, Cohen, Nielsen, Gravelle and Werder, 1967), and cow (Dutcher, Larkin and Tumilowicz, 1965; Dutcher, Larkin, Tumilowicz, Marshak and Szekely, 1966).

It is no longer disputed that viruses are the specific etiologic agents of certain animal Lks (Gross, 1961). While there is some evidence to suggest that similar agents probably play a role in human Lk as well as other neoplasms, no convincing

evidence has yet been obtained to satisfy the belief that what has been demonstrated in animals is also true for man. Attempts have been made for many years to transmit human Lk either to animals or to other human beings (reviewed by Falkner, 1938). The production of Lk by transfusions even of very large quantities of untreated human leukemic blood has, in fact, never been achieved, as was recently attested by Bierman, Byron, Kelly, Dod and Black (1957). Various hematologic abnormalities (leukemic, Lk-like) and tumours have been produced in chick embryoes, guinea pigs, mice and hamsters by the injection or implantation of filtered or unfiltered serum, plasma, blood or marrow cells from cases of human Lk, both acute and chronic (Torrioli and Torrioli, 1951; Magrassi, 1951; Magrassi, Leonardi, Negroni and Tolu, 1951; Mas y Magro, 1951; Giordana, 1952; Bergolz, 1960; Grace and Mirand, 1960). Some of these abnormalities have been serially passed through further embryoes or animals, and the authors concluded that the presence of a virus was proved by their experiments. It must, however, be emphasized that none of the lesions which were produced bore any real resemblance to the human Lks from which the material has been obtained.

Dmochowski (1964, 1966) has demonstrated the occurrence of virus-like particles in the tissues of human cases of Lk and lymphoma. Anderson (1965) has reported on the occurrence of similar particles in high-speed centrifugates of plasma from leukemic patients. Tissue cultures derived from human Lk cases (Moore, 1966) as well as from Burkitt lymphoma (Pulvertaft, 1964; Epstein, Henle, Achong and Bass, 1965) contained as yet unidentified Herpes-like virus particles.

The occurrence of a Herpes-like virus in continuous peripheral blood cultures established by different investigators in widely separated parts of the world is mystifying. Are these particles somehow related to the etiology of Lk and/or the Burkitt lymphoma, or are they just harmless passengers which have evolved or been selected quite by accident due to the development of a new cell culture technique?

The main argument against the significance of this virus in Lk is that it represents a morphologically and biochemically different virus type from those which comprise the RNA tumour virus group known to be the causes of avian and murine Lks and which occur in feline, canine, bovine and now human tissues as well.

The major problem in attempting to establish the viral etiology in man hinges upon the isolation of a candidate virus, reproduction of the disease in some suitable alternate host, perhaps the monkey, and some sero-epidemiological and electron microscopic evidence to unite these entities. Lk of the larger mammals are closer to the human Lk, but unfortunately they are far less investigated than avian or murine Lks (Boiron, Thomas and Mahouy, 1968).

During the last 10 years many reports have described particles that morphologically resemble viruses found in the blood and tissues of patients with Lk and other malignant processes (Dmochowski, Grey, Sykes, Shullenberger and Howe, 1959; Dalton, Potter and Merwin, 1961; Sorensen, 1961; Ota, Suzuki and Higashi, 1963; Almeida, Hasselback and Ham, 1963; Zucker-Franklin, 1963; Burger, Harris, Anderson, Bartlett and Kniseley, 1964; Porter, Dalton, Moloney and Mitchell, 1964; Benyesh-Melnik, Smith and Fernbach, 1964; Dmochowski et al., 1965; Sorensen, 1965; Harris, 1966; Levine, 1967; Viola, Dalton, Mitchell and Moloney, 1967; Tanaka, Bell and Brindley, 1967). In most studies the number of cases reported was small, there was little attempt at quantitation of the particles, and the techniques varied.

Most recently, Newell, Harris, Bowman, Boone and Anderson (1968), reported an extensive investigation of 'virus-like' particles in the plasmas of 225 patients with neoplasms of the blood-forming organs along with a group of controls. The authors used the term 'virus-like' for the particles on the basis of their centrifugation characteristics, rather than their electron-microscope morphology alone. The authors employed negative staining method for the particles which fall within the same density range in cesium chloride gradients as all known leukemogenic animal viruses and Herpes virus. There was no significant difference between control and disease groups in the type or number of particles seen. The authors concluded that the virus-like particles seen in the plasma of patients with Lk and allied disorders were unrelated to their disease. The source of the plasma virus-like particles remains unidentified. They were not related to the severity of illness, the white-cell count or the platelet count. Benyesh-Melnick et al.,(1964) and Prince and Adams (1966) have independently shown that cellular debris from whole blood can contribute particles to the plasma that are identical with virus-like particles.

No virus has yet been found that indubitably actuates tumours in man, yet this is not to say that viruses play no part in initiating them occasionally. Now and again a human cancer arises where a virus has persistently wreaked other cellular injury. The virus causing "fever-blisters" repeatedly next to the mouths of persons notably susceptible to its action provides an instance in point, cancer sometimes arising after a while from the epidermal tissue long kept in a disturbed state. One sees the same course of events occasionally on skin where the severe virus causing herpes zoster ("shingles") has left tissue permanently damaged. Yet these instances tell no more, as concerns causation, than do the cancers that now and then arise on the skin of old people where this was burnt in youth. Sunlight provides yet other examples, cancer arising from skin that it has kept inflamed instead of tanned. In all these instances the tumours have been merely initiated. No virus actuating any of them has been found. Experimental proof has, however, still to be brought forth and may be more difficult to obtain than seemed likely in the first flush of optimism which followed the discovery of the Gross virus, over a decade now.

2.4.3. RADIATION

The ionizing radiations constitute the only group of agents which have been unequivocally shown to be able to cause Lk in man as well as in experimental animals. When Lk is induced by radiation in man, the disease is usually of the myeloid variety, but in mice lymphoid tumours or LLks are nearly always produced, and only a few strains develop MLk with any frequency.

The application of x-rays or other ionizing radiations produce a characteristic series of changes in the blood of experimental animals and man. There is an almost immediate reduction in the number of lymphocytes; the granulocytes may show a brief rise, but then decline within one or a few days of exposure. The degree of depression in the leukocyte count is greatest and speed of recovery is slowest when doses have been given which will ultimately prove lethal. The effect of radiation on the red cell count is different from that on the leukocytes. There is an early diminution in the number of reticulocytes, with a recovery after varying time intervals. The red cell count itself, however, declines much more slowly, and anemia, if it occurs at all, becomes clearly measurable

only two or more weeks following irradiation, and is transient following all but the largest doses. The platelets show a transient or more prolonged fall which parallels that of granulocytes (Dameshek and Gunz, 1964).

The first clear experimental demonstration of the leukemogenic action of x-rays in mice was provided by Krebs, Wagner and Rask-Nielson (1930), who exposed 5,500 mice to sub-lethal doses and found the incidence of Lk among the irradiated mice to be 6 times that among controls. Furth and Furth (1936) confirmed these observations in a number of different strains of mice, using one or several doses of 300-400 r of x-irradiation. A great deal of later work from many centres (Henshaw, 1944; Kaplan, 1947, 1949, 1950; Upton et al., 1954; Mole, 1958; Upton, Wolff, Furth and Kimball, 1958; Mewissen, 1959) has shown that all types of ionizing radiation, including gamma radiation from radium, fast and slow neutrons from atomic reactors, and radiation from P^{32} are capable of inducing Lk in mice, although there are marked genetic differences in susceptibility in different strains. Other common lab animals, rats, guinea pigs and rabbits, appear to be

relatively resistant, to judge from the restricted studies carried out with these species.

Recently much effort has been spent in defining the details of irradiation, especially dose size and rate of administration and their influence in the induction of Lk. An important result has been the finding that the size of the dose is not the only factor determining the number of Lks induced under otherwise standardized conditions, but that the rate of administration and degree of fractionation of a given dose are also of great importance (Mole, 1960). Indeed, repeated exposures are more effectively leukemogenic than a single larger dose, and the induction rate increases with increase in total dose, but although lymphopoiesis may be depressed by doses as low as 25 r, Lk is rarely induced by doses much less than 200 r in sum. Whether the relation between dosage and Lk incidence is curvilinear or straightline at low levels has not apparently yet been established with certainty, although the studies of Kaplan and Brown (1952), and of Finkel (1958), suggest the existence of a minimal effective dose.

The incidence of radiation-induced Lk, like that of the spontaneous disease, may be influenced by several factors. Among these, thymy and cpd E administration inhibit leukemogenesis, while adrex increases susceptibility (Law, 1947; Kaplan, 1950; Woolley, 1950; Kaplan, Marder and Brown, 1951; Woolley and Peters, 1953).

2.4.3.1. Mechanism of Radiation-Induced Leukemogenesis

2.4.3.1.1. In Mice

The mechanism of Lk induction by radiation remains uncertain, and much of the work related to this problem has been done on Lks originating as thymic tumours, a common mode of onset of both spontaneous and induced forms of the disease in mice. Kaplan (1949), working with C57 black mice, found that irradiations of the upper half of the body led to the development of Lk in 4% of the mice, irradiation of the lower half in only 2%, while whole-body exposure induced Lk in 64%. The implications of this observation have been further clarified by the finding that the direct irradiation of the thymus, with the remainder of the body shielded, did not provoke Lks of thymic origin, while a protective effect against the leukemogenic

action of the whole-body irradiation could be achieved by shielding one hind leg (Kaplan, 1949, 1951; Kaplan and Brown, 1951). Similar inhibition can be brought about by spleen shielding (Lorenz, Congdon and Uphoff, 1953), or, even more remotely, by injecting homologous bone-marrow suspensions intravenously into unshielded totally irradiated mice (Kaplan, Brown and Paull, 1953). A close relationship must exist between these inhibitory effects of organ shielding or tissue injections on leukemogenesis and the widely studied protective action of organ shielding and injection of spleen and marrow homogenates against acute radiation injury and death in mice. The intimacy of this relation is emphasized by Cole, Nowell and Ellis (1956), who found that x-irradiated mice, protected from acute radiation death by intraperitoneal inoculation with homogenates of isologous mouse splenic tissue, showed a marked decrease in lymphoma incidence as compared with non-irradiated control mice, whereas in the experiments of Furth, Upton, Christenberry, Benedict and Moshman (1954), unprotected survivors of the same strain of mice (LAF_1), after exposure to radiation from a nuclear detonation showed an increase in both thymic and generalized lymphomas. It seems reasonable to conclude that the spleen homogenates provided protection against both the short-term

danger of acute radiation death and the long-term risk of developing Lk or lymphoma.

In all the investigative work, organs whose shielding has proved protective and tissues which enhance survival are invariably rich in hemopoietic elements and primitive multipotential cells. Injections of thymus cell suspensions have failed to afford protection from acute radiation death, nor do they promote the regeneration of thymus and lymph nodes after radiation injury, as bone marrow and spleen cells do (Brown, Hirsch, Nagareda, Hochstelter, Faraghan, Toch and Kaplan, 1955; Cole and Ellis, 1955). Cole et al., (1956) therefore argued that induced thymic regeneration in irradiated mice protected by bone-marrow injection is the result of an indirect mechanism secondary to the establishment of the marrow graft. A similar indirect mechanism might also inhibit the later production of Lks and thymic lymphomas. Certainly, the efficacy of partial body shielding and tissue transplantation in preventing radiation leukemogenesis is difficult to explain on the basis of simple repopulation of damaged areas, since the majority of tumours arise in the thymus rather than in the spleen or bone marrow, and there is no evidence of thymic repopulation by cells from the graft.

The weight of the evidence at present suggests that protection from radiation death can be achieved by shielding hemopoietic tissue during the period of exposure, or later injecting hemopoietic cells, so that the multiplication of the shielded or injected cells can bring about replacement of the blood-forming tissues destroyed by radiation.

One of the most intriguing discoveries was the finding that removal of the thymus lowered the susceptibility to the induction of lymphoma, while splenectomy lowered the susceptibility to GLk (Upton et al., 1958). Transplantation of unirradiated thymus into irradiated thymectomized mice caused lymphoma to arise in the graft. This indirect action of x-rays points to a humoral (or possibly viral) agent effective in radiation leukemogenesis and must be accounted for in any explanation of its mechanism.

Mice of the RF strain treated with small amounts of whole-body x-irradiation have shown a marked increase in the incidence of MLk (Upton, Jenkins and Conklin, 1964), a disease that closely resembles CGLk of man in its clinicopathologic expression. Radiologic and host factors influencing the development of

this disease have been described (Upton et al., 1964). Upton, Jenkins, Walburg, Tyndall, Conklin and Wald (1966) have discussed the induction of this and other Lks in RF mice by radiation, chemicals and viruses, and have made some preliminary observations on the influence of microflora.

Previous experiments have suggested that radiation-induced MLk has a complex etiology, involving radiation, virus, and other factors (Upton et al., 1966). The reduction in the incidence of MLk associated with a germ-free environment does not appear to be related to the absence of an essential virus component. Virus-like particles have been observed in leukemic tissue of germ-free mice, including RFM (Walburg, Upton, Tyndall, Harris and Cosgrove, 1965), and other strains (Kajima and Pollard, 1965). These observations suggest that Lk viruses are transmitted from parent to offspring in a manner which does not permit separation of virus from offspring by the "hysterectomy-caeserian section" technique used to develop germ-free animals.

The pathogenesis of radiation-induced MLk in RF mice remains unclear, but the available

evidence suggests that Lk virus is essential for the development of the disease and that the virus is present in both germ-free and conventional mice. It is tempting to speculate that radiation releases the virus, as has been demonstrated in other strains of mice (Haran-Ghera, 1966; Haran-Ghera and Kotler, 1967) and that this occurs in both germ-free and conventional animals.

Finally, Kaplan (1967) in an elegant review on the natural history of the murine Lk, stated that mice of C57 black and other low-Lk strains normally harbor a latent leukemogenic virus, which is probably transmitted vertically prior to the time of intrauterine implantation, and which can persist in the tissues of such naturally infected strains throughout life without producing any discernible ill effects, unless the animals are exposed to appropriate doses of irradiation or certain chemical agents, which appear to trigger a change in the host-virus relationship leading to the development of Lks.

2.4.3.1.2. In Humans

To summarize what is known about the relationship of human Lk to ionizing radiation, it is

unquestionable that radiation can cause and has caused Lk. Some hundreds of reasonably well-authenticated cases were known to have followed the administration of moderately large or large quantities of radiation given either as single or fractionated doses to the whole body, or to large parts of the body. The evidence for these statements is contained in a number of official publications (National Research Council, 1956; Medical Research Council of Great Britain, 1956, 1960; United Nations, 1958), as well as in numerous research papers. Whether small doses of radiation can cause Lk is, however, doubtful, and no definite answer can be given as long as it is unknown if there is a threshold to radiation leukemogenesis. Of these, the relatively heavy doses used in radiotherapy have the greatest chance of producing Lk, but the risk of these doing so is quite small when compared to the great capacity of this form of treatment for relieving the symptoms of grave disease and for prolonging life. No firm evidence exists to show that diagnostic radiation has caused Lk, although in exceptional cases, patients have received doses of diagnostic radiation comparable to those used in radiotherapy. There is nothing to indicate that the extremely small quantities of radiation so far provided by non-medical sources (fall-out) are leukemogenic.

In the etiology of Lk, radiation undoubtedly plays a part, but is likely to be a small one. Even the most pessimistic estimates have produced no support for the suggestion that a rise in the population exposure to radiation can account for the rise in Lk incidence which has taken place in large parts of the world in past decades. Direct investigations of case histories have made it seem likely that not more than 1 Lk patient in 20 has ever been exposed to significant doses of radiation in the past (Dameshek and Gunz, 1964).

2.4.4. CHEMICAL LEUKEMOGENS

Chemicals have long been suspected of being leukemogenic. Because so many substances are known to be myelotoxic, it was natural to assume that anything which is capable of injuring the marrow might also produce a neoplastic change in it. It is possible to list numerous early agents used for Lk therapy (arsenic, benzol, etc.) and proceeding via the sulphonamides and antibiotics to practically any of the multitude of chemotherapeutic drugs introduced during the past 15 years. Results in the exploration of

this vast field of potential leukemogens have been meager, the very multiplicity of substances forming a serious contender for the status of an accepted leukemogen. However, there seems little question that cases of Lk have occurred more frequently in persons with a history of heavy occupational exposure to benzol than among the general population (Hunter, 1939; Oldfelt and Knutson, 1948; Bernard and Braier, 1951; Vanucchi, 1957). The most interesting fact about these cases is the apparent need for a very heavy exposure to the agent, without which no leukemic changes have as yet been noted. This is analogous to the situation concerning the majority of radiation-induced Lk in man and suggests rather strongly that leukemogenesis, in at least some cases, may be a two-stage process: the first being an initial depression of marrow function, perhaps with structural alterations (destruction, going on to hypoplasia), and the second, the transformation of the altered cells into neoplastic ones. Such a series of events would correspond closely to that known to occur in many forms of experimental carcinogenesis (Brues, 1958), but it must be stressed that there is no sign as yet to show that either all or anything like the majority of Lks arise as a result of a two-stage process.

The other putative leukemogens which have made their appearance in the literature have generally reflected the therapeutic fashions of the days (e.g., phenylbutazone: Bean, 1960). Because of their ubiquity for over two decades, the sulphonamides and antibiotics have been often suspected, but the few statistically acceptable studies have failed to provide definite confirmation (Abbatt and Lea, 1948; Stewart, Webb and Hewitt, 1958). This does not mean that chemical agents should now be regarded as above suspicion, for among the many different varieties capable of damaging the bone marrow, there will be some which could cause Lk as well. There is indeed some evidence, both from animal experiments and clinically, that aplastic anemia of chemical origin may be followed in some instances by ALk. Whether this is due to an abnormality of growth inherent in the preparative process or to some other mechanism such as the development of abnormal (enzyme-deleted) clones of leukocytes is largely speculative at this time.

Most of these drugs are so widely and commonly used, and the development of Lk so rare among patients taking them, that any possible leukemogenic

action must certainly be extremely weak, or perhaps operating on a background of predisposition already present in the patient from some other cause.

Not only drugs but other substances containing potential carcinogens such as road and industrial fumes, solvents, cleaning materials, fuels, etc., are still in the running as possible leukemogens.

2.4.5. AGE, SEX AND HORMONES

Age appears to influence susceptibility to leukemogenic agents, perhaps as a result of intrinsic changes in hemopoietic tissues or as a consequence of altered hormonal activity. Kaplan (1948) showed that C57 black mice developed lymphoid tumours and Lk after whole-body x-irradiation much more frequently when less than 6 months' old than when over this age. Similarly DBA/2 mice became leukemic in response to methylcholanthrene only when treated early in life, although older mice might have their susceptibility increased by removal of the gonads (Kirschbaum, 1957).

Females have a higher incidence of Lk in mice, especially in certain high-Lk strains (Richter

and McDowell, 1935), and in AKR mice, spontaneous leukemogenesis may be inhibited by androgens (Murphy, 1944).

It is difficult to assume that the etiological factors will be the same for all types of human Lk. The difference in the age and sex incidence of lymphatic and myeloid Lks is so striking that it seems almost certain that they differ in causation. Lymphatic Lk is a disease of the extremes of life, but more particularly of old age. It is also a disease of males. Myeloid Lk is a disease of middle span of life, and it affects males and females equally. The percentage of myeloid forms in Lk occurring after x-irradiation is much higher than in spontaneous Lk in man. Some people believe that acute and chronic Lks are different diseases, and certainly their response to treatment is very different (Witts, 1957).

Whereas there is evidence in some strains of mice that estrogenic hormones are leukemogenic (Gardner, Dougherty and Williams, 1944), that the incidence of spontaneous Lk is higher in females than in males (Murphy, 1944), and that it can be reduced by ovariectomy (Furth, 1946), there is nothing to suggest that the female sex hormone may play a similar part in human Lk. Indeed, such data as are

available all point in the opposite direction, for all forms of Lk are commoner in human males than females, and in CLLk this sex difference is particularly apparent (Bierman, 1951; Ledlie, 1953; Shimkin, Lucia, Oppermann and Mettier, 1953). This fact suggests that sex hormones may play a part in the etiology of human Lk, but there are no data to elucidate the mechanism of any such effect.

No other hormones are known to be leukemogenic in man. The CSs, instead, are lymphocytolytic in animals (Dougherty and White, 1943, 1944) and ACTH, E and its derivates have temporary antileukemic effect in man. This subject will be discussed in greater detail later.

2.4.6. BLOOD GROUPS IN RELATION TO LEUKEMIA

The genetic relationship suggested by Videbaek (1947) to exist between Lk, pernicious anemia and cancer might be expected to involve an association between Lk and the ABO blood groups, since both pernicious anemia and gastric carcinoma are more common in persons of blood group A (Aird, Bentall and Roberts, 1953; Buckwalter, Wohlwend, Colter, Tidrick and Knowler, 1956). A considerable number of leukemic

patients has now been studied with regard to ABO and other blood groups, but no unusual distribution of groups has been observed (Tinney and Watkins, 1941; Best, Limarzi and Ponder, 1949; Lucia and Hunt, 1952; Kay and Shorter, 1956; Buckwalter et al., 1956; Walther, Raeburn and Case, 1956). This negative finding provides an additional argument against the existence of a pernicious anemia-leukemia genetic relationship.

2.4.7. REGULATORS OF WHITE BLOOD CELL COUNTS

The physiologic agents which control the proliferation of the white blood cells are largely unknown, but it is quite clear from the narrow limits in which the levels of these cells are held under normal conditions and in response to various stimuli, that such regulators must exist. In the case of red cells the presence of at least one regulator, erthyropoietic hormone ('erthyropoietin'), has been demonstrated (Stohlman, Cronkite and Brecher, 1955; Gurney, Goldwasser and Pau, 1957; Jacobsen, Goldwasser, Fried and Plzak, 1957; Gordon, 1959), and its mode of action is being investigated (Lajtha and Oliver, 1960). Substances normally inhibiting the growth of leukocytes

have been postulated by Osgood (1957), and their presence may be inferred from the events which take place in tissue cultures. Thus, as soon as myelocytes are removed from the blood of patients with CGLk, they begin to divide actively, although no sign of division could be demonstrated before their removal (Osgood and Brownlee, 1936; Gunz, 1948). This burst of activity does not depend upon a stimulating substance present in the artificial culture medium but seems to be initiated simply by the removal of the cells from their normal surroundings. It can be concluded that excess proliferative activity in cultures is possibly a result of the absence of a normal growth inhibitor. The regulation of lymphocyte production by means of a LSF manufactured in the thymus, and of lymphocytolysis by means of adrenocortical hormones has been suggested by Metcalf (1958, 1960, 1966), as discussed earlier.

The theory put forward by Furth is that some human (and also in a few mouse) Lks, the initial change lies not in the cell itself, but in the normal growth regulators which in some so far unknown way, permit or "stimulate" cells to proliferate abnormally. At this stage, the cells appear normal

and the process can still be regarded as potentially reversible. The transition from dependent to fully autonomous growth, although variable and gradual, is, however, inevitable and sooner or later changes will develop, so that these neoplasms are no longer influenced by any normal agents. This concept accords well with events which are known to occur, for example in CGLk with its terminal acute phase characterized by the appearance of new abnormal cell strains unresponsive to any kind of treatment. As yet, however, there is no positive evidence in confirmation of this theory; nevertheless, experience of the actual course of many cases of human Lk suggests that here — in the field of host-tumour relationship — is a promising field of future research.

2.4.8. SUMMARY

To summarize, leukemogenesis in the widest sense, involves a genetic problem and the various alternative theories can be included in the over-all picture of the genetic change. Among these are the theories attributing Lk to somatic mutations, to gene deletions (with or without an immunologic element), to

virus actions, to metabolic alterations, or to endocrine imbalance. What is basically altered is the cellular genotype.

The mechanism of leukemogenesis is obviously of great complexity although it is likely that a certain simplification will be introduced into the subject as a result of what Furth (1959) calls a 'meeting of the ways' between workers in different fields. None of the theories of carcinogenesis or leukemogenesis can evidently claim to contain the whole truth, although probably all state some of the essence of the malignant transformation. A synthesis must eventually be achieved, but as yet no one can guess the direction of the approach which will lead towards it.

2.5. THERAPY

2.5.1. CHEMOTHERAPY

2.5.1.1. Introduction

Rapid progress during the last two decades in the development and trial of chemotherapeutic agents have provided an almost bewildering variety of

antimetabolites and cytotoxic drugs, which, together with the adrenocortical hormones, are claiming increasing attention from the investigators and physicians engaged in treating leukemic patients.

The cytotoxic drugs appear to be active by entering the cell and interfering with essential metabolic processes by virtue of their chemical structure. Some of these agents, the antimetabolites and the antivitamins proper, are structurally similar but biologically antagonistic to metabolites or vitamins of physiologic importance. More specifically, these agents are sufficiently similar in structure to an essential metabolite to tend to replace it in biologic systems, thus bringing about a conditioned deficiency of the metabolite (Goodhart, 1955). Antagonists are competitive with the naturally occurring substances, competing for essential enzyme systems with which the metabolite normally reacts.

The antagonists which are used in the treatment of Lks are all designed to compete with substances required for the synthesis of nucleoproteins, and most of them for the nucleic acid rather than the protein moiety.

2.5.1.2. Antimetabolites

2.5.1.2.1. The Folic Acid Antagonists

Extensive nutritional and microbial studies over a period of nearly 20 years culminated in the identification of and synthesis of pteroglutamic acid, or folic acid, by Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, Subba Row, Waller, Cosulich, Farrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith (1946). The substance, one of the B group of vitamins, is closely concerned in cell metabolism, particularly in the process of cell growth and division. Deficiency leads to macrocytic anemia, often with megaloblastic erthyropoiesis, to leukopenia, and to certain gastro-intestinal changes.

Amethopterin, aminopterin, and other folic acid antagonists apparently work in identical fashion against leukocytic proliferation. Originally found to be of value in the treatment of ALk of childhood, these agents continue to have almost their only use in that condition. When given alone, they produce complete remissions in somewhat less than 50% of the cases of ALk in childhood; in association with ACTH or the CSs, complete clinical hematological, and bone

marrow remissions occur in at least the majority of cases (Drageon, 1956; Dameshek and Gunz, 1964). In the ALk of adults, the results are far less satisfactory, and only occasional complete remissions are obtained, usually at the expense of an extreme toxic reaction. These materials have but little value in the CLks, although they have been used occasionally in the treatment of the granulocytic variety.

The acquisition of resistance in mouse Lks by amethopterin is thought to result from the selective over-growth of individual resistant cells produced by random mutation (Law, 1952). That is, they are able to continue converting folic acid to citrovorum factor much more efficiently than are sensitive cells in similar circumstances (Nichol, 1954).

2.5.1.2.2. Purine Antagonists

Many synthetic analogues of naturally occurring purines and pyrimidines have been prepared. These substances might be expected to act as anti-metabolic antagonists in the early stages of nucleic acid synthesis in the body, and many of them have been

subjected to clinical and experimental trials in a variety of neoplastic diseases in the search for an agent which would influence neoplastic cell metabolism selectively without causing severe damage to normal cells.

Elion, Hitchings and Vanderwerff (1951), by substituting a mercapto group in the 6-position of the purine ring, produced two new cpds, 6-mercaptoguanine (6-MG) and 6-mercaptopurine (6-MP) and these were subjected to testing in animals. Of these, 6-MP was found to be less toxic (Philips, Sternberg, Hamilton and Clarke, 1954), and very effective against animal tumours (Clarke, Philips, Sternberg and Stock, 1954). The cytologic disturbance induced by the drug was so pronounced that the majority of the cells lost their characteristic viability when transplanted to normal mice. Clinical tests on Lks and allied diseases confirmed the potency of 6-MP and its relative lack of toxic effects (Burchenal, Murphy, Ellison, Sykes, Tan, Leone, Karnofsky, Craver, Drageon and Rhoads, 1953).

Six-MP is by far the most widely used purine antagonist which has so far been available. A very large number of others have been tested

experimentally, and some of them in human Lk, i.e., 6-methylmercaptopurine, 6-mercaptguanine or thioguanine, 8-azaguanine, 6-chloropurine, and thioguanosine. Among the prymidine analogues are 5-fluorouracil, 5-fluorodeoxyuridine and 5-fluorodeoxycytidine, 6-azauracil and 6-azathymine. None of these substances appear to have any essential advantage over 6-MP, and many are much more toxic. Six-MP is useful in both childhood and adult ALk (Dameshek and Gunz, 1964).

Patients with CGLk have also shown some good responses to this drug with occasional satisfactory remissions maintained by continuous therapy. In CLLk, on the other hand, no evidence of response has been obtained (Hayhoe, 1960).

2.5.1.2.3. The Actinomycins

The mode of action of this group of antibiotics, derived from cultures of streptomyces, is uncertain, but it appears likely that they have an antimetabolic effect. Azaserine — Actinomycin C and D have shown considerable anti-tumour activity in experimental animals, and have some clinical applicability in lymphomas and solid tumours, but they appear

to be of little value in Lks (Farber, Toch, Sears and Pinkel, 1956; Pinkel, 1959; Tan, Drageon, and Burchenal, 1959).

Daunomycin is a newly introduced antibiotic which has inducer activity in children with advanced Lk refractory to conventional therapies. Its maintenance activity appears to be less impressive (Tan and Tasaka, 1966; Holland, 1968).

2.5.1.3. Alkylating Agents

Chemotherapy for malignant disease has travelled a long path since Gilman and Philips (1946) first used alkylating agents in patients. The large number of cpds used for chemotherapy since then, and the variable success achieved with these cpds, point strongly to the fact that the ideal chemotherapy for malignant disease has not been developed. Many of the cpds which have been used for chemotherapy are variations of the original alkylating agents used by Gilman and his colleagues. Other classes of cpds have been used in the treatment of malignant disease, but in the treatment of lymphomas and CLKs, the alkylating agents are still most widely used.

The term alkylating agents comprise a large group of organic substances which, being unstable in solution, break down with the liberation of active alkyl radicals capable of combining with and frequently of inactivating molecular groupings of physiologic importance. The earliest alkylating agents of biologic interest were the nitrogen mustards, which possess two active alkyl groupings.

2.5.1.3.1. Nitrogen Mustards

The employment of nitrogen mustards, and later of other newly synthesized alkylating agents, was an indirect outcome of research dating originally from the First World War, during which mustard gas was used extensively in the field. It was thought at the time that this substance (ββ-dichlorodiethylsulfide) exerted its vesicant actions by releasing hydrochloric (HCl) acid intracellularly, and a few reports appeared which described deleterious effects of the gas on hematopoietic tissues in distant sites (Gilman and Philips, 1964). Following the outbreak of World War II, research on toxic chemicals was resumed, and among new cpds for potential military use were nitrogen analogues of mustard gas. It was then rediscovered that both S and N mustard, in addition to their local

vesicant action, had pronounced systemic effects, and that those tissues appeared particularly susceptible which showed a maximum proliferative activity, notably the hematopoietic and lymphatic organs and the gastro-intestinal mucosa. The results of the investigators on the mode of action of the mustards began to appear in press from 1946 onwards, and research has proceeded steadily since then.

There is yet no complete explanation for the physiologic activity of the nitrogen mustards and other alkylating agents. The possibility exists that they affect cells by enzyme inhibition, for it is known that many enzymes are sensitive to their action (Ross, 1958). The respiration of bone marrow slices is inhibited by nitrogen mustards, but this effect is prevented by the addition of choline whose structure is closely similar (Barron, Bartlett, Miller, Meyer and Seegmiller, 1948).

Of much greater importance than enzyme inhibition is probably the reaction between the alkylating agents and nucleic acids (Sykes, Philips and Karnofsky, 1956). <u>In vitro</u>, both S and N mustards react with nucleoproteins, the precise outcome depending

on the relative concentrations of the substances (Berenblum and Schoental, 1947).

The action of the nitrogen mustards in human Lks and leukosarcomas is indirectly mediated by the same mechanisms as in experimental animals and in vitro. The proliferative cells of the marrow, spleen and lymph nodes, both normal and abnormal, are injured and destroyed, with changes in the blood reflecting those in the hematopoietic organs, i.e., lymphopenia, neutropenia and thrombocytopenia. Algenstaedt (1959, 1961) observed that the long-term effects on the marrow produced by the alkylating agents in Lk resemble those of x-rays.

2.5.1.3.2. Triethylene Melamine (TEM)

Triethylene Melamine has proved most generally useful in neoplastic states. The effects of TEM in vivo appear to parallel very closely those of the nitrogen mustards, producing cytotoxic changes of varying severity in proliferating tissues, particularly hematopoietic and lymphoid tissues. TEM, the first of the oral "mustards", is now used but seldom, chiefly because of its striking myelotoxic effect.

2.5.1.3.3. Chlorambucil

Chlorambucil is a phenylbutyric derivative of nitrogen mustard. It is a drug which can be given by mouth and probably because of this, received wide use early in the development of chemotherapeutic agents. It is usually given in a dose of 2 to 20 mg/kg per day. It has been thought by some to be relatively specific for CLLk. Although the concept of specificity can be questioned, chlorambucil is an excellent drug for the treatment of this disease. The effect of chlorambucil in a given case of CLLk is a variable one, perhaps because of the variability of the leukemic disease from case to case. In most instances, a definite response occurs, and complete remissions are possible (Dameshek and Gunz, 1964). White blood cell counts can be brought down and tumour masses reduced in size with chlorambucil. Again, there is question as to whether or not a great deal is accomplished in the treatment of CLLk with respect to prolongation of survival. Boggs, Sofferman, Wintrobe and Cartwright (1966) gave a very pessimistic viewpoint concerning this. These authors reviewed a series of 130 patients seen between 1945 and 1954. They considered that there is still

no definite evidence that any form of Lk-directed therapy prolongs the life of the patients with CLLk.

Drugs related to nitrogen mustard, such as TEM, triethylenephosphoramide, and chlorambucil have been used in the treatment of lymphomas, but there is no evidence that they are more effective. While they depress the bone marrow in the same way as the nitrogen mustard, they do not produce the severe nausea and emesis characteristic of nitrogen mustard. TEM and chlorambucil can be taken by mouth, whereas nitrogen mustard must be given intravenously. Nitrogen mustard is generally preferred when a rapid effect is desired, and chlorambucil when there is no immediate hurry.

2.5.1.3.4. Endoxan or Cytoxan

This relatively new drug can be given orally or by injection and has a range of activity rather similar to that of chlorambucil (Gross and Lambers, 1958; Matthias, Misiewicz and Scott, 1960; Korst, Johnson, Frenkel and Challenger, 1960; Wall and Conrad, 1961). It is particularly useful in Hodgkin's disease and lymphosarcoma, but less so in CLLk.

2.5.1.3.5. Busulfan (Myleran)

This alkylating agent used orally appears to have a selective effect in granulocytic proliferations, particularly of the chronic variety (Haddow and Timmis, 1953; Haut, Altman, Cartwright and Wintrobe, 1955; Galton and Till, 1955; Unugur, Schulman and Dameshek, 1957; Haut, Abbott, Wintrobe and Cartwright, 1961), a statement which has not been universally accepted (Miller, Diamond and Craver, 1959). It is probable that only relatively small doses of busulfan will act selectively on myeloid cells (Galton, Till and Wiltshaw, 1958), while larger ones affect all hematopoietic cells. It is in fact possible to obtain remissions with busulfan in CLLk (Sykes, 1958) but a precise analysis has shown that high doses of the drug are needed, that far fewer remissions are produced than with chlorambucil and that a decline in the lymphocyte count is frequently associated with thrombocytopenia (Rundles, Grizzle, Bell, Corley, Formmeyer, Greenberg, Huguley, James, Jones, Larsen, Loeb, Leone, Palmer, Riser and Wilson, 1959). However, busulfan used with caution produces useful remissions in the various myeloproliferative disorders (Dameshek, Granville and Rubio, 1958; Oishi, Swisher and Troup, 1960; Killmann and Cronkite, 1961).

The apparent specificity of busulfan in granulocytic proliferations represents one of the most encouraging features of the chemotherapy of Lk since it indicates the possibility of achieving some degree of selectivity for other materials.

2.5.1.3.6. Urethane

Urethane (ethyl carbamate) was among the earliest of the drugs which ushered in the renaissance of chemotherapy in the treatment of Lk. In CGLk, urethane was found to be capable of reducing the leukocyte count and raising the erthyrocyte count, with involution of the enlarged spleen and a reduction of mitoses in the myeloid and an increase of those in the erthyroid cells of the bone marrow (Moeschlin, 1947). According to Moeschlin, urethane acts in Lk by a selective depression of the mitotic activity of myeloid cells and others have found mitotic abnormalities in urethane-treated leukemic marrow (Schulze, Fritze and Müller, 1947), as well as pyknosis, karyolysis and karyorrhexis of leukemic nuclei (Berman and Axelrod, 1948).

Administration of urethane to normal animals produces a rapid fall in the lymphocyte and a

rise in the neutrophil count. In leukemic animals, urethane was reported to produce a decrease in leukemic cells with normalization of the blood and marrow, and sometimes a prolongation of life, as well as failure to "take" of a proportion of transplanted Lks (Murphy and Sturm, 1946, 1947; Engstrom, Kirschbaum and Mixer, 1947; Law, 1947; Kirschbaum and Lu, 1947; Kirschbaum, Judd, Lu, Engstrom and Mixer, 1948). These effects have been explained as resulting from mitotic depression (Dustin, 1947; Guyer and Claus, 1947). However, other authors (e.g., Haddow and Sexton, 1946) believed that urethane acted by increasing cellular differentiation. Actual mitotic stimulation followed by mitotic abnormalities was found by Green and Lushbaugh (1949), Rosin (1951), and Rosin and Goldhaber (1956). These experiments were conducted with large doses of urethane which caused considerable toxicity or even death of the animals. The therapeutic action of urethane is very slow and sustained in experimental Lk, but when used in human Lk, it generally produced acute, short-lived effects. It appears doubtful if experiments with such large doses can throw much light on the mechanism of drug action in the human patient.

Any modification of the molecule reduces its leukopenic and antileukemic effect in mice (Skipper, Bryan, Riser, Welty and Stelzenmuller, 1948). From about 1947 to 1953, urethane was used for the treatment of CGLk (Sherwood, 1948), but with the emergence of busulfan (myleran), its use in that condition has almost completely lapsed.

2.5.1.4. Arsenic

Inorganic arsenic depresses leukopoiesis and has been used for many years in the treatment of chronic myelocytic Lk. Many patients with this disease may derive considerable benefit from the continued treatment with arsenic. A state of mild chronic arsenic poisoning must be induced to achieve a satisfactory therapeutic level, and because of this drawback, and the availability of new, more effective and less troublesom chemotherapeutic agents, arsenic is little used in Lk at the present time.

2.5.1.5. Periwinkle Alkaloids

2.5.1.5.1. Vinblastine

Among the newer drugs, vinblastine sulfate has shown signs of becoming a preferential

chemotherapeutic agent. Although its mode of action is not clear, it does not appear to be an alkylating agent (Ezdinli and Stutzman, 1968).

Since the discovery of the periwinkle alkaloid vinblastine (vincaleukoblastine) in the late 1950's, and its description as a cancer chemotherapeutic compound (Noble, Beer and Cutts, 1958; Johnson, Armstrong, Gorman and Burnett, 1963), a number of reports have appeared concerning its efficacy in neoplastic diseases in general (Warwick, Darte and Brown, 1960, 1961; Midwest Cooperative Group, 1962; Hodes, Rohn, Bond, Yardley and Carpening, 1962; Obrecht, 1964; Cooperative study, 1965; Bleehem and Jelliffee, 1965; Armstrong, 1966), and in Hodgkin's disease in particular (Bernard, Jacquillet, Boiron, Weil and Soto, 1964; Mathé, Schweisguth, Schneider, Amiel, Cathan, Schwarzenberg, Burle and Smadja, 1964; Stutzman, Ezdinli and Stutzman, 1966; Wilson and Louis, 1967). It has also been established that vinblastine has an effect on a number of Lk strains in mice and rats. Thus, it increases the life span of mice inoculated with Lk L1210. In a dose of 0.8 mg/kg daily, vinblastine can cure mice with Lks P1534 and AKR (Cutts, Beer and Noble, 1960).

Vinblastine is apparently an anti-metabolite. Its effects on leukemic cells *in vitro* can be reversed by a number of metabolites, for example, tryptophan, glutamic acid, etc. (Johnson, Wright, Svoboda and Valantis, 1960).

Sohier, Wong and Aisenberg (1968) reported the use of this drug in 35 cases of advanced Hodgkin's disease over a period of $3\frac{1}{2}$ years, and achieved a high response rate with many remissions of long duration. The side effects of vinblastine were not severe. The same authors reported that vinblastine is able to produce useful and prolonged remissions in such patients who are unsuitable for radiotherapy and resistant to alkylating agents. The authors concluded that although vinblastine does not alter the unfavourable ultimate prognosis of advanced Hodgkin's disease, it is a very valuable drug in a difficult therapeutic situation.

2.5.1.5.2. Vincristine

Vincristine sulfate, a dimeric indole-indoline alkaloid of the periwinkle plant, has an anti-neoplastic effect in Lk and in a variety of childhood neoplasms including lymphoma, Wilm's tumour,

neuroblastoma, embryonal adenocarcinoma, Ewing's sarcoma, malignant teratoma and retinoblastoma (Karon, Freireich and Frei, 1962; Rohn and Hodes, 1962; Selawry and Delta, 1962; Tan and Adams, 1962; Evans, Farber, Brunet and Marino, 1963; Sutow, Thurman and Windmiller, 1963; Johnson, Armstrong, Gorman and Burnett, 1963; James and George, 1964; Heyn, Beatty, Hammond, Louis, Pierce, Murphy and Severo, 1966; Sutow, Berry, Haddy, Sullivan, Watkins and Windmiller, 1966; Windmiller, Berry, Haddy, Vietti and Sutow, 1966). In a recent report on 94 patients, Haggard, Fernbach, Holcomb, Sutow, Vietti and Windmiller (1968) further established the previous observation that vincristine is an effective therapeutic agent for induction of remission in Alk, even when refractoriness to other chemotherapeutic agents had developed.

Armstrong (1968), reviewing the work of several groups, concluded that vincristine definitely proved quite effective for inducing remission in ALk of children.

2.5.1.6. Combined Chemotherapy

Several chemotherapeutic agents may be administered simultaneously (combined therapy proper)

or successively, when one agent is replaced by another at the end of a course of treatment, or in a relapse to avoid drug resistance. Simultaneous administration of two or more agents aims at utilizing cpds with different mechanisms of action and is justified if the effect produced by (usually reduced) doses of the agents exceeds the sum of effects of each of the agents used, or the effect of one of the agents administered, in its full dose. Such a combination is of clinical value if the toxic effect of the combination does not exceed that of their sum. But the purely additive effect of two cpds administered in reduced doses is also of clinical value if the toxicity is lower than when a full dose of one (or both) of the components is used. It is clear that these conditions are not easy to observe, and it is very difficult to study them clinically since it is necessary to have several groups of patients.

In experimental studies, combination of drugs have been found to exist in which an additive therapeutic effect is accompanied by reduced toxicity. Potentiation of the therapeutic effect without increased toxicity has also been shown to occur (Goldin and Mantel, 1952; Sorokina, 1957; Burchenal, 1964).

Several investigators (Law, 1952; Frei, Holland, Schneiderman, Pinkel, Selkirk, Freireich, Silver, Gold and Regelson, 1958; Frei, Freireich, Gehan, Pinkel, Holland, Selawry, Haurani, Spurr, Hayes, James, Rothberg, Sodee, Rundles, Schroeder, Hoogstraten, Wolman, Traggis, Cooper, Gendel, Ebaugh and Taylor, 1961; Burchenal, 1964; Burchenal and Murphy, 1965; Boiron, Jacquillet, Weil and Bernard, 1965; Tyrer, Kline, Venditti and Goldin, 1967; Holland, 1968) investigated the efficacy of combinations of anti-leukemic agents and whether such combinations could improve the frequency and duration of complete remission in ALLk and allied diseases. The authors observed that the complete remission-induction rate approaches 60% and above. Combinations of effective agents (such as, prednisone and vincristine, and prednisone and 6-MP) produce at least an additive increase in complete remission rates over that which can be achieved when the agents are used individually. Patients who do not achieve complete remission with initial treatment have a significantly shorter survival. In ALLk of children, prednisone, vincristine, and prednisone and vincristine combined, necessarily need maintenance therapy. Also, the duration of remission is doubled or extended with

maintenance therapy (Holland, 1968).

Recently, Tyrer et al., (1967) reported on the effectiveness of combination therapy with cytosine arabinoside and methotrexate in mice inoculated with a mixture of cytosine arabinoside and methotrexate-resistant Lk L1210 cells. Earlier, cytosine arabinoside-resistant L1210 variant cells have been reported, but these resistant tumours were not cross-resistant to methotrexate and cytosine arabinoside respectively. The sensitivity of each of these resistant variants to the alternative drug raised the question of the extent to which a mixture of the resistant tumours might respond to single drug or combination therapy. The drugs, alone and combined, were given daily from day 6 after inoculation of the resistant tumours separately, or as a mixture. The mixture of resistant cells failed to respond to the individual drugs. However, in two experiments, optimal combination therapy increased by 170% and 120%, the life span of mice with the mixture of resistant tumours (Henderson, 1969). The effectiveness of combination therapy against the mixture of resistant cells suggests that each drug in the combination exerts an independent effect by selectively inhibiting drug-sensitive cells in the mixture.

Bodey, Hart and Freireich (1968) observed that cytosine arabinoside is capable of inducing remissions in patients with ALk. In the mouse Lk L1210 activity of cytosine arabinoside can be enhanced by changes in schedule and by combination with alkylating agents. Major toxicity was limited to myelosuppression, nausea and vomiting, during drug administration.

It can thus be concluded from these studies of several groups, that the effect of a combination of drugs in inducing remission has been found superior to the separate components; and in most instances, a greater remission-induction rate resulted than that anticipated from additive effects alone.

2.5.1.7. Dose Schedule and Therapeutic Effect

In the treatment of the Lks and other neoplasma with drugs which produce remissions, two main problems eventually present themselves: the subject may succumb to the cumulative toxic effects of the therapy or the malignant cells become resistant to the therapeutic agents. In this connection, several authors have demonstrated that it was possible

to significantly increase survival by postponing the time when resistance develops without increasing the toxicity of the therapeutic agent.

Such studies have been conducted by Goldin and Mantel (1952), Goldin, Venditti, Humphreys and Mantel (1956), Heyn, Brubaker, Burchenal, Cramblett and Wolff (1960), Freireich, Karon and Frei (1964), Selawry and James (1965), Burchenal and Murphy (1965), Tan and Tasaka (1966), Sullivan, Fernbach, Griffith, Haddy, Vietti and Watkins (1966), Holland (1968), and others.

These studies have emphasized the importance of dose schedule and therapeutic effects in the subjects. Their work illustrates the principle that in the treatment of neoplasia the optimal schedule of therapy and the optimal dose are not fixed, but may be influenced by the stage of development of the disease. They also emphasized the dual need in chemotherapy for recognition of the stage of disease and for the development of appropriate therapies for each stage.

Mantel, Greenhouse, Venditti and Humphreys (1954) performed some elegant experiments on

the specificity of action of an anti-leukemic agent aminopterin. The authors reported that the specificity of action of a drug expressed in terms of its relative effect on host and tumour is not a fixed property of the drug, but may be altered by the manner in which the drug is employed. The anti-leukemic specificity of action of aminopterin was influenced by (1) the time of administration of the drug with reference to the stage of development of the tumour, and (2) the schedule of treatment. Demonstration of enhanced anti-tumour activity with the drug is contingent upon the ability to increase the inhibitory effect of the drug on the tumour relative to the toxic effect of the drug on the host. Thus, the anti-leukemic specificity of action of aminopterin, defined in terms of the relative effect of drug upon host and tumour was demonstrated to vary with the time of treatment following tumour implantation and the schedule of treatment.

Goldin, Venditti, Humphreys, Dennis, Mantel and Greenhouse (1954), conducted several experiments in order to determine whether the anti-leukemic specificity of 6-MP may be altered by the schedule of

treatment. The authors reported that with either aminopterin or amethopterin, daily multiple treatment was not as effective as multiple treatment spaced 4 days apart. The authors suggested that with the longer interval between treatments (every 4 days), the drug treatment may be extended for a longer period of time, thus permitting more extensive increase in survival time.

In another study, Goldin Venditti, Humphreys and Mantel (1954, 1956) studied the survival time of mice with the transplanted leukemia L1210. The authors reported that tumours in the initial stage should be treated with higher dosages of amethopterin and less frequently than tumours in the later stages of development. By following such a programme it was demonstrated that advanced tumour is susceptible to therapy.

Goldin and his associates (1954, 1956) discovered the superiority of the intermittent amethopterin dose schedule in animals with a low body burden of leukemic cells, whereas the same phenomena did not occur when 9 days have elapsed after transplantation of L1210 and the leukemic population was high. Furthermore, a single treatment with an effective leukemic agent on day 7 followed by methotrexate allowed greater effectiveness than when the

antifol was given without the priming dose (Venditti and Goldin, 1964). Goldin et al., also failed to find major increased effect from intermittent 6-MP (Holland, 1968).

More recently, Kline, Gang, Waravdekar and Venditti (1967) reported a comparison of daily and intermittent treatment with cytosine arabinoside in the chemotherapy of mouse Lk L1210. The authors observed that a therapeutic advantage may be provided with an intermittent schedule employing repeated courses of daily treatment with high doses interspersed with periods of no treatment as compared with continuous daily treatment with low doses. The authors suggested that the superior response with intermittent therapy resulted from the ability of the host to tolerate more total drug, thereby increasing the degree of damage to the leukemic cell population.

2.5.2. ACTH and CORTICOSTEROIDS

The use of ACTH and CSs play an important role in the treatment of Lk. Discussion of this subject is deferred until Chapter III.

2.5.3. RADIATION THERAPY

2.5.3.1. Introduction

The efficacy of x-rays in the treatment of certain forms of Lk and allied diseases was demonstrated early in this century (Senn, 1903), and for nearly 50 years radiation therapy provided the chief means of control of CLks. Acute Lks failed to respond and were sometimes exacerbated by x-ray treatment. The striking ability of irradiation, whether external or internal, to induce remissions in CLks but not in the acute ones, the development of general remissions after irradiation of quite small areas, the eventual production of a resistant state, and many other phenomena observed during the clinical management of leukemic patients have stimulated a wealth of experiments on the mode of action of radiations in Lk.

A typical favourable response to radio-therapy in CLk consists in a fall in the leukocyte count, occasionally preceded by a short-lived rise, in a rise in the erythrocyte count and the hemoglobin level, an involution of the pathologically enlarged organs, and an increase in the patient's

general well-being. It has been generally assumed that this somewhat complex chain of events is elicited by the same mechanism which brings about the destruction of normal hematopoietic tissue in experimental animals, namely, a direct interference with mitosis in the developing leukemic cell. There are, however, certain difficulties in accepting so relatively simple an explanation. Thus, the dose of radiation necessary to produce remissions in susceptible patients with Lk tends to be much smaller than those which will result in visible destruction of normal blood-forming cells. Remissions, at least in early cases, usually last much longer than the time required for the recovery of experimentally irradiated marrow, and, most puzzling of all, a similar response can be obtained by irradiation either of the whole body with x-rays or radioactive isotopes, or solely of local organs like the spleen (Brauner and Gottlieb, 1939; Moeschlin, 1947; Berlin and Lawrence, 1951; Dameshek and Gunz, 1964).

While it must be accepted that remissions in Lk occur because ionizing radiation causes anatomic changes in the affected tissues, their precise extent is still uncertain. There remains the

question exactly how such changes are brought about. Thus, treatment of spleen may cause the disappearance of enlarged leukemic nodes or skin lesions (Piney and Riach, 1932; Grilli, 1938) or the subsidence of priapism in CGLk (Craver, 1933). It has therefore been assumed that x-rays must have a distant as well as a local effect, but proof of such an effect has been most difficult to obtain. Clearly there are more unknown than known facts concerning the action of ionizing radiation in Lk. There is little doubt that their basic effect is on the reproduction of leukemic cells.

Patients with the ALks are not primarily radiotherapeutic problems, while those with CLks, particularly CLLk and CGLk, are benefitted greatly by irradiation. Treatment must be tailored to meet the particular manifestations of the disease in each patient. Both the local and general signs and symptoms must be evaluated. Subsequent conduct of therapy is guided entirely by the patient's response. Thus, Dameshek (1955) has repeatedly stated that he had discarded radiotherapy for TEM in the treatment of CLLk. However, data suggesting the superiority of TEM

have not been published (Moss, 1965). Radiotherapy quite justifiably remains the modality preferred by many in the treatment of the chronic forms of both granulocytic and lymphocytic Lk.

2.5.3.2. The Therapeutic Use of X-Rays in the Leukemias

2.5.3.2.1. In ALk

None of the patients with ALks are helped by either splenic or whole-body irradiation — in fact, they may be harmed. However, such patients may be helped by limited irradiation of symptom-producing infiltrations. Local therapy given in the recommended doses rarely produces clinically important changes in peripheral blood counts (Moss, 1965).

Terminally, CGLk may assume most of the characteristics of AGLk. Thus toxic symptoms together with all of the symptoms of an exhausted marrow develop. The leukemic cells acquire a radiosensitivity similar to that of the remaining hemopoietic tissues. Then, as Levitt said, "The effect of irradiation is merely to flog an already exhausted marrow, and the damage caused by attempts at treatment often hastens the progress of the disease".

2.5.3.2.2. In CLk and Lymphosarcoma

Radiation therapy plays a major role in the treatment of CLks. In it we find a highly flexible approach permitting excellent total body treatment when indicated, as well as the best available local treatment. Irradiation is indicated with the appearance of weakness, anemia, weight loss, malaise, fever, progressive lymphadenopathy, and splenomegaly. An elevated leukocyte count alone is usually not an indication for therapy, but the majority of patients with a rising leukocyte count above 50,000 per ml will require irradiation. Some authors claim that remissions have been produced with total body irradiation which have not been surpassed by another method, while others have given up its use (LaDue and Molanda, 1964). Splenic irradiation is probably best used when total irradiation has failed to satisfactorily shrink a splenomegaly. On the other hand, cases resistant to local irradiation seldom responded well to total irradiation. Radio phosphorus and total body irradiation probably produce equal results when applied with equal care. Over-treatment may be fatal, while under-treatment may be followed by early relapse. The guides for adequate treatment in the CLks are almost

entirely biological. Most important are the decrease in anemia, increase in strength and weight, and return of the hemogram to near normal.

Radiation therapy is the principal and most reliable form of treatment for the majority of the patients with lymphosarcoma (Molander and Pack, 1964).

2.5.3.3. Radio Phosphorus in the Treatment of Leukemia

Lawrence, Scott and Tuttle, in 1939, first described the use of P^{32} in the treatment of acute and chronic Lks. Like most other agents, its efficacy in ALks was found to be negligible. Experience in treatment of CGLk over the past 20 years, however, has shown that remissions comparable to those obtained by conventional roentgen ray therapy are usually possible. Lawrence and his colleagues claimed prolongation of life in patients treated with P^{32}, whereas most investigators do not find this to be true. The experience of most therapists is that the life span of such patients, from the onset of Lk to death, is from 3 to 4 years (Hahn, 1964).

The rationale of the use of P^{32} in this condition is based on the integrated delivery of radiation with minimal radiation sickness. A major disadvantage is the relatively long half-life of the nuclide, which makes evaluation of the patient's response rather slow and difficult. Another disadvantage is the lack of specificity in the distribution of P, since it is incorporated into almost every cell in the body. This largely offsets the relatively small amount preferential uptake by the bone marrow.

In the treatment of LLk the use of P^{32} has not proved as satisfactory as in the myelogenous form. This is not surprising, in view of the distribution of the nuclide in the body tissue. Radio phosphorus has proved of no value in the treatment of monocytic Lk.

2.5.3.4. Irradiation by Other Agents

Isotopes other than P^{32} are rarely used in the treatment of Lk. There have been trials of radioisotopes of arsenic, sodium and gold, but none of these isotopes have any decisive advantage over P^{32} or x-rays.

Radium has been applied over the spleen in patients with CLk by means of a 4-gm. radium pack. Although effective, this form of treatment is not superior to local x-irradiation. Lingreen gave radioactive sodium to 5 patients with CLk with fair results, but concluded that these results were less satisfactory than those usually achieved with P^{32}. Radium chloride has been given intravenously but has not proved to be desirable (Hamilton and Stone, 1937). Radioactive cobalt (Co^{60}) is valuable as a source of high voltage rays on a wider scale and has been in use for the treatment of cancer of the cervix, but there is no report of its use in any form of Lk.

2.5.3.5. Irradiation and Bone Marrow Therapy

The ultimate in therapy of ALk would be the 'complete but safe' eradication of all cellular origins of Lk. While this has not been accomplished to date in humans, the results of animal experimentation have suggested that it may be possible in the future. Leukemic mice have been given lethal doses of x-ray — enough to destroy both the leukemic tissues and the normal marrow present (Lorenz and Congdon, 1955; Lindsley, Odell and Tausche, 1955;

Barnes and Loutit, 1957; Trentin, 1957; Congdon, 1957; Odell, Tausche, Lindsley and Owen, 1957).

Results of such experiments have prompted Dameshek (1957) to state that: (1) lethal irradiation can be survived, provided normal marrow is injected; (2) marrow injected intravenously seek out normal marrow sites, apparently escaping the pulmonary barrier; (3) irradiation strips the immunologic defenses to such a degree that not only hemotransplants but even heteroplants can be effective.

Many reports have appeared in the past few years giving details on high-dose radiation treatment of human Alk combined with marrow transplantation (e.g., Mathé and Bernard, 1959; Thomas, Lochte, Cannon, Sahler and Ferrebee, 1959; McGovern, Russell, Atkins and Webster, 1959; Atkinson, Mahoney, Schwartz and Hesche, 1959; Beard, Bernhard, Ross and Conlin, 1959; Murphy, 1959), and a valuable summary of the present situation was published by Miller and Diamond (1961).

The present position in this field may be summarized as follows: It has been possible for human beings to withstand doses of radiation

which would in the ordinary course of events be fatal because of the destruction of blood-forming tissues. This has been achieved through marrow transfusions which, when obtained from identical twins, were capable of establishing themselves in the patient and of taking over hematopoiesis; when other relatives or unrelated donors were used, the homologous grafts rarely "took". In the few cases where "takes" of homologous material occurred, there followed secondary disease which was itself fatal in some instances. In a proportion of the cases in which homologous marrow transplants did not establish themselves, they were, nevertheless, of benefit by providing a temporary supply of normal blood cells, until the patient's own immunologically competent cells (lymphocytes) recovered sufficiently from the irradiation to produce antibodies and so eliminate the foreign hematopoietic tissue. In other cases, the patients died from the radiation effects or the Lk, without any evidence of activity of the transfused cells.

In no case was the Lk cured, relapse occurring with distressing rapidity even when an initial remission had followed the therapy. It appeared that even with the highest radiation doses

used — which were well in the supralethal range — some leukemic cells survived and were capable of continued proliferation. This unfortunate fact was in line with other experimental work. Shrek, Leithold and Friedman (1957) showed that x-ray doses below 4000 r were unable to kill all the cells in suspensions of human leukemic lymphocytes irradiated in vitro; and several groups of workers (Hewitt and Wilson, 1959; Burchenal, Oettgen, Holmberg, Hemphill and Reppert, 1960) failed to cure mouse Lks or make them non-transplantable with doses of 2500 r or even higher. Some uncertainty still remains in view of the original work of Barnes, Carp, Loutit and Neal (1956), who produced cures in mice. Barnes et al., reported the treatment of x-irradiation in CBA/H inbred 4-months' old strain of mice inoculated with leukemic tissues and x-irradiated a week after such an inoculation. The authors reported 25 animals were cured out of 35.

Later, Barnes and Loutit (1957) reported the treatment of a generalized Lk of the CBA mouse by means of whole-body irradiation by x-rays. A dose of 950 rads given at a high dose rate — the LD_{98} for the normal CBA mouse — was insufficient to eliminate all the malignant cells. When recovery

from radiation syndrome was affected by the administration of isologous myeloid tissue, the animals died after about one month usually with overt Lk. When the radiation syndrome was treated with homologous bone marrow, the animals often survived for longer periods but died in a wasted condition without signs of Lk. This might indicate destruction of the residual leukemic cells by the homografts or failure of these cells to grow in an animal poorly nourished because of delayed effects of radiation damage of the gut.

These findings have not yet been adequately explained, although it is possible that success in eliminating leukemic cells was, partly due to genetic differences between the donors and recipients of the Lk (Hewitt and Wilson, 1959), and that a developing immunologic resistance was enough to help decisively in the recovery of these animals. At any rate the experimental arrangements were not strictly comparable with conditions in which a cure of spontaneous human Lk was attempted. It appears unlikely that Lk will be found to be curable by radiotherapy combined with marrow transplantation. Possibly some leukemic cell strains may eventually be found to be more sensitive to heavy chemotherapy than radiotherapy.

2.5.4. COMBINATION OF CHEMOTHERAPY AND RADIATION THERAPY

Chemotherapy and radiation therapy happily supplement each other. In fact, the most characteristic feature of chemotherapy is that it may act on tumour nodes at any site of the body even if a particular lesion has not been demonstrated clinically. In contrast, radiotherapy concentrates the whole force of its action on a definite tumour node but, as a rule, is not capable of effectively influencing lesions scattered in the body, especially if they have not been clinically demonstrated.

It thus follows that the most desirable form of combining both types of treatment is that of directly concentrated radiations on 1-2 tumour nodes of large size and using chemotherapy for the smaller nodes in other regions and for the regions not revealed clinically.

The difficulty of combining chemotherapy and radiation treatment is that both ionizing radiations and the majority of anti-tumour drugs have a depressant influence on hemopoiesis. Therefore, one cannot combine intense irradiation of many regions of the body and use of the maximum tolerated doses of

anti-tumour drugs. However, the most desirable form of combining both methods is perfectly possible since irradiation is given from a few fields and its total dose does not reach large values.

2.5.5. ANCILLARY METHODS

The treatment of Lk has been facilitated to some extent, and the lives of leukemic individuals prolonged not only by the methods outlined above, but by various ancillary methods that have been introduced in recent years. These include the antibiotics to control infections, the use of platelets transfusions in thrombocytopenia, and splenectomy in some cases of CLk requiring numerous transfusions. This concept, Farber (1957) has called "total care", and represents the desire to give the patient every conceivable physical aid. This concept is the outcome of a somewhat more optimistic approach towards the treatment of Lk than was present only a few years ago.

2.5.6. THE FUTURE OF THERAPY

The future of the therapy of Lk may be in several directions — either in the development

of newer and more potent chemicals, or in methods to attack the agents or the abnormal metabolic factors responsible for the intense proliferation. It is also conceivable that prophylaxis may some day be applied either by careful attention to various possible etiologic factors as radiation exposure or by the development of (hypothetical) vaccines against the more or less hypothetical viral agents that may cause Lk. It is well to reflect in these sad days when there is no cure for ALk that our therapeutic programmes, however good they may seem to us now, are undoubtedly in their infancy and that many new and better methods will almost surely be developed in the years to come.

CHAPTER III

ADRENOCORTICAL LYMPHATIC TISSUE INTERRELATIONSHIPS

1. THE EFFECT OF ADRENAL CORTEX ON NORMAL LYMPHATIC TISSUE

1.1. INTRODUCTION

The literature dealing with the influence of lymphocytes in particular has grown considerably during the last two decades. In discussing the problem it should be emphasized that it is extremely difficult, if not impossible, to consider the effects of hormones in isolation, since there is so much interaction between them. It is also difficult to distinguish a direct hormonal effect and a secondary result of the metabolic changes to which it gives rise.

During this period of development, the adrenal gland has received most attention in connection with the control of lymphoid tissue. From the time when Addison (1855) first noted a marked increase in the white cells of the blood, evidence has steadily accumulated to indicate a close connection between lymphoid tissue and the suprarenal cortex. In Addison's disease there is usually to be found a marked lymphocytosis. Large numbers of lymphoid

follicles may develop in the bone marrow (Hedinger, 1907), though they are very infrequent in normal marrow (Sundberg, 1955; Yoffey and Courtice, 1956).

In 1905, Hammar observed an intensive decrease in the size of the thymus during starvation or malnutrition and contrasted this acute type of atrophy to the gradual involution of lymphatic tissue which occurs during ageing. Subsequent publications by Hammar in 1921 and 1936 confirmed the involution of lymphatic tissue in man and animals. Since then the literature has been enriched with a great deal of information about acute and age involution of lymphoid tissue. At an early stage it became obvious that endocrine factors have a profound influence on the lymphoid tissue, but the full significance of this hormone dependence is still far from clear.

1.2. THE EFFECT OF ADREX ON LYMPHATIC TISSUE

The afore-mentioned, and many other clinical observations of this type, suggested strongly that the adrenal cortex exerted somehow a depressant effect upon the thymo-lymphatic system, and experimental studies seem to fit in very well with this idea. Thus Jaffé (1924) found in rats that the thymus

hypertrophied after adrex, while Marine, Manley and Bauman (1924) obtained similar results in rabbits and in fact noted that if the adrex had been performed when the thymus was already undergoing involution, regeneration occurred. Later, Simpson, Dennison and Korenchewsky (1934), and Reinhardt and Holmes (1940) repeated and confirmed such findings. In other studies it was shown that adrex not only increases the size of the thymus, but it results in an increase in circulating lymphocytes and a decrease in circulating PMN neutrophils (Zwemer and Lyons, 1928; Corey and Britton, 1932).

Reinhardt and Holmes (1940) found that the thymus and lymph nodes of rats were much heavier in adrexed animals maintained 45 days on 1% NaCl than those of normal controls or controls receiving NaCl but not adrexed. Also, Simpson, Li, Reinhardt and Evans (1943) found the thymuses of adrexed rats to average 58% greater than those of control animals, allowed the same food intake.

Rapela (1944) followed the weight changes of the lymphatic organs of animals adrexed at different periods of life and concluded that although the lymphatic organs of operated animals increased in

size as compared to intact controls, the time of onset and rate of lymphatic tissue loss during age involution was not altered by adrex. Furthermore, he concluded that adrenocortical secretions exert a constant moderating influence on lymphatic tissue throughout life.

1.3. THE EFFECTS OF ADRENAL CORTICAL HORMONES ON LYMPHATIC TISSUE

The thymus and lymphatic structures have repeatedly been observed to involute following administration of adrenal cortical hormone or of ACTH to animals with intact adrenals, and among the first reports in this regard were those of Selye who described the adaptation syndrome whereby a wide variety of agents (exercise, fasting, cold, ultraviolet rays, etc.) function as a non-specific stress to produce an "alarm reaction" of which one of the striking changes is acute involution of the thymus and to a lesser extent of other lymphatic tissues. In 1938, Ingle and Mason demonstrated loss of weight of thymus glands in rats in which cortin was implanted subcutaneously in the form of solid pellets. Ingle et al., (1938) administered cortin and purified adrenal cortical preparations to rats that were then killed at

intervals. The thymus gland showed marked progressive atrophy in those rats treated with cortin in doses of 5-10 cc daily, each cc representing about 75 gm of whole adrenal gland.

Ingle (1938) also demonstrated substantial involution of the thymus in the intact rat after administration of large amounts of cortin. Wells and Kendall (1940) found that the average thymus weight of 6 rats fed large doses of a highly potent non-crystallized fraction of adrenal cortex was 27% less than of 20 controls while the average thymus weight of 6 rats receiving B was 63% less than that of control. Neither DOC nor its acetate administered in drinking water, or subcutaneously, produced involution of the thymus in these studies. Ingle (1940), in a similar study employing DOC, reported that there was no significant regression of the thymus in 5 rats. However, thymic regression of mild degree occurred when the daily dose of DOC was raised to 10 mg.

Finally, Ishidate and Metcalf (1963) confirmed the earlier reports by publishing cytological and histological data which revealed that cpd E inhibited mitosis and destroyed lymphocytes within 3 hours of its administration.

1.3.1. SECONDARY HYPERPLASTIC EFFECTS ON LYMPHATIC TISSUE PRODUCED BY THE CORTICOSTEROIDS

The hypoplastic effect of the CSs on lymphatic tissue is followed by a secondary hyperplasia, characterized by mitosis of maturing reticular lymphocytes and restoration of normal structure (Dougherty and White, 1945).

However, Weir and Heinle (1950) could not bring about lymphoid tissue regression in mice by the administration of cortisone; nevertheless, they did not accept Dougherty and White's (1945) suggestion that this was the case because of the secondary hyperplasia.

Supporting Dougherty and White, Yoffey and Baxter (1946) gave daily injections of an aqueous cortical extract for one month to rats, and found that it gave rise to a slight but definite hyperplasia of lymphoid tissue. The idea of a reactive hyperplasia was not borne out, however, by similar experiments with ACTH, associated with depletion of lymph nodes and thymus but with no evidence of increased lymphocyte breakdown.

Furthermore, that the adrenal cortex may indeed have a stimulating action on lymphoid

tissues, in addition to the depressive effect of the CSs, has been shown by Santisteban (1959), who found that the adrenals were essential for the normal restoration of the thymus after stress-induced involution.

Finally, Ishidate and Metcalf (1963) confirmed the earlier reports that the administration of cpd E to the mouse causes acute weight loss in the thymus, followed, after a delay, by active regeneration.

1.4. THE ROLE OF THE ADENOHYPOPHYSIS

The foregoing experiments proved that adrenal cortical hormone is capable of producing gross and histologic changes of an involutionary nature in lymphoid and thymic structures. That the anterior pituitary through its hormone ACTH, the normal regulator of adrenocortical secretion, also plays an important role in the involution of lymphatic tissue under conditions of stress was demonstrated by the experiments of Selye (1936, 1937, 1940), in which thymic involution did not occur following stress unless the pituitary was present. In 1938, Evans, Moon, Simpson and Lyons reported marked thymic weight loss in normal rats after ACTH administration, while

in the same year Ingle (1938) demonstrated that cortin administration produced thymic atrophy in hypoxed rats whose adrenal cortices were maintained at normal size by regulated amounts of ACTH. Later, Noble and Collip (1941) were able to confirm the consistent presence of thymic atrophy after the administration of corticotrophin to normal and hypoxed rats. Simpson et al., (1943) found that the administration of purified ACTH to 6 normal rats resulted in a striking reduction in weight and size of the thymus gland and the cervical lymph nodes, as compared to the weight and size of these structures in 6 untreated controls. This was also radically apparent in 5 treated hypoxed animals as compared to 5 untreated hypoxed controls, but was not true in 10 adrexed animals when these were compared with untreated adrexed controls. Similarly, Dougherty and White (1943) showed quite clearly that ACTH injected in CBA strain mice produced a decrease in weight of the inguinal, axillary and mesenteric lymph nodes and of the thymus. Sayers, White and Long (1943) reported similar findings. Still later, Yoffey and Baxter (1946), Robertson (1948), and Baker et al., (1951) showed that ACTH administration resulted in weight loss and atrophic changes in lymphoid tissue. Ringerz,

Fragraeus and Berglund (1952), and especially Dougherty and White (1944, 1945), studied the alterations in lymphatic tissue following the administration of ACTH and the CSs.

Finally, Moon (1947) reported the observation of complete atrophy of the thymus in spayed rats given ACTH. This was in marked contrast to the findings in untreated spayed controls.

1.5. MECHANISM OF ACTION OF ACTH AND CORTICOSTEROIDS ON LYMPHOCYTES

Dougherty and White (1944) found that in mice, rats and rabbits, single injections of pituitary ACTH, aqueous or oily cortical extracts, cpd B or E, produced, within a few hours, lymphopenia and a neutrophilia which they regarded as non-specific, as well as a slight decrease in red cell count and hemoglobin concentration. DOC, on the other hand, was without effect on the blood lymphocyte. At this point they endeavoured to find some reason for the lymphocytolytic and other degenerative changes in the lymphocytes. They noted that the lymphopenia and lymphoid tissue changes were associated with an increase in the serum proteins, which they then decided were

partly antibody globulin, and they concluded therefore that lymphocytes were rich in antibodies.

In 1945, Dougherty and White conducted an elaborate and intensive study, and described marked histologic changes in thymic and lymphoid structures of rabbits and mice following the administration of ACTH and a variety of adrenal cortical preparations and steroids. The changes did not occur in adrexed animals treated with ACTH, nor did they occur in animals given DOC alone. These histologic alterations were grouped by these authors into three stages: (1) the stage of degeneration, characterized by pyknosis of medium and small lymphocytes, edema of lymphatic structures, cessation of mitosis and diminished numbers of lymphocytes in nodes. This stage lasted approximately 6 hours; (2) the stage of repair, beginning at 6 hours and characterized by phagocytosis and the presence of increased numbers of histiocytes and giant cells; (3) the stage of recovery, beginning at about 9 hours in mice, and characterized by mitosis of remaining lymphocytes, maturation of reticular lymphocytes and restoration of normal structure.

When Dougherty and White (1944, 1945) first noted the action of steroids on lymphoid tissue,

they attributed it in the main to a direct action on the lymphocyte and the result was manifested either by the lymphocytes giving off cytoplasmic buds, which they believed to represent the liberation of antibody, or by nuclear damage resulting in pyknosis and cell death.

The consequences of these cellular changes were a dissolution of lymphocytes in thymus and lymph node cells and the peripheral lymphopenia suggested as being due to a failure of delivery of normal numbers of lymphocytes to the circulation from the lymphoid organs (Dougherty and White, 1945). Subsequently, Dougherty (1960) pointed out that destructive effects of CSs on lymphoid cells were not lytic, but karyorrhectic. The authhor suggested the term lymphocytokaryorrhexis to describe the histological alterations seen in lymphoid tissue consequent to exposure to certain CSs.

Yoffey and Baxter (1946), Robertson (1948), and later Baker et al., (1951) attributed the depressant effect of ACTH mainly to the inhibition of mitosis. The formation and subsequent detachment of buds from the cytoplasm of lymphocytes had previously been described by Downey and Widenreich (1912).

Further, there may be other factors than lymphocytolysis responsible for the lymphopenia. Thus, Yoffey, Metcalf, Herdan and Nairn (1951), found that in the guinea pig a single dose of ACTH or of an aqueous ACE was followed after 6 hours by an appreciable increase in the marrow lymphocytes. Subsequently Hudson, Herdan and Yoffey (1952) found that after 7 daily injections of ACTH there was an increase in total marrow cellularity, and though there was no significant increase in the marrow lymphocytes, there was also no diminution. Yoffey, Ancill, Holt, Otwn-Smith and Herdan (1954) then gave repeated injections of cpds E, F and A to guinea pigs; again there was no diminution in marrow lymphocytes, but there was a marked increase in the erythroid population. Compound A differed from the other two substances in that it actually gave rise to a lymphocytosis in the peripheral blood. Fruhman and Gordon (1955), applying a somewhat different technique, obtained a similar type of result in the bone marrow of the rat after the administration of corticosterone. Ehrich and Seifter (1953), reviewing the breakdown of large numbers of lymphocytes, suggested that the significance of lymphocytolysis may be in the delivery of purines and pyrimidines and other split products of nucleoproteins, thus furnishing not only building stones,

but also energy for the synthetic processes.

1.5.1. *IN VITRO* EFFECTS OF CORTICOSTEROIDS ON LYMPHATIC TISSUE

A number of observers studied the action of steroids upon lymphocytes *in vitro*. Heilman (1945) reported that cpd E provoked lymphocyte migration somewhat, while slightly increasing the number of degenerating lymphocytes. Hechter and Stone (1948) noted a rapid decrease in lymphocyte numbers with aqueous cortical extracts, while Reiss, Martens and Schrek (1949) reported similar findings after adding cpds B, F and E acetate. However, Delaunay, Delaunay and Lebrun (1949) observed no significant action of cpd A in this regard. Feldman (1950, 1952) reported a rapid cell damage by adding an oily ACE in relatively large amounts. The authors observed 90% of lymphocytes dead after 3 hours. Hechter and Johnson (1949) found that adding homogenates of adrenal tissue auguments lymphocytolysis, recalling findings such as those of Herlant (1950), or Ickowicz (1953), in the intact animal. Trowell (1953) found that in the concentrations used, cpd E killed lymphocytes, though very much more slowly than *in vivo*. His

cultures grew on the surface of cotton wool in an atmosphere of pure oxygen.

However, a number of conflicting reports have also appeared. Thus, some find that incubation of the cells for a period of time up to 17 hours in a medium containing E had no effect upon the cells (Robertson, 1948; Baldridge, Kligman, Lipnik and Pillsbury, 1951). Others, however, find that when the period is extended, then lymphocyte death does occur (Schrek, 1949; Trowell, 1955). Hechter and Johnson (1949) found that although cell lysis may be brought about rapidly if ACE is used, but after 1 hour of culture very little cell death occurs if cpd E is employed. Elves, Gough and Israels (1954) found that the synthetic hormone, prednisolone, will bring about a considerable fall in the number of cells present in cultures after 24 hours. These discrepancies are no doubt due to variation in culture times and dose of hormone used.

Delaunay et al., (1949) suggest the further possibility that the karyoclasic action of cortical cpds (and adrenaline) was secondary to vascular changes in lymphoid tissue.

The low activity of E *in vitro* may be due to an inability of isolated thymocyte to convert it to cpd F, a reaction postulated to be necessary for cpd E activity *in vivo* (Bush and Mahesh, 1959). The relative steroidal activities obtained with the *in vitro* thymocyte system resembled those reported by others for inhibition of growth of lymphoma cells (Gabourel and Aronow, 1962), and mouse fibroblasts (Ruhmann and Berliner, 1965) cultured *in vitro*. Of interest is the fact that except for E, the relative activities shown by the present experiments correlate well with the known thymolytic potencies of the steroids *in vivo* (Makman, Nakagawa and White, 1967).

Moreover, Makman et al., (1967) reported that DOC, testosterone, Prog and a variety of other steroids without thymolytic potency *in vivo* were also inactive *in vitro* when tested at a concentration of 10^{-5}M.

However, it is evident that for the most part steroid substances do not appear to have as marked or speedy an action on lymphocytes *in vitro* as *in vivo*, which suggests the possibility that when

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However, it is evident that for the most part steroid substances do not appear to have as marked or speedy an action on lymphocytes *in vitro* as *in vivo*, which suggests the possibility that when

administered *in vivo*, metabolic derivatives or interaction with other substances may be responsible for the results observed.

1.5.2. THE IMMUNE RESPONSE

1.5.2.1. The Lymphocyte and Immune Responses

Ehrich (1929) found that in animals injected subcutaneously with a staphylococcus of low virulence, the original lymph node underwent an initial purulent inflammation, and following this, there was a period of lymphatic hyperplasia accompanied by a lymphocytosis in the peripheral blood. During the second and third days, the existing lymphoid nodules of the cortex regressed and showed degenerative changes. At the same time new nodules developed. During the period following there was enlargement of the cortex which culminated in a state of diffuse hyperplasia, the predominating cells being the small lymphocytes. These cells were thought to have been produced in newly formed "pseudo-secondary nodules" from larger cells attached to the walls of the blood vessels. These cells, which were actively dividing, had large round nuclei with fine chromatin and

nucleoli and cytoplasm, which was deeply basophilic. There were transitional forms between these cells in the small lymphocytes. No record was made of any plasmacytic reaction in these nodes, but it is highly probable that these cells were not in fact lymphocytic precursors, but primitive cells of the plasmacytic series. Intense proliferation also occurred in the medullary cords. Germinal centres did not appear, however, until after the blood lymphocytosis and therefore were not considered to be lymphopoietic.

After intravenous inoculation of staphylococcus, extensive lymphoid hyperplasia was found not only in the nodes, but in the spleen and thymus as well — the so-called 'status thymico-lymphaticus' (Ehrich, 1929). Greatest activity in these animals was, however, found in the spleen and the thymus later underwent a temporary regression. It was thought that large numbers of lymphocytes involved were produced in the nodes and spleen, but this work would seem to suggest that many of them could be derived from the thymus and arrive in the nodes by migration, a suggestion that is borne out to some extent by radioactive marker studies. In these animals there was also a marked mesenchymal reaction in other

organs, particularly in the liver and lungs; in the interstitial tissue of these organs there was proliferation of lymphocytes and plasma cells (Elves, 1966).

This sequence of events giving the primary immune response has also been observed by several other workers, and is now well established (Conway, 1938, 1939; Ehrich and Harris, 1942; Habel, Endicott, Bell and Spear, 1949; Harris and Harris, 1949; Marshall and White, 1950; Ringertz and Adamson, 1950; Gyllensten, Ringertz and Ringertz, 1956; Elves, 1966).

1.5.2.2. Anterior Pituitary - Adrenal Cortex and the Immune Response

The regulatory influence of the pituitary-adrenal cortical secretory mechanism on the structure of lymphoid tissue, the importance of these structures in host resistance, including immune phenomena, have encouraged studies of the nature, function of lymphoid tissue, and the biochemical mechanisms underlying the cellular alterations in the lymphocytes consequent to their exposure to elevated concentrations of adrenal steroid. The demonstration that normal

lymphoid tissue contains a protein with an electrophoretic mobility identical with that of serum gamma-globulin (White and Dougherty, 1945, 1946; White, 1948), and that lymphocytes from immunized animals have titrable antibody (Dougherty, Chase and White, 1944; White, 1948, 1958) which is released when the cells undergo lysis, emphasized the role of lymphocytes in immune phenomena, including antibody production. A subsequent recognition that thymus is probably the site of origin of the first immunologically competent cells and that this structure is of fundamental significance in host resistance and host self-recognition (Good and Gabrielsen, 1964), have contributed additional importance to studies of lymphoid tissue structure and function.

There is a great deal of evidence now accumulated which suggests an active role for the small lymphocyte in graft rejection. If it is the small lymphocyte which is a principal mediator of homograft rejection, the process should be seriously upset as a result of procedures resulting in the death of lymphocytes. The effect of such agents as x-irradiation and CSs on the lymphatic system has proved quite beneficial in this respect. Transplantation immunity

may be suppressed by E, either administered systemically (Billingham, Krohn and Medawar, 1951; Scothorne, 1956, 1957), or locally to the graft (Billingham et al., 1951; Billingham, Krohn and Medawar, 1951a). Shepro, Eidelboch and Patt (1960) found that E-treated hamsters were unable to reject grafts of human tumours. Both Shepro and his associates, and Scothorne (1956) found that in E-treated animals, grafting was not followed by the usual changes in the lymphoid tissues. Germinal centres were not increased, nor did the characteristic large lymphoid cells appear.

From the foregoing it is thus clear that the lymphocyte is necessary for the immune response and any form of manipulation which results in a depletion of lymphocytes, either by removing the existing small lymphocytes, or by removing the source of new cells, will lead to a state of immune unresponsiveness. The question of how the lymphocyte participates in immune response is still an enigma. Some recent work is tending to throw some light on the problem. The small lymphocyte itself has not been found to contain antibody although they are capable of protein synthesis (Elves, 1966).

1.6. METABOLIC EFFECTS

Adrenocortical steroids alter rates of metabolism of amino acids, protein and fat, primarily by exerting an inhibitory effect on glucose metabolism. Their anti-inflammatory, general metabolic, and direct effect on adipose tissue also appear to be directly related to inhibition of glucose metabolism. Generally, direct effects of adrenocortical steroids on cells are inhibitory and appear to be related to their ability to alter rates of synthesis of high energy cpds for synthetic purposes. Secondary metabolic effects of exogenously administered adrenocortical steroids such as amino acid metabolism, fat metabolism, increased plasma triglyceride synthesis and increased CO_2 production, can be prevented by administration of glucose. Primary effects, i.e., increased plasma and urinary glucose and glycogen concentrations are accelerated or markedly enhanced by glucose injection.

Of the metabolic changes induced by adrenal steroids, those in protein and nucleic acid metabolism are among the most striking and earliest. Particularly interesting is the fact that the alterations produced in protein and nucleic acid metabolism

following corticoid administration, while extensive in magnitude, are at the same time, opposite in direction in the lymphatic tissue from what they are in the liver. Thus, shortly following injection into rats of cpd E or F, there is an acute stimulation of protein and nucleic acid synthesis in liver (White and Dougherty, 1947; Roberts, 1952; Lowe, Rand and Venkataraman, 1958; Korner, 1960; Leon, Arhenius and Hultin, 1962; Kenney and Kull, 1963; Garren, Howell and Tomkin, 1964; Kidson and Dirby, 1964; Lang and Sekeris, 1964a, 1964b; Peña, Dvorkin and White, 1964, 1966; Barnabei, Romano and DiBitonto, 1965; Greenman, Wicks and Kenney, 1965; Kenney, Wicks and Greenman, 1965), and a similar early but diminished degree of formation of these macromolecular constituents in lymphoid tissue, notably the thymus (Fiegelson, 1964; Peña et al., 1964, 1966; Brinck-Johnsen and Dougherty, 1965; Gabourel and Fox, 1965; Stevens, Colessides and Dougherty, 1965, 1966; Nakagawa and White, 1966; Makman, Dvorkin and White, 1966a).

It was demonstrated that adrenal cortical secretory activity has a marked influence on the mobilization of protein from lymphoid tissue of the mouse (White and Dougherty, 1947; White, 1949).

On the basis of studies with administered radioactive glycine, it was suggested (White, Hoberman and Szego, 1948; Hoberman, 1950) that the action of adrenal cortical steroids *in vivo* on tissue protein was not one of accelerating mobilization of protein from peripheral sources to the liver, but rather an anti-anabolic influence exerted at the level of the cellular pool of free amino acids. The net change, a loss of cellular protein from peripheral tissues including the lymphoid organs was a result of this anti-anabolic action of the steroid hormone.

Peña et al., (1964, 1966) reported that cell-free homogenate prepared from thymic tissues of rats injected with F 3 hours prior to sacrifice, showed an impaired capacity to incorporate amino acid to microsomal or ribosomal protein. Preliminary studies (Nakagawa and White, 1966) have indicated that this impairment was accompanied by a diminished RNA polymerase activity of thymic nuclei. Makman et al, (1967) concluded that the exposure of thymocytes to F *in vivo* or *in vitro* results in inhibition of RNA and protein synthesis, and a decreased transport of nucleic acids and protein precursors into thymocytes incubated *in vitro*. These two effects are apparently dissociable.

These inhibitory effects of F are manifest only in the presence of an energy source in the medium. Thus, cpd F injection results in an early decrease in activity of thymic DNA-dependent RNA polymerase followed by a diminished degree of synthesis of ribosomal protein. Inhibition of protein synthesis could, in turn, lead to (a) less RNA polymerase enzyme, (b) less membrane transport protein, (c) less DNA polymerase enzyme and less DNA template. It is also possible that the primary action of F in thymus is to inhibit ribosomal protein synthesis in a manner not mediated by a prior RNA synthesis. In support of this suggestion are the recently reported studies of Peña et al., (1966), who reported a marked early inhibitory effects of in vivo exposure to F on nucleic acid and protein synthesis.

Recently, Knutson and Lundin (1966) have suggested that reutilization of DNA in lymphoid tissues, particularly in the thymus, is a normal mechanism and that it is increased under the influence of adrenal cortical steroids.

Evidence assembled in recent years supports the suggestion that hormonal augumentation of

protein synthetic ability by ribosomal preparations from a number of tissues is reflected in an accelerated rate of RNA synthesis, including the RNA with a rapid turn-over, inferred to be messenger RNA. Also after cpd F injection, at a time when the activity of a cell-free amino acid incorporating system in liver was markedly stimulated, this activity was decreased in a comparable system from thymus (Peña et al., 1964, 1966).

1.7. SUMMARY AND CONCLUSIONS

The degree to which various cellular elements of lymphoid tissue respond to stress is controlled by the pituitary-adrenal cortex relationship. The sequence of alterations produced in the structure of lymphoid tissue as a result of increased adrenal cortical secretion is the basis of some of the important functions of lymphoid tissue.

Lymphoid tissue must contribute to the normal defense of the "stressed" organism in at least three ways:

(1) by an increased release of globulin from lymphocytes, thus providing precursor for CHO synthesis, and also perhaps for serum albumin formation in the liver. An increase

of protein in the blood would aid in maintenance of normal blood volume;

(2) by releasing antibody globulin in the immunized animals;

(3) by an increased production of macrophages in the lymphoid structure.

A dose of any active GCC or of ACTH in an animal with intact adrenals leads very rapidly to an edema of the lymphoid tissue. Indeed, this effect can be observed within an hour of administration of the steroid. With continued steroid administration, the lymphoid cells progressively diminish in number, although the reticular tissue of the lymphoid follicles is not significantly affected. Surviving lymphocytes show evidence of degeneration and there is a marked fall in the number of mitosis visible. Eventually a severe lymphoid atrophy occurs and all lymphoid tissue throughout the body, including the spleen and thymus, takes part in the general lymphoid involution. There is thus brought about a very considerable diminution in the bulk of lymphoid tissue available to form antibodies. Not only is lymphoid tissue atrophied, but steroids provoke an early and pronounced lymphocytopenia.

Cortisol depressed multiplication of lymphoblasts — thus leading to their maturation — and

kills lymphocytes. Cortisone does not have this effect. Within the cell an equilibrium exists between the active cortisol and inactive cortisone. The interconversion between these two CSs is enzymatic and requires either DPN or TPN as co-enzymes. This similar relationship holds for prednisolone and prednisone.

2. THE EFFECT OF THE ADRENAL CORTEX ON MALIGNANT LYMPHATIC TISSUE

2.1. EARLY EXPERIMENTAL WORK

The use of the hormones, ACTH and cpd E, as a palliative treatment of Lk is an outcome of much earlier work on the ACTH and CSs on lymphoid tissue as cited in the previous section. That the adrenal gland may play an important role in the pathogenesis of lymphoid Lk has in fact been demonstrated by several studies.

In the last two decades much of the attention has been focused on the effects of manipulation of the pituitary-adrenal axis on the peripheral leukocyte numbers. Thus it is now clear that administration of adrenal cortical steroids, particularly those oxygenated at C-11, or activation of the adrenal cortex by ACTH, or as a result of stress, almost

invariably evokes a peripheral lymphopenia, eosinopenia and neutrophilia (Dalton and Selye, 1939; Dougherty and White, 1943, 1944, 1945; Hills, Forsham and Finch, 1948). The processes responsible for this triad of response have not been resolved fully, and it is apparent that the mechanisms differ to some extent, depending on whether the treatment is applied acutely or over a sustained period of time. The peripheral PMN leukocytosis, an almost invariable component of the response to CS or ACTH treatment, is probably the result, at least in part, of an increased production of these elements within the bone marrow (Quittner, Ward, Sussman and Antopol, 1951; Yoffey et al., 1954).

It was the lymphocytic suppressing properties of the adrenal factors that probably first suggested their use in the treatment of animal blood dyscrasias. Thus, Murphy and Sturm (1944) observed the ability of adrenal cortical factors and pituitary extracts, containing ACTH activity, to inhibit the growth of transplanted LLk cells in rats. Similarly, adrex was found to increase greatly the susceptibility of rats to this type of transplantable tumour (Sturm and Murphy, 1944). Along the same lines, favourable

results were reported with E in inhibiting the growth of malignant lymphoid tumours in mice (Heilman and Kendall, 1944). Law and Spiers (1947), by similar means, induced regressions in the enlarged lymphatic organs of mice affected with spontaneous LLk. On the other hand, adrex has been shown to increase the incidence of LLk in mice (Law, 1947) while 11-oxygenated corticoids delay the incidence of spontaneous (Kaplan et al., 1951; Upton and Furth, 1953; Woolley and Peters, 1953) and radiation-induced LLk (Kaplan et al., 1951).

2.2. EFFECT ON HUMAN LEUKEMIA

2.2.1. ACTH AND CORTICOSTEROIDS

Reports soon appeared attesting the ability of ACTH and cortical steroids to induce remissions in leukemic states in human beings (Pearson, Eliel, Rawson, Dobriner and Rhoads, 1949; Pearson and Eliel, 1950, 1951; Rambert, 1950; Farber, Schwachman, Toch, Downing, Kennedy and Hyde, 1950). It is now recognized that ACTH and oxysteroids are useful members in the armamentarium available for the palliative treatment of such diseases as CLLk, lymphosarcomas and acute MLks. The mechanism of action of

pituitary and adrenal factors in these situations is not as yet fully known. In the LLks, it seems probable that the ameliorative effects must stem, to some extent, from the destructive (catabolic) effects of the steroids on the lymphoid growths, as well as from the inhibition of the anabolic reactions responsible for the synthesis of both cytoplasmic and nuclear cpds important in the fabrication of lymphoid cell structure (see earlier sections in this chapter). Correction of a maturation defect in the granulocytic elements, probably mediated indirectly, may provide the basis for whatever beneficial effects accrue with the cortical factors in the acute MLks. It has been suggested that the steroids may operate by mobilizing factors such as metabolites or enzymatic components which are lacking in the leukemic state (Furth, 1951; Pearson, Li, Maclean, Lipsett and West, 1955).

A discussion of the mechanisms of hormone action in cellular proliferative processes should include the ability of endocrine factors to alter certain fundamental aspects of metabolism. Thus adrex in the rat results in a significant increase in the glycolytic activity of the bone marrow associated

with an increase in the myeloid-erthyroid ratios and in the relative immaturity of the granulocytic forms (Gordon, 1954, 1955). Treatment with ACE lowers the rate of glycolysis and restores the morphological features of the marrow to normal. Since chemical changes must of necessity precede the morphological ones in cellular differentiation processes, it is possible that the ameliorative effects of the steroids in Lk may derive from their capacity to modify abnormal metabolic activity (e.g. glycolytic-respiratory ratios) in the blood-forming cells. Possibly, adrex leading to tissue ischemia, sparks a glycolytic mechanism within the blood-forming organs that is conducive to excessive leukocytic and diminished erythrocytic proliferation.

Cortisol inhibits mitoses among the lymphoblasts and causes a reduction in the volume of their cytoplasm, thus leading to rapid maturation of the immature cells. If the lymphoblasts oxidize F to E, thus converting it into an inactive cpd, immaturity of the cell is maintained. The effect of F on medium-sized and small lymphocytes causes a further shedding of cytoplasm, decrease in size of the nucleus and finally karyorrhexis. In all these effects E has

less than 1/70 of the activity of F and even this low activity may be due to the conversion of E to F within the cell.

Normal lymph cells cannot catabolize E or F: they can only convert the one into the other. In contrast normal fibroblasts and leukemic cells metabolize both steroids fairly rapidly and within a few hours much of the given CS is metabolized into ineffective compounds. The liver also takes part in these catabolic processes of E and F, though most of the CS appears to be catabolized by the mesenchymatous tissue.

These observations by Dougherty and White (1943, 1944, 1945, 1947), are supported by clinical findings on patients treated with CSs. If the patient responds, a reduction in the number of mitoses among the leukemic cells in the bone marrow can be demonstrated. There is no specific delay in any phase of the mitotic division, but the cells are prevented from entering mitosis, hence the lowered mitotic rate. Numerous small lymphocytes with pyknotic nuclei appear in the circulation, as was observed repeatedly in children with ALk, when they responded.

In fact, at this stage of a response, the blood film may give the impression of a subacute or almost CLLk owing to the preponderance of small lymphocytes. This observation applies to any of the CSs, whether E itself or prednisone or triamcinolone is used.

The question present itself: can the failure to respond to CSs be correlated with the conversion of the active 11-OH-, into the less active 11-keto- form? This may apply to patients who either never responded to the steroids or became 'resistant' to them after an initial remission. Little is known about the state of the two co-enzymes (DPN or TPN) involved in this enzymatic conversion, that is, whether the reduced or the oxidized form is prevalent in the leukemic cells. If the oxidized forms of the co-enzymes are prevalent, the equilibrium between E and F would be shifted in favour of the oxidized (inactive) E; on the other hand, if the reduced co-enzymes are prevalent, the equilibrium between the two forms of CSs would be shifted in favour of the reduced (active) F. Both co-enzymes are involved in many other metabolic processes which affect the state of co-enzymes and thus the position of the equilibrium between E and F. A similar relationship holds for prednisolone and prednisone.

Basic to the understanding of the influence of hormones in Lk would be a knowledge of the reasons why greater success is obtained in children afflicted with the acute MLks and why refractoriness to these agents develops in most cases despite their continued administration. The liver and the adrenal undoubtedly participate in this phenomenon for it is well recognized that these organs operate, under both normal and pathological conditions, in converting active steroids into others with little or no physiological activity.

Inasmuch as the ACTH and the CS were known to produce their chief effect on lymphoid tissue they were first employed in cases of CLLk and of lymphosarcoma. Rapid involution of the enlarged organ followed, but few striking hematologic remissions. No success was achieved in acute monocytic or myelomonocytic or in CGLk, but the best remissions, rather surprisingly, ensued in acute lymphocytic and granulocytic Lk, no difference being noted in the response between these two forms. On the other hand, the possibility that ACTH and the CSs actually accelerate the course of the AGLk had been broached by Rosenthal, Saunders, Schwartz, Zannos, Perez-Santiago and Dameshek (1951); and Haut, Altman, Cartwright and

Wintrobe (1955). However, this does not seem to hold true when massive doses of CS are used (Granville, Rubio, Unugur, Schulman and Dameshek, 1958). It was further found that remissions were temporary and that resistance was rapidly acquired, but nevertheless the advent of the hormones had opened a distinct new phase in the chemotherapy of Lk. Whereas previously most therapeutic agents had been general poisons which damaged cells particularly in mitosis, ACTH and E were normally produced in the body, or closely related to those normally secreted by the body, and were furthermore not myelotoxic — in fact, were possibly myelostimulatory. A considerable body of facts is known about the physiologic effects of these substances, but the precise mode of their action in Lk has not yet been elucidated.

Remissions following E or ACTH administration may be complete or incomplete, the former comprising a restoration of the blood and marrow to a normal composition. Careful quantitative studies of the marrow under treatment (Cramer, 1952; Gasser and Cramer, 1953; Marmont, 1955) have shown that the first change is a striking diminution of mitoses in the blast cells. No definite alteration in the cellular

distribution appears for the first week, after which there is an increase in the numbers of normal erythroid and myeloid precursors and in their mitotic rate which rises to a peak. Findings such as these, together with reticulocytosis and rise in platelets during remissions (Rosenthal, 1951), have produced the suggestion that the hormones, while inhibiting leukemic, and especially lymphocytic leukemic cells, may indeed stimulate normal cells to proliferate (Schulman, 1951; Gasser and Cramer, 1953). Such a view is difficult to test experimentally, but the facts which are known about the mode of action of the hormones makes it appear unlikely that they can stimulate cellular proliferation. Thus, normal human bone marrow shows no change in total cellularity, morphology or cell distribution during steroid therapy (Rosenthal et al., 1951); long-term administration of ACTH to rats, however, causes a great diminution of mitotic activity and cellularity in the marrow, spleen and thymus (Robbins, Coöper and Alt, 1955). Stress, which leads to excess adrenocortical function, produces a pronounced fall in mitotic activity (Bullough, 1952, 1955, 1962, 1965), and while certain transplanted animal neoplasms may show an unusually rapid proliferation under E treatment, this effect has been

attributed to inhibition of the cells which normally produce antibodies to the transplanted tumour, rather than to an actual growth stimulation (Green and Whiteley, 1952). In vitro, E has a direct destructive effect on lymphocytes (Heilman, 1945) but not on normal human marrow or the granulocytes of CGLk (Krippaehne and Osgood, 1955). Other references on the in vitro effect of E on lymphocytes were given earlier (see section 1.5.1. in this chapter).

It would appear likely that the changes in the hematopoietic organs and the blood which have been observed to follow hormone administration in vivo are an expression of the general metabolic alterations which are a well-known accompaniment of such therapy. Some loss of BW is often found, unless there is marked water retention, but an extreme muscular wastage following large doses of E is observed. There is a pronounced negative P and N balance which correlates well with the fall in the leukocyte count and the improvement in the patient's general condition. As E is withdrawn, a striking retention of P occurs (Adams, Valentine, Bassett and Lawrence, 1952). Uric acid excretion is high during the fall in leukocyte count (Snelling, Donohue, Laski and Jackson, 1951), and this is followed a few days

attributed to inhibition of the cells which normally produce antibodies to the transplanted tumour, rather than to an actual growth stimulation (Green and Whiteley, 1952). In vitro, E has a direct destructive effect on lymphocytes (Heilman, 1945) but not on normal human marrow or the granulocytes of CGLk (Krippaehne and Osgood, 1955). Other references on the in vitro effect of E on lymphocytes were given earlier (see section 1.5.1. in this chapter).

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later by an increased creatinine excretion which appears to accompany the disappearance of blasts from the marrow. Those findings might be interpreted as being the result of the anti-anabolic action of E and ACTH — an action which would consist in a failure to make available the "building blocks" required for proliferation of the pathologic cells, with cell death as a consequence, rather than in an actual cell destruction.

2.2.2. PREDNISONE, PREDNISOLONE AND 9-ALPHA-FLUORO-HYDROCORTISONE

The synthetic steroids prednisone and prednisolone, introduced in 1955 (Bollet, Black and Bunim, 1955; Dordik and Gluck, 1955; Margolis, Barr, Stolzer, Eisenbreis and Martz, 1955) are closely related in structure to E, but have increased potency with minimal salt-retaining properties. Their effects in Lk appear to be very comparable to those of ACTH and E and the relative freedom from salt and water retention when they are used has led most clinicians to prefer them, especially when long-term or high-dosage treatment is envisaged. The anti-leukemic activity of prednisone and prednisolone is from 2 to 5 times that of E. Whichever steroid is used,

continued maintenance treatment at a decreased dosage may be given after remission has been induced, and this is probably the best course of action when the improvement is partial. When complete remission occurs, the treatment may be withdrawn until signs of relapse appear, in the hope that the inevitable development of a resistant state may be postponed. Although such remissions commonly last only a few weeks, occasional cases remain in full remission for many months. Since infections are frequent in leukemic patients and liable to be exacerbated by steroid therapy, antibiotics are usually given concurrently with the steroids.

Attempts have recently been made to influence the course of myeloblastic Lks and chronic myeloid Lks in a terminal myeloblastic phase by massive doses of steroid hormones. Complete clinical and hematological remissions were induced in some cases by from 3 to 5 gm of E in a single injection, or 1 to 3 gm daily by mouth for 2 to 4 days. (Bernard and Deltour, 1953; Bernard, Bilski-Pasquier and Deltour, 1954). Secondary effects in this series of patients were apparently not marked or dangerous despite the huge dosage employed. Similar studies have been reported in

which 9-alpha-fluoro-F, a halogenated derivative of F, was used. Biological studies of this cpd, which was synthesized initially by Fried and Sabo (1953), showed that its activity was between 10 and 25 times that of E as regards glycogenesis, Na and K regulation and other metabolic effects (Liddle, Pechet and Bartter, 1954). Hill and Vincent (1955) used this material in the treatment of 16 cases of ALk. The authors reported that there was evidence of increased erthyropoietic activity, with peripheral erthyroblastosis in several cases, while in others the rapidity of the platelet increase suggested a direct stimulatory effect.

Little significant difference exists in the biologic potency of prednisolone compared to F on either tumour inhibition or glycogenic activity in C3H mice (Glenn, Richardson, Bowman and Lyster, 1960). In another study using C3H mice, Glenn, Miller and Schlagel (1963) observed that inhibitory effects of C_{21} steroids on tumour growth are apparently in the presence of or after continued administration of steroid. Withdrawal of steroid leads to resumption of tumour growth. Steroids merely induce temporary restraining influence on cellular processes, and effects are reversible after their withdrawal.

2.3. SUMMARY

Although various effects of ACTH and the CSs make them invaluable for the everyday treatment of the Lks and of the various complications resulting from treatment with myelotoxic chemicals and ionizing radiation, nevertheless, it should be kept in mind that the CSs, although not myelotoxic, may be harmful in other respects. Their continued use, particularly in the elderly, leads to serious osteoporosis and not infrequently to pathologic fractures. There is furthermore the danger of increased susceptibility to infections which may be a very serious problem, particularly in the late stages of CLLk, where the severe neutropenia and the frequent hypogammaglobulinemia make infection even more likely. With large or massive doses of the CSs, as in ALk, gastritis and peptic ulcer may come to the forefront as problems. Large doses of the CSs may also bring abnormal psychological reactions (depression, extreme hyper-irritability, insomnia, etc.), and these may constitute a limiting factor to their further use. With prednisone, as opposed to E, K depletion and Na retention are generally minimal, even in large dosage, although some patients accumulate considerable edema, requiring diuretics.

The remissions induced by the CSs may be complete or partial and may persist for years or for only a few weeks, depending on the extent of the leukemic process, its degree of malignancy and such other factors as possible reactions induced by the steroids themselves.

The management of ALk, particularly in childhood, must be considered amongst the most distressing chapters of human experience. Here we have youngsters — usually very bright and attractive — for whom a remission can be promised and almost always takes place. During this reversal in course, the child feels and looks well, eats and plays like a normal youngster, is to all intents healthy — but one knows that his days are numbered. In a few months, or a year, rarely two years, the child will be dead. This is the tragedy of our present-day treatment: remissions can be induced; complete reversal of the proliferating process is not only possible but highly likely; but ignorance of how to maintain the reversal permanently remains.

In CLLk, the place of steroids is even less evident. The effect on lymphatic tissue is

likely to be unimpressive unless very large doses are used, and this is undesirable in any chronic disease unless dramatic suppressive effects are being obtained. Acute exacerbations are less likely in the chronic form of Lk, but a progressive anemia may prove troublesome. This may usually be due partly to hemolytic anemia.

In recent years, significant increases in survival were achieved by combination therapy and by utilizing certain dose schedules. This was discussed in sections 2.5.1.6. and 2.5.1.7 of Chapter II.

2.4. SEARCH FOR SYNTHETIC ANALOGUES OF THE CORTICOSTEROIDS

2.4.1. INTRODUCTION

As indicated earlier, the CSs play an important role at the present time in the therapy of various forms of Lk. Because it is often necessary to administer these hormones in high dosage or for long periods of time, side effects, particularly those involving Na and water retention and K excretion, become apparent and often sufficiently serious to

limit further therapy with these substances. For reasons such as this, a search for synthetic analogues of the CSs was undertaken with the result that cpds were obtained whose anti-leukemic activity was several times that of the naturally occurring CSs coupled with a relative freedom from salt and water retaining properties. The search for such cpds will be described in this section.

The steroid originally administered in the first extensive trials was cortisone acetate (Hench, 1948), and for several years this was the only adrenal steroid available for clinical trials. This was not a great disadvantage for E, which is readily and almost completely absorbed when given by mouth, is freely converted into the natural and active steroid F within the body. Cortisol acetate is less soluble than E and is less efficiently absorbed. However, E itself, is probably inactive, exerting its therapeutic effects only by virtue of its conversion into F.

The first synthetic modification produced was, in fact, F itself (Wendler, Graber, Jones and Tishler, 1950), the hormone normally secreted by

adrenal cortex. Although it is the main CS in the human (Peterson, 1956), its therapeutic or pharmacological activity is only about 25% greater than that of E. Because of this, it was assumed at that time that F was the ultimate in potency and that no further improvement over nature was possible. However, the great interest which was shown in these two steroids eventually brought to light more potent CSs and a very large number of pharmacological actions, some of which were desirable, such as the anti-inflammatory effect, and some less desirable or definitely disadvantageous, such as the Ca-depleting effect on bone and the Na-retaining effects (Cope, 1965).

2.4.2. ALPHA HALOGENATION

Thus, during the years 1953 and 1954, Fried and Sabo (1953, 1954) were exploring various methods of synthesizing F for commercial production. One method being used involved the production of 9a-Br-F-acetate, which could later be reduced to form F-acetate. The high activity of this brominated cpd was unexpected and it led to a full exploration of the properties of a full range of steroids in which a halogen was attached to the 9-carbon. They found that

certain halogenated intermediate cpds, especially the 9a-Cl- and the 9a-Fl- derivatives of F showed a high degree of glucocorticoidal activity. That they also possessed very strong mineralocortical activity was shown by Borman, Singer and Numerof (1954).

2.4.3. C_1 - C_2 DEHYDROGENATION

Another major advance was the introduction of two new analogues now called prednisone and prednisolone, but originally introduced in 1955 (Herzog, Payne, Jernek, Gould, Shapiro, Oliveto and Hershberg, 1955), with the names metacortandracin and metacortandralone respectively. These two cpds differed only from their respective parent cpds, E and F, in having a double bond between the 1- and 2-carbon atoms.

Thorn, Renold, Morse, Goldfien and Reddy, in 1955, compared the effects of prednisone and prednisolone with therapeutically equivalent quantities of E and F, and found them not very different. They concluded that prednisolone and prednisone have definite Na-retaining activity and that it is of approximately the same order of magnitude as that of F.

But wider clinical experience soon led to the more general recognition that, whereas prednisone and prednisolone were neither of them freed completely of the Na-retaining effect, yet this was nevertheless appreciably reduced and, as a result, edema was a much less frequent complication than when E was used in equivalent therapeutic dosage. The Na-retaining effect is a particular disadvantage in all those subjects who have or are prone to develop congestive heart failure.

Because of their relatively small Na-retaining effects relative to GCC or anti-inflammatory effects, prednisone and prednisolone have achieved wide and generally justified popularity for CS therapy, and have maintained this against many of the more recently introduced analogues.

It is well to be quite clear that prednisone and prednisolone both obey the general rule that a steroid which is effective as an anti-inflammatory agent will inhibit the patient's own adrenal cortex. This has been well recognized since the introduction of these cpds. Bunim, Pechet and Bollet (1955) observed a fall in 17-KS as a result of their

administration, so also did Orr, di Raimondo, Flanagan and Forsham (1955).

The next encouraging development was the finding that the effects of different modifications were additive and that the prednisone and the 9a-Fl changes could be combined in the same molecule with enhanced effect.

This success by two separate methods of enhancing the potency of CS, by introducing a double bond between 1- and 2-carbon atoms (C_1 - C_2 dehydrogenation) as in prednisone, and by introduction of a fluorine atom on 9-carbon atom, as in 9a-Fl-F, naturally raised the possibility that introduction of both modifications into the same molecule would still further improve the potency by a summation of the separate effects. Accordingly, the cpd delta-1-9a-Fl-F was prepared by Fried, Florey, Sabo, Herz, Restivo, Borman and Singer (1955), and by other groups. In practice it has been found not to differ very greatly in its action from the simpler 9a-Fl-F. Thorn et al., (1955) compared the two steroids and found that both cpds showed essentially the same degree of potency in suppressing urinary CS excretion, an effect indicative of their similar glucogenic activity.

A somewhat different assessment of its potency relative to 9a-Fl-F was obtained by Li, Bergenstal, Shricker and Graff (1956). This group concluded that delta-1-9a-Fl-F had protein catabolic and eosinopenic activities twice as great as did 9a-Fl-F. These GCC effects were 5 times as great as in prednisone. They found it valuable for replacing the MCC deficiency in Addison's disease.

This steroid, of course, by virtue of its high GCC activity, is very potent in inhibiting ACTH secretion and in inducing adrenal suppression. It shares this property with 9a-Fl-F. Both these cpds, can therefore be used to produce almost complete suppression of adrenal F production (Liddle, 1956). These steroids can also be very useful clinically for providing maintenance in patients seriously suspected of impaired adrenal function, so that the response to ACTH can be studied without hazard to the patient or interference by the maintenance steroid with the urinary adrenal steroid output derived from endogenous sources.

2.4.4. 2- AND 6-METHYL ANALOGUES

Hogg, Lincoln, Jackson and Schneider (1955) introduced an interesting variant of F, 2-methylcortisol. That this cpd possessed intense MCC activity was shown by Liddle and Richard (1956), and by Byrnes, Barnes, Bowman, Dulin, Morley and Stafford (1956). The former group also showed that this cpd was a GCC and produced an eosinopenia, and that it could suppress adrenal-cortical activity. But in these actions it did not differ appreciably from non-methylated parent cpd F.

With the discovery of the profound effect which methylation of the 2-carbon has on the pharmacological action, it was natural to try the effects of this modification on the already potent steroid 9a-Fl-F. The resultant cpd 2-methyl-9a-Fl-F was tested by standard assay methods on rats by Byrnes and his co-workers (1956) and found to be a cpd of quite exceptional potency. In glycogen deposition tests it was found to be about 40 times as potent as F. When tested for Na-retaining power on rats, it was found to have about 35 times the potency of 2-methyl F, and so far as could be judged, about 90 times the potency of DOC-acetate. It thus revealed

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itself as the most potent Na-retaining and K-depleting MCC so far discovered. Swingle, Brannick and Parlow (1955) found it very potent in reviving adrexed dogs from severe insufficiency.

The introduction of a methyl grouping on the 6-carbon atom was done by Spero, Thompson, Magerlein, Hanze, Murray, Sebek and Hogg (1956). A complementary study of the 6a-methyl-CSs was made by Liddle (1958) in the human subject, and broadly similar results were obtained to the experiments of Dulin, Barnes, Glenn, Lyster and Collins (1958) in lab animals. It was soon evident that the effect of introducing the 6a-methyl radicle was not a constant one. With some steroids it enhanced potency; with others it brought no change, or even reduced it.

Several investigators discovered that the side effects of 6-methyl prednisolone were much the same as those to be expected from the use of other CSs (Goldberg, 1958; Yunis and Harrington, 1958; Soffer, Wolf, Moses, Silbersweig and Gabrilove, 1958). Many, however, noted the almost complete absence of Na-retention.

In general, the slightly higher

potency is not a real advantage, and no significant difference has emerged between this steroid and the prednisolone from which it is derived.

2.4.5. 16-HYDROXYLATION AND METHYLATION

The introduction of a OH-group on the 16-carbon atom of the CS molecule showed a beneficial influence. Bernstein, Lenhard, Allen, Heller, Littell, Stolar, Feldman and Blank (1956) using a microbial method, introduced 16-OH grouping into F. Earlier, Hirschman, Hirschman and Farrel (1953) showed that 16-hydroxylation of DOC eliminates entirely the Na-retaining potency of this steroid.

2.4.5.1. Triamcinolone

Bernstein et al., (1956) prepared the cpd 16a-OH-9a-Fl-delta 1-cortisol and found it to be a very potent GCC with no detectable Na-retaining activity at all. Indeed, it tended to cause a Na diuresis. This is the steroid now called triamcinolone.

Freyberg, Bernstein and Hellman (1958) made a careful analysis of its value and of the

adverse effects resulting from its use. It was found to have an equal potency with prednisone. Triamcinolone diacetate seemed to be slightly better.

2.4.5.2. Dexamethasone

The introduction of a methyl group on the 16-carbon atom was made possible by Arth, Johnston, Fried, Spooner, Hoff and Sarrett (1958). Later, Oliveto (1959) found that the introduction of this radicle into the molecule increased by about 3 times the anti-inflammatory power as assayed in the rat, giving the case of 16a-methyl-9a-fluroprednisolone, a cpd 190 times as potent as F. Those activities are, however, not quite so high in the human subject, and, judged by anti-arthritic potency, the latter cpd is about 26 times as active as F.

Boland (1958, 1960) considers that the overall incidence of adverse reactions from dexamethasone is about the same as with prednisolone when equivalent doses of the two drugs are given. The large doses of dexamethasone also caused a markedly negative Ca balance, the loss being much more in the feces than the urine (Bunim, Black, Lutwak, Peterson and Whedon, 1958).

2.4.6. SUMMARY

In summary, cortisone and its synthetic analogues, when administered systemically, exert their influence upon the mesenchymal tissue, inhibiting its new formation (Schiller and Dorfman, 1957). The changes which they cause in the tissue are reduced fibroblastic activity, reduced fibrocyte counts, reduced quantity of fibrin, inhibited endothelial regeneration, inhibited formation of collagen fibrils, and reduced vascularization. Moreover, the capillary permeability is reduced, as is also the leukocyte and macrophage counts, and in general an inhibitory action upon the reticulo-endothelial cells. Their effect on lymphatic tissue has already been described in an earlier section of this chapter.

Corticoid preparations have no direct effect upon micro-organisms, but the above-mentioned action may promote the spread of an infection and at the same time facilitate the action of antibiotics upon micro-organisms.

CHAPTER IV

MEASUREMENT OF HORMONES IN BLOOD BY METHODS INVOLVING PROTEIN BINDING

1. INTRODUCTION

In experimental as well as in clinical endocrinology, it is often necessary to assess the ability of an endocrine organ to perform its function. This may sometimes be done post mortem, but it is frequently undesirable for the experimental animal and never really satisfactory for the human subject. Methods for determining hormones and their metabolites in body fluids have therefore been extensively developed. The higher concentrations frequently found in urine, and its ready availability in large quantities, inevitably led to the development first of methods for determining hormones in urine. However, the conversion of some hormones to a large number of metabolites, which are then excreted in the urine, frequently made these analyses either complicated and time-consuming or difficult to interpret in terms of endocrine function. A further handicap is that the quantities of metabolites excreted can only represent the average excretion and the average endocrine activity during

any given time and when the organ is subject to short bursts of abnormal activity, analyses only of urine collected during a period may not be helpful.

For this reason many workers have preferred to determine the hormone content of the blood in the belief that this may give information as to the functional state of the endocrine organ at the precise time when the sample is collected. This somewhat naïve view has not been entirely confirmed in practice, for the concentration of a substance in the blood is the resultant not only of the rate at which it is secreted into the blood, but also of its distribution into the tissue, its metabolism into other compounds and the rate of its removal. For this reason, it seems that, except when the hormonal content of a systemic blood sample is compared with the content of the endocrine organ involved, possible in certain animal experiments and sometimes on human subjects during surgical operations such as adrex, a complete picture of endocrine organ function is likely to be obtained only by investigating both blood and urine.

2. THE PROTEIN-BINDING OF ANTIGENIC HORMONES (IMMUNOASSAYS)

Historically, the immunoassays of hormones were the first of the protein-binding assays to be developed and therefore will be discussed first.

Until recently, assay of hormones in blood was achieved in most instances by bioassay techniques which were difficult to perform and gave results which were neither precise nor easily reproduced. The use of immunological methods has now revolutionized the technique of assay of blood protein hormones. Such methods are specific, sensitive, precise, and are readily applicable to the assay of large numbers of samples at a time.

Originally employed for the immunoassay of insulin (Yalow and Berson, 1959), this method has been applied to the measurement of glucagon (Unger, Eisentraut, McCall, Keller, Lanz and Madison, 1959), human growth hormone (Utigar, Parker and Daughaday, 1961, 1962; Hunter and Greenwood, 1962, 1962a; Glick, Roth, Yalow and Berson, 1963; Roth, Glick, Yalow and Berson, 1963), parathyroid hormone (Berson, Yalow, Aurbach and Potts, 1963), and

ACTH (Felber, 1963; Yalow, Glick, Roth and Berson, 1964), in plasma. Although the general principles apply in all cases, technical details vary with the different hormones because of individual characteristics. Success in the application of the method for a new hormone (or other substance of biologic interest) may depend upon exploiting or overcoming the unusual properties of the hormone.

The concentrations of the protein hormones in plasma do not generally exceed about 10^{-10} M when the secreting gland is at rest, but concentrations may rise 10-50 fold following appropriate physiologic stimulation or in the presence of a hormone-producing tumour. Since the concentration of serum proteins is of the order of 10^{-3} M, it is evident that both a high degree of sensitivity and a high degree of specificity are required for the detection and measurement of protein hormones in plasma.

It is an important factor in determining the sensitivity and precision of an immunoassay to select an appropriate antiserum and concentration of labelled hormone to use in the assay. Labelled hormone of high specific activity is desirable because the higher the specific activity of the labelled

hormone preparation, the lower the concentration of labelled hormone required to provide the desired concentration of radioactivity. This leads to a greater sensitivity and greater precision of the assay.

There are several reasons for desiring high sensitivity. In the first instance a certain minimum degree of sensitivity is required simply for detection of the low concentration of hormone usually present in the circulation. However, there may be a need for increasing the sensitivity still further by a factor of 10 or 20 for what may be a rather unique reason, namely, the tendency of nearly undiluted plasma to damage labelled hormones. In the case of ^{131}I-labelled insulin, at least part of this damage is attributable to the reduction of the disulfide linkages. The precise nature of the other changes that take place in labelled insulin and of those that occur in other labelled hormones lacking disulfide linkages has not been clarified, but the net effects are the release of a small fraction of the radioactivity as iodide, and the production of labelled components that bind more or less indiscrimmately to normal plasma proteins, particularly alpha-globulin (Berson, Yalow, Bauman and Rothschild, 1956). Damage to the labelled hormone following incubation

with plasma is considerably less troublesome when the plasma is diluted significantly. Vigorous attempts have been made to improve the sensitivity to a level that permits the assay of hormone in plasma diluted at least at 1:10 to 1:20 (Berson, Yalow, Glick and Roth, 1964). At this dilution, damage to ^{131}I-insulin usually remains below 4 to 5% and to ^{131}I-labelled human growth hormone (HGH) below 10%. The authors also reported that in order to minimize the damage by incubation, the assay is routinely carried out at 4°C.

Since the principle and the application of the radioimmunoassay of plasma insulin was first described, the assay of some 18 peptide hormones has been described utilizing this principle. The principle has been applied to non-antigenic hormones also (e.g., steroids using the specific binding proteins of the plasma), and these will be discussed later.

2.1. PRINCIPLE

Immunoassay methods depend upon highly-specific reactions between a protein hormone and its antibody. This technique is therefore limited to

hormones which are antigenic. Differences between immunoassays are due mainly to differences in methods used to measure the antibody-antigen reaction.

For the detection and assay of hormones, or of anything else by immunological means, it is necessary to have a specific antiserum. In theory, specifically sensitized animals in sufficient numbers for assay and that would give an immediate or delayed-type hypersensitivity reaction would suffice for detection, but for practical purposes what is needed is the antiserum.

Standard immunological methods for the detection of antibody-antigen reactions such as precipitation reactions oannot be applied to the immunoassay of most hormones in blood because the concentration of antigen is too low. Human chorionic gonadotrophin is an exception, and useful assays for this hormone by complement fixation reactions are possible (Brody, 1966). However, the use of antigens have made possible the measurement of many protein hormones present in the blood in minute amounts with a high degree of precision.

The principle depends on the competition between radioactively-labelled and unlabelled hormone for the specific binding sites of the antibody, i.e.,

Antibody	Free Hormone		Antibody-Bound Hormone
Ab	+ H	$\rightleftharpoons$	Ab-H
Ab	+ ^{131}I-H	$\rightleftharpoons$	Ab-^{131}I-H

In the presence of a constant concentration of labelled hormone (^{131}I-H), the higher the concentration of unlabelled hormone (H), the less radioactively-labelled hormone will be bound to the antibody (Ab). After separating the bound from the free hormone, the quantity of labelled hormone bound to antibody can be determined. Using suitably small, known quantities of a hormone, a standard curve is prepared where it is assumed that the antibody-bound hormone has been completely separated from the free hormone and that the radioactivity has been measured in the antibody-bound hormone. The concentration of hormone in unknown samples can be read off the curve directly.

In the radioimmunoassay of hormones in blood it is customarily assumed that the radioactively-labelled and unlabelled hormones behave identically, that the circulating hormone is chemically and immunologically identical with the standard hormone preparation, that the amount of hormone bound by the antibody is independent of the concentration of the hormone and also that different antisera made against a particular antigen will give a similar quantitative response in the immunoassay system.

In an immunoassay, the most highly purified protein hormone preparations are not homogenous. Several times recrystallized insulin may contain many minor impurities (Mirsky and Kawamura, 1966), and it is often difficult to exclude trace amounts of trypsin and glucagon. It is also possible that a hormone with several minor modifications in its amino acid sequence may exist in the gland.

The extent to which contaminating or denatured proteins interfere will depend upon the type of immunoassay used. An advantage of the radioimmunoassay is that antibodies to contaminating proteins and small amounts of impurity in the standard preparation can be ignored providing that the radioactively-labelled

hormone is free from these impurities.

2.2. METHODS OF SEPARATING FREE AND ANTIBODY-BOUND HORMONES

2.2.1. ELECTROPHORESIS

Paper electrophoresis was first employed by Berson, Yalow, Bauman, Rothschild and Newerly (1956), for the separation of free-labelled from labelled insulin bound to antibody. This method was later made the basis for the radioimmunoassay of insulin (Yalow and Berson, 1960). The separation depends on the electrophoretic properties of insulin-binding antibody, which at pH 8.6, in veronal buffer moves towards the anode away from small quantities of free insulin which remain bound to the paper strip at the point of application. If ^{131}I-insulin mixed with antibody in appropriate amounts is subjected to electrophoresis on paper, two peaks of radioactivity are obtained, corresponding to free and bound insulin. Radioactivity may be determined by scanning, though corrections may have to be made for radioactively damaged insulin. The nature of the paper used for the electrophoresis is crucial to the success of the assay.

2.2.2. DOUBLE ANTIBODY PRECIPITATION METHODS

In these, hormone bound to antibody is separated from unbound hormone by precipitating with a second antibody. Originally used to separate insulin from antibody by Sköm and Talmage (1958), this method was developed into an assay for insulin by Hales and Randle (1963) and by Morgan and Lazarow (1963). In both these methods, antibodies against insulin are allowed to react with a rabbit anti-guinea-pig gamma-globulin. In the method devised by Hales and Randle, the fine precipitate is filtered on a millipore filter, and counted. In the Morgan and Lazarow procedure, a visible precipitate is obtained by the addition of normal guinea pig serum and the addition of rabbit anti-guinea-pig gamma-globulin. The precipitate is spun down and counted.

The advantage of this method is that labelled hormone of rather lower specific activity may be then employed in the electrophoretic techniques. These methods are somewhat easier to use for large numbers of samples, and attempts have been made to automate them. But, the insertion of an additional precipitation step provides further opportunity for error.

These methods have now been widely used for growth hormone (GH), glucagon, placental lactogen, gonadotrophins, thyroid-stimulating hormone (TSH) and vasopressin.

2.2.3. SALT PRECIPITATION OF GLOBULIN

This has been used by Grodsky and Forsham (1960) for the radioimmunoassay of insulin. In this method, globulins are precipitated by sodium sulphite, and thence separated from free insulin. Sodium chloride in ethanol has been used to fractionate TSH bound to globulins by Odell, Wilber and Paul (1965).

2.2.4. ADSORPTION ON CHARCOAL AND SILICA

Charcoal coated with dextran has been used to separate free and antibody-bound hormone in the case of insulin (Herbert, Lau, Gottlieb and Bleicher, 1965), and GH (Lau, Gottlieb and Herbert, 1966) in guinea pig serum.

Coated charcoal appeared to be a simple alternative to ultracentrifugation and gel-filtration for separating free from bound insulin at

physiologic pH and ionic strength (Chao, Karam and Grodsky, 1965). It is not known how useful this method may be for estimations in serum. The use of talcum powder and precipitated silica for a similar purpose has been suggested by Roselin, Assan, Yalow and Berson (1966).

2.2.5. ION EXCHANGE RESINS

The use of the resin amberlite GC 400 has been described by Meade and Klitgaard (1962) for the immunoassay of insulin. Values obtained by this method were in close agreement with those obtained by other methods.

2.3. SPECIAL PROBLEMS RELATING TO THE IMMUNOASSAY OF HORMONES IN BLOOD

2.3.1. FORM OF HORMONE IN THE BLOOD

A potential difficulty in the immunoassay of proteins in serum is that the chemical form of the hormone in blood may differ from that in the gland of origin. It is always possible that hormones may be chemically modified by tissues, and may be immunologically altered. So far, however, differences of this kind have not been shown to be important.

2.3.2. PRE-EXISTING ANTIBODY

This may be a serious practical problem. It has been encountered when attempts are made to assay insulin in the blood of insulin-treated diabetics. There may be interference both from the very large amounts of insulin which are present (which dissociate from the antibody), as well as from the antibody to heterologous insulins which have been injected. A number of methods have been proposed to overcome the difficulty though none are entirely satisfactory.

2.3.3. INTERFERENCE BY GAMMA-GLOBULINS

Hales and Randle (1963) suggested that circulating gamma-globulins might interfere with the second antibody precipitation step in "double antibody" assays. This interference appears to result from competition between gamma-globulins in the serum being assayed and the guinea pig gamma-globulins for precipitating antibody. This was confirmed by Kuzuya and Samols (1964), who demonstrated cross-reactions between human gamma-globulins and precipitating antibody. Lack of effective precipitation resulted in

falsely-high insulin values being obtained. Pre-precipitation in this way seems to be very satisfactory.

2.3.4. INTERFERENCE BY COMPLEMENT

In addition to gamma-globulins, a heat labile factor has been described in rat serum which interferes with the second antibody precipitation step in double antibody-type assays (Morgan, Sorenson and Lazarow, 1964). This factor also appears to be present in human serum (Sheldon and Tyler, 1965). If this material is not inactivated before assay, certain sera may give falsely-high hormone values. Addition of ethylene-diamine tetracetate (EDTA) routinely to precipitating antibody appears to overcome this problem.

Certain heparin preparations may give falsely-high hormone levels when used in excess in a double antibody method for insulin assay (Soeldner and Slone, 1965).

2.3.5. DEGRADATION OF HORMONES BY PROLONGED INCUBATION WITH SERA

It was noticed by Berson et al., (1956), that there may be a breakdown of ^{131}I-insulin on

prolonged incubation with serum. There are present in sera, enzymes which might easily initiate the proteolysis of hormones. Leucine amino-peptidase can readily attack insulin (Smith, Hill and Borman, 1958). It is not known whether other hormones in blood might be similarly attacked, though it should be noted that hormones like glucagon are more readily broken down by proteolytic enzymes than insulin. In practice breakdown of this kind can be minimized by carrying out various steps in the immunoassay at 4°C.

2.3.6. IMMUNOASSAY OF HORMONES IN EXTRACTS OF SERUM OR PLASMA

To avoid the difficulties encountered in assaying hormones in untreated samples of serum or plasma, the assay may be carried in extracts of blood containing the hormone. In one method of assaying insulin, a preliminary acid-ethanol extraction of serum is always used (Grodsky and Forsham, 1960). A preliminary extraction of serum GH has also been employed by Hunter and Greenwood (1964) to concentrate the hormone for assay. The sensitivity of the radioimmunoassay for insulin, GH and chorionic gonadotrophins is now sufficient for direct measurements of blood levels. Even using the most potent antisera and

radioactively-labelled hormones of high specific activity it may not be possible to make direct measurements of blood levels of such hormones as ACTH and vasopressin.

2.4. PRECISION

A check of the precision of any assay technique is required in order to determine its true reliability and sensitivity relative to other assays. One way of achieving this is to calculate the index of precision which is defined as the ratio of the standard deviation (SD) of the values obtained for the standards over the slope (b) of the standard curve (Loraine and Bell, 1966)

$$SD = \sqrt{\frac{\Sigma (x-\bar{x})^2}{N-1}}$$

and

$$b = \frac{\Sigma (x-\bar{x})\ (y-\bar{y})}{\Sigma (x-\bar{x})^2}$$

Where x = independent variable
y = dependent variable
$\bar{x}$ = mean of the independent variable
$\bar{y}$ = mean of the dependent variable
N = number of observations
Σ = sum of

The reliability of radioimmunoassays is good compared to other bioassays. The index of precision was 0.016 - 0.056 for the radioimmunoassay of insulin (Hales and Randle, 1963) compared to 0.25 - 0.32 for other types of bioassays for insulin; similarly for chorionic gonadotrophin a range of 0.011 - 0.064 was found using the radioimmunoassay (Wilde, Orr and Bagshawe, 1967) compared to 0.10 - 0.55 in other types of bioassays.

2.5. THE IMMUNOASSAY OF INDIVIDUAL HORMONES

Immunoassays have been developed for a large number of antigenic hormones, many of which are in the process of active modification. These include assays of insulin (Randle and Taylor, 1960; Stewart, 1960; Yalow and Berson, 1960; Grodsky and Forsham, 1960; Hales and Randle, 1963; Morgan and Lazarow, 1962, 1963); ACTH (Yalow et al., 1964; Demura, West, Nugent, Nakagawa and Tyler, 1966; Imura, Sparks, Tosaka, Hane, Grodsky and Forsham, 1967); growth hormone (Utiger et al., 1961, 1962; Hunter and Greenwood, 1962, 1964; Parker, Utiger and Daughaday, 1962; Glick et al., 1963; Hartog, Gaafar, Meisser and Fraser, 1964; Tchobroutsky, Rosselin,

Assan, Dronet and Derot, 1965; Morgan, 1966; Schofield, 1966; Cerassi, Della Casa, Luft and Roovete, 1966; Lazarus and Young, 1966; Catt, Niall and Tregear, 1966; Schalch and Reichlin, 1966; Birge, Peake, Mariz and Daughaday, 1967, and others); glucagon (Sköm and Talmage, 1958; Unger et al., 1959; Grodsky, Hayashida, Peng and Geschwind, 1961; Lawrence, 1966; Schalch, 1966); human chorionic gonadotrophin hormone (Bagshawe, Wilde and Orr, 1966; Wilde et al., 1967); thyroid-stimulating hormone (Odell, Wilber and Paul, 1965; Freychet, Rosselin, Assan, Dolais, Tchobroutsky and Derot, 1966); pituitary gonadotrophins (Franchimont, 1966a, 1966b; Bagshawe et al., 1966; Midgley, 1966; Wilde et al., 1967; Hunter, 1967); vasopressin and oxytocin (Permutt, Parker and Utiger, 1966); parathormone (Sherwood, Potts, Care, Mayer and Aurbach, 1966); prolactin (Kwa and Verhofstad, 1967; Arai and Lee, 1967), and lactogen (Kaplan and Grumbach, 1965). Further details of these methods can be found in the above-mentioned references.

2.6. CLINICAL APPLICATION OF IMMUNOASSAY

The most obvious clinical application for any hormone assay is in the evaluation of abnormally

elevated or depressed hormone levels. Radioimmunoassays can provide diagnostic aids in the evaluation of insulin-secreting tumours of the islets of Langerhans, GH-secreting tumours of the pituitary and parathyroid hormone-secreting tumours of thyroid glands. Absent or subnormal concentration of plasma hormone after appropriate physiologic stimulation is evidence for inadequate gland function.

Another important clinical consideration is that improved understanding of the pathogenesis of endocrine disorders may follow a clear-cut appraisal of plasma hormone levels.

Whereas there have been available other assays for plasma insulin (albeit less specific, less accurate and more tedious than immunoassay) there has been none other for assay of glucagon, HGH or parathyroid hormone in plasma.

The radioimmunoassays have also led to a deeper understanding of the physiologic regulation of several of the peptide hormones (Berson and Yalow, 1966).

The regulation of metabolic processes by hormones is, at present, imperfectly understood. It is hoped that radioimmunoassay of hormones can contribute to an increased understanding of the complex inter-relationships between hormone function and distinct metabolic activities in specific tissues.

3. THE PROTEIN-BINDING OF NON-ANTIGENIC HORMONES

3.1. INTRODUCTION

A number of assays for non-antigenic hormones have been developed which are based on the principle of competitive protein binding (Murphy, 1968, 1969), and although they were developed independently of the immunoassays for peptide hormones, the principle of competition between labelled and unlabelled hormones for sites on a binding protein is the same. In this section, attention will be given to some of the factors involved in protein binding (PB) and inasmuch as this thesis is concerned chiefly with the determination of the CS by this technique, the PB of CSs will be emphasized.

Many substances in the blood are known to be associated with plasma proteins. Some of

these associations are highly stereospecific as for example globin-heme, certain CSs, thyroxine (T_4) and possibly some peptide hormones (insulin and some pituitary hormones) and their carrier-globulins, enzymes and substrates and antigen-antibody complexes, iron and transferrin, copper and ceruloplasmin and zinc and alpha-globulins. Other associations are much less specific as in the case of the binding of epinephrine, norepinephrine and many steroids including CSs. Szego and Roberts (1946a, 1946b, 1947) reported that 58 to 67% of plasma estrogen was associated with proteins, mostly with albumin. An understanding of the nature of this physical state is a prerequisite for interpreting the biological activity of hormones such as CSs in the plasma, distribution within the body, metabolism and excretion (Daughaday, 1959). The word "ligand" is often used to denote the smaller molecules.

The forces involved in protein binding are poorly understood. Complementariness, or the spatial arrangement of the molecules, permits a strong attraction resulting from the combination of many weak short-range interactions. The exact fit of one molecule into the other is, therefore, very important with respect to the specificity and affinity

of such binding. These reactions obey the law of mass action, and one practical application of such reactions is that any protein which has a high specificity and affinity for a particular ligand can be used to measure the ligand's concentration if a suitable isotopic form of the ligand is available. With the advent of isotopes of hormones in 1954, there appeared rapid advances in the study of hormone-protein interactions.

3.2. METHODS OF SEPARATION OF PROTEIN-BOUND AND NON-PROTEIN-BOUND LIGANDS

Like many other biologically important substances, steroids are present in plasma both in simple solution and in association with protein, these two forms being in dynamic equilibrium. Although special techniques are needed to separate the two components, they are probably easily distinguished at the cellular level. Certainly, only the non-protein-bound (NPB) component can diffuse through protein-impermeable barriers and it is generally assumed that even if plasma protein comes in contact with steroid target structures, steroid complexed on it is unable to associate directly with those structures. Thus, steroid "diffusing potential" and probably its

biological activity, is a function of the NPB concentration in the plasma, not of the total concentration. This is not affected by the consideration that dissociation of the steroid-protein complex might be essentially instantaneous; moreover, since real biological situations are usually non-equilibrium states, any appreciable dissociation time will act to reduce further the activity of the steroid.

In the study of PB, several techniques have been used to separate the protein and non-protein phases (Klotz, 1953). These include precipitation, electrophoresis, equilibrium dialysis, ultrafiltration and filtration. Ultrafiltration is nearest to the ideal, since apart from the addition of radioactive tracer, the equilibrium present in the plasma is undisturbed; however, it may not be as sensitive as other methods since the ultrafiltrate is small in volume and contains the lower concentration of unbound steroid. Equilibrium dialysis is easier to perform and the volume of the non-protein phase can be adjusted to suit the investigation, but diffusion into this added volume disturbs the original equilibrium. Electrophoresis has provided valuable quantitative data about PB assays but its use in quantitative

is limited by dissociation of the complex as the protein band moves. Although gel filtration has a similar disadvantage, separation can be achieved quickly so that with suitable precautions this dissociation can be made negligible.

The methods of equilibrium dialysis, ultrafiltration and the ultracentrifugation essentially gave the same information, provided the conditions of temperature and electrolyte concentrations were maintained constant.

In the non-equilibrium techniques, such as electrophoresis and gel filtration, the relationship between bound and unbound steroid to the protein may be disturbed. These methods have yielded useful information where the bond between protein and steroid is a strong one, but are unsuited to the study of weaker protein-steroid interactions.

3.3. THE BINDING OF CIRCULATING CORTICOSTEROIDS BY CORTICOSTEROID-BINDING GLOBULIN (CBG) AND ALBUMIN

Daughaday (1956) showed that 94% of unconjugated 17-OHCS and 65% of 17-OHCS glucuronosides were bound to plasma proteins and discussed the

limiting effect of this on renal excretion. Before this, the extent of PB had been considerably underestimated.

Bush (1957) performed equilibrium dialysis experiments at 37°C using ^{14}C-cortisol at several different concentrations and showed that there were two binding species, one dominant at concentrations less than about 20 µg/100 ml, but approaching saturation at this concentration, the other having a much greater capacity but lower affinity for cortisol.

Daughaday (1956, 1958), after measuring free or bound steroid with one or more of the above-mentioned techniques following the addition of a variety of biologically significant steroid hormones to plasma in increasing concentration, observed the peculiar behaviour of F, B and Prog. He found that the binding between F and the plasma protein is markedly decreased by rather small amounts of total F added to plasma. Corticosterone behaves in the same way and Prog to a much lesser extent. The binding of estrogens and testosterone is little affected by the concentration of these hormones.

These observations and others led to the suggestion that there are at least two distinct CS binding systems of plasma (Daughaday, 1956, 1958): one with high affinity for CSs, and low binding capacity and a second steroid binding system with less structural specificity and lower binding affinity but with a greater total capacity to bind steroid hormones of a number of types. Direct support for this hypothesis was provided by electrophoretic separation of the two binding systems (Daughaday, 1958; Slaunwhite and Sandberg, 1959). When such separations were carried out in conventional alkaline buffers, the high-affinity CS binding proteins migrated with the globulins, and the high-capacity, low-affinity steroid binding system was associated with albumin. Thus, the high-affinity CS binding protein, also postulated as an acid glycoprotein (due to its behaviour in acetate buffer at pH 5.2), was called corticosteroid-binding-globulin or CBG.

By the electrophoresis of plasma proteins after the addition of tracer quantities of ^{14}C-cortisol, Daughaday (1958a, 1958b, 1958c) was able to show that at very low physiological concentrations of F in plasma, the steroid travelled with the

alpha-globulin fraction, but that when the F concentration was higher and exceeded the upper physiological range, the greater part of the steroid moved under electrophoresis with the albumin peak.

Slaunwhite and Sandberg (1959) dialysed the dilute plasma against matched solutions of human serum albumin, thus showing the binding effect only of the globulin, which they named "transcortin". They reported that the transcortin fraction binds cortisol 6,000 times as strongly as does albumin. They also reported that at 4°C transcortin had a high affinity for F but was present in low concentration. The authors separated transcortin from plasma proteins and suggested that it was an alpha-globulin.

3.3.1. PURIFICATION AND GROSS AMINO ACID COMPOSITION OF CBG

Doe and Seal (1962) developed a technique for isolation and purification of CBG, which yields a product of apparently high purity. Later, they modified this procedure and reported the parallel isolation of CBG, thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA) from a single plasma sample.

CBG, TBG and TBPA, all seem to be about the same size and have the same gross amino acid composition. CBG differs in having a high concentration of aspartic acid, glutamic acid and valine. Evidence has been obtained that CBG contains hexose, hexosamine, fucose and sialic acid. Studies of F binding by the isolated purified CBG indicate that only a single binding site exists per protein molecule (Seal and Doe, 1966; Daughaday, 1967).

3.3.2. SPECIFICITY OF THE CBG BINDING SITES

The large alterations in the binding of CS to CBG which may be brought about by small changes in steroid structure, as described above, indicate that the stereochemical configuration of the steroid and the specific steric structure of the protein determine this type of binding, although the exact mechanism is not clear.

Several workers (Chen, Mills and Bartter, 1958; Daughaday, 1958; Slaunwhite and Sandberg, 1959; Westphal and Ashley, 1959; Florini and Buyske, 1961; De Moor, Deckx and Steeno, 1963) have demonstrated that CBG in human plasma has the highest affinity for F

and B of the corticosteroids. The minimum requirements for a full-binding are a 3-keto, delta 4-5 conjugated system in ring A, and a 20-ketone group in the side chain. In addition, the OH-groups at positions 11β, 17a, and 21 contribute to the strength of CBG-steroid bond.

The nature of the oxygen group at C-11 is most important. The oxidation of 11β-OH group of F to an 11-keto group (E) or replacement by an 11a-OH group markedly decreases binding affinity by CBG. The decreased binding of Aldo as compared to B may be related to the involvement of the 11β-OH-group in the hemiacetal form of Aldo.

The configurational specificity of binding of steroids by proteins reveals that the steroid molecule is essentially planar. In the conventional representation hydrogen or other substituent groups on the top surface of the molecule are β while those on the underneath surface are alpha.

Westphal and Ashley (1959) have presented considerable evidence that the lower, or 'alpha' surface of Prog is involved in binding by

albumin. This surface permits access to the 3-keto, 4,5 double bond and probably to 20-keto groups, all of which contribute to the strength of binding by albumin.

Consideration of the structural requirements for binding of steroids by CBG in whole plasma also supports the general notion of an approach of the 'alpha' surface of the steroid molecule to the protein.

Florini and Buyske (1961), working with synthetic analogues of the CSs discovered that the 6a-methyl, 9a-fluoro, 16a-OH and 16a-methyl substituents on B molecule all decreased the competition of such analogues with F for binding to CBG. Dehydrogenation at 1,2 position in ring A of the steroid reversed this effect. They concluded that the lesser plasma binding of the synthetic substituted CSs accounted in part for their enhanced biological activities.

"P" and "B" compete at the same binding sites of human transcortin which binds "F" more intensely (Daughaday, 1958; 1960; Slaunwhite and Sandberg, 1959; Mills, 1961; De Moor, Heirweig,

Heremans and Declerk-Raskin, 1962; Seal and Doe, 1965). In contrast, Keller, Sendelbeck, Richardson, Moore and Yates (1966) found that in the rat plasma, B is bound more firmly than is F. The same authors also showed that the binding of F is more intense in rats than in humans, and less intense in dogs than in humans.

Aldo is bound to plasma proteins to a much smaller extent than is F (Daughaday et al., 1961; Meyer, Layne, Tait and Pincus, 1961; Davidson, De Venuto and Westphal, 1962). The binding is greater at 4°C than at 37°C (Daughaday et al., 1961). Meyer et al., (1961) reported some binding protein of plasma other than albumin, involved in the binding of Aldo.

In pregnancy, there is no increase in the binding of Aldo by the plasma, despite the great increase in the concentration of CBG. On the other hand, the administration of Enovid increases the binding of Aldo as well as F (Daughaday, 1967).

There is relatively little information concerning the binding of other CSs. Chen, Mills and Bartter (1961) showed by ultrafiltration that epd E, S, B and DOC were all bound to a greater extent by plasma than by 5% human albumin solution; the first

three of these exhibited a pattern of change of equilibrium with increasing concentration similar to that of cortisol. Prog was apparently bound only to albumin. Murphy (1967) and Grad and Khalid (1968) studied the competitive effect of various steroids on F binding and found that for F precursors this was related to the degree of hydroxylation, confirming the earlier findings of Daughaday (1958). Thus, B, cpd S and 21-deoxycortisol were strong and approximately equal competitors, 17-OH-Prog and DOC were weaker competitors, while Prog was weaker still. The androgens and estrogens showed very little binding. Reduction of the ring A or substitution in the 11-position by oxo or alpha-OH groups profoundly reduced binding competition.

3.3.3. THE BINDING OF CIRCULATING CS BY ALBUMIN

Albumin is the most important binding protein of plasma for steroids other than CSs, and the contribution of albumin and other secondary binding systems in plasma is not insignificant. At body temperature albumin binding is even more important, accounting for nearly one-third of bound F at baseline levels of cortisol. Thus, Yates and Urquhart (1962)

have estimated that under physiologic conditions about 10% of plasma F is unbound, about 60% is bound by CBG and the rest, 30%, by the albumin.

Binding of CSs by CBG differs from the binding of steroids by albumin in that the strength of binding increases with OH-substituents at positions 11β, 17α and 21, whereas these groups suppress binding by albumin. The augumentation of binding by CBG with the latter two OH-groups confers a rather slight disadvantage of F over the corresponding deoxy-compound. It should be remembered that these substituents decrease the binding of steroids by albumin and would thereby increase the amount of steroid available to compete with F for binding in CBG.

Seal, Makey and Doe (1964), and Seal and Doe (1966) support this view by reporting that the binding of Prog by isolated CBG is only slightly less than that of F, wherease it competes much less effectively with F for the binding sites in whole plasma.

3.3.4. EFFECT OF DILUTION ON THE PROTEIN BINDING OF CORTISOL

Slaunwhite and Sandberg (1959) reported a decrease in binding of CS on diluting whole

plasma one-fifth with 0.9% saline.

Farese and Plager (1962) compared the concentration of F in whole blood and plasma, and observed that the concentration of F in red blood cells was no greater than that which might be expected on the basis of simple diffusion and that there was no specific binding by the red cells. This is substantiated by the work of Murphy who also reported that undiluted plasma is more strongly bound than the whole blood, possibly because the red cells act in the same way as dilution to decrease binding (Murphy, 1967).

Slaunwhite and Sandberg (1959) also observed a change in binding by the addition of F, and showed that for each plasma concentration the binding decreased with increasing addition of F. When other steroids were added, a similar decrease in binding was observed and the amount of decrease depending on the specific steroid added.

3.3.5. EFFECT OF TEMPERATURE AND pH ON CBG BINDING OF CORTISOL

The strength of F binding by CBG is markedly influenced by temperature below that which

leads to irreversible inactivation. This was first clearly shown in ultracentrifugation studies by Chen et al., (1958) and later confirmed by Mills, Schedl, Chen and Bartter (1960), Florini (1961), De Moor et al., (1962) and Doe, Fernandez and Seal (1964). At 4°C only about 1-2% of human plasma F is unbound at basal steroid levels. At 37°C this percentage increases to 5-8%. The effect of rise in temperature is to reduce the association constant between CBG and F, thus decreasing the affinity of CBG for F without changing the number of binding sites. The binding activity is lost precipitously when pH is less than 5 while the binding ability remains uniform at pH 5 to 10 (Daughaday, 1967).

The strength of F binding in human plasma is also inversely related to temperature (Slaunwhite and Sandberg, 1959; Daughaday and Mariz, 1960; Seal and Doe, 1963; Slaunwhite, Schneider, Wissler, Sandberg, 1966); at 4°C binding is considerably stronger than at 37°C. Preheating human plasma for 20 minutes at 60°C appears to inactivate CBG at least partially, and binding becomes less extensive (Daughaday, Adler, Mariz and Rasinski, 1962). Similar

temperature effects were found for B binding in rat plasma.

3.3.6. THE EFFECT OF HORMONES ON THE CIRCULATING CBG LEVEL

3.3.6.1. Role of Estrogens

Elevated CS levels following estrogen therapy have been observed in clinical studies (Peterson, Nokes, Chen and Black, 1960; Wallace and Carter, 1960). That this is due to an increased CS-binding capacity of human plasma in pregnancy or following prolonged treatment of patients with either natural or synthetic estrogens is now well known (Sandberg, 1959, 1960; Slaunwhite and Sandberg, 1959; Daughaday, 1959, 1962; Murphy, 1963).

Herman, Schindle and Bondy (1960), Peterson et al., (1960) and Wallace and Carter (1960) reported that in pregnant or estrogen-treated subjects, the response of plasma corticoids (PC) to ACTH was also greatly increased. Wallace and Carter explained these results on the basis of increased CS-binding protein. Daughaday et al., (1961) confirmed these findings by a direct determination of CBG from the estrogen administered subjects.

McKerns (1957) showed that the effect of administered estrogens on the CBG level was not due to a direct influence on the adrenals, and similarly Sandberg and Slaunwhite (1960) later showed that estrogen administration increased CBG levels in the absence of adrenal glands. They also showed that male hormones or their synthetic analogues were ineffective in elevating plasma CBG or CS levels. The increased CBG levels result from stimulation of synthesis rather than from interference with metabolism of CBG (Sandberg, 1964).

Presently available studies have provided significant information on the presence and properties of a similar protein in the dog (Plager, 1963); sheep (Lindner, 1964); rat (Westphal, 1964), and some 20 other species (Seal, 1963). However, plasma from dogs or sheep seemed to contain little CBG-like activity, and the administration of estrogen in these species is without effect on plasma-binding activity for cortisol (Plager, Knoop, Slaunwhite and Sandberg, 1963; Lindner, 1964).

The greater intensity of B binding in the plasma of female rats compared to that seen in

plasma samples from male rats, and the increase in binding observed after estrogen treatment in males, suggested that endogenous estrogens in the female animals might be responsible for the sex difference in binding (Keller et al., 1966).

Keller et al., (1966) also found that the concentration of unbound CS is increased by estrogen treatment in the rat; this is similar to that obtained in dogs by Plager, Schmidt and Stanbitz (1964).

3.3.6.2. Role of ACTH and Corticosteroids

Sandberg and Slaunwhite (1960) observed that CS binding and CBG capacity were decreased by ACTH, surgical stress and intravenous cortisol.

Westphal (1964) has shown an increase in levels of CBG in rats 10 days after adrex and hypox, an increase which remained at a constantly elevated level for one year. In the mouse, the elevated levels of CBG becomes apparent 4-6 weeks after adrex (Westphal, 1964). Treatment with B has an effect opposite to that of adrex; that is, the binding activity was diminished. Dexamethasone produced essentially identical results in female rats as B did in males (Keller et al., 1966).

Doe, Zinneman, Flink and Ulstrom (1960) found that the amount of bound F was normal while the unbound F was greatly elevated in patients with Cushing's syndrome.

Keller et al., (1966) investigated whether any property of the corticosterone-binding macromolecules follows the circadian variations in total plasma CS concentration. Binding parameters were determined at the time of the diurnal minimum of total B concentration, in the morning, and again at the time of the diurnal maximum, in the later afternoon. Although the total B concentration increased approximately 3-fold in normal male animals in the afternoon, over the morning levels, the net binding capacities differed very little.

3.3.6.3. Role of Thyroxine

Labrie, Raynaud, Pelletier, Ducommun and Fortier (1965, 1968) showed that the chronic administration of graded doses of T_4 to intact rats resulted in progressive increase of adrenal weight, evidencing a corresponding enhancement of ACTH secretion. Graded doses of T_4 concurrently resulted in step-wise increases

of total plasma B concentration, without significant alterations in the absolute level of the unbound fraction. This strongly suggests that the unbound, as opposed to the total, cpd B concentration is the variable under feedback control and that the binding capacity of transcortin is responsible in the rat for the adjustment of the secretion rate of B to changes in thyroid activity. From a comparative assessment of the effects of adenohypophyseal, adrenal cortical, and gonadal hormones, it is inferred, furthermore, that T_4 alone has a direct enhancing effect on the binding capacity of transcortin and that the stimulating effect of estrogen and Prog is exerted through the pituitary-thyroid axis.

3.3.6.4. Miscellaneous Factors Influencing CBG Concentration in the Circulation

Decreases have been observed in patients with decreased serum albumin concentrations (Doe, 1964), and in a family with a genetic CBG deficiency (Doe, 1965), but not in association with any endocrine deficiencies (Seal and Doe, 1965). Earlier, Daughaday (1958) reported impaired CS binding in severe liver disease and several other conditions associated with the abnormalities of plasma proteins.

3.4. THE FUNCTIONAL SIGNIFICANCE OF PROTEIN BINDING

The physiological significance of the binding between proteins and small molecules is not well understood. Thus, the functional significance of the binding of iron to transferrin, copper to ceruloplasmin and zinc to alpha-globulins, is still quite obscure. For at least some hormones, binding increases their solubility in aqueous media such as plasma, decreases their excretion through the kidney, thus conserving body stores, and by regulating their passage into the liver and other tissues, controls rates of degradation and utilization.

The precise function of protein-binding of F specifically, is also obscure. Since the solubility of F in water far exceeds any concentration found in the plasma, transport is unlikely to be a primary role in respect of the more polar steroids. It is possible that CS-binding properties of CBG are purely incidental or that CBG-steroid complex has a function not directly concerned with steroids. As just mentioned, conservation is certainly a result of protein binding.

Careful studies of the renal excretion of F have been correlated with the extent of protein binding by Beisel, Di Raimondo and Forsham (1964). These workers found that the excreted F could best be expressed as a function of the plasma unbound F, and the clearance of F can be regarded as a process of glomerular filtration of the unbound F with considerable passive reabsorption of F by the renal tubules (Daughaday, 1956; Beisel, Cos, Horton, Chao and Forsham, 1964).

Florini and Buyske (1961) showed that the binding of F by CBG exerts a major influence on equilibrium concentration of F across capillary barriers. The low levels of CBG in cerebrospinal fluid (CSF) and in certain extravascular fluids partially explain the lower concentrations of unconjugated 17-OHCS in these fluids (Sandberg, Eik-Nes, Nelson and Tyler, 1954).

Also, Sandberg and Slaunwhite (1959) reported that bound F was physiologically inactive and later Wallace and Carter (1960) confirmed these findings. Therefore, protein-bound F acts as a circulating immediate access store without raising the biologically effective F concentration. Inasmuch as this reserve is large compared with the non-bound material, the latter

will tend to be buffered and remain relatively constant when the total pool size varies. Any significance of albumin-bound F is even less clear. Its concentration is more than double that of the unbound F. Where protein barriers exist, this fraction of the total plasma F must be non-diffusible. Albumin is the most important binding protein of plasma for steroids other than CSs.

3.5. ASSAYS OF NON-ANTIGENIC HORMONES BASED ON COMPETITIVE PROTEIN BINDING ASSAYS

3.5.1. CORTICOSTEROIDS

3.5.1.1. Equilibrium Dialysis

The principle of competitive protein binding (CPB) as applied to the assay of a steroid, was first developed by Murphy, Engelberg and Pattee (1963) for the determination of F. In this method, equilibrium dialysis for 40 hours was used to separate the free and protein-bound fractions. Transcortin, or CBG, in the test plasma is first destroyed by heating or by precipitation with ethanol. The addition of increasing amounts of unlabelled F to an equilibrium dialysis system containing standard plasma and a constant

amount of ^{14}C-cortisol (or ^{3}H-cortisol), caused a proportional decrease in the percentage of radioactive F bound to the plasma proteins. The steroid content of test samples was obtained by comparison with the standard curve. This method requires 1 ml of test plasma for F concentrations in the normal range, and the authors reported a standard deviation of $\pm$ 1 µg over the range of 0-10 µg%. The authors also reported mean recovery value of 91.1 $\pm$ 13.7 (SD), with a range of 68 to 120%.

Jones and Mason (1966) compared this technique with the method of Nelson and Samuels (1952) as employed by Harwood and Mason (1956). The coefficient of correlation was found to be 0.92; the values obtained from the isotope dialysis procedure were about 25% lower.

Sensitivity depends upon the use of ^{14}C-cortisol (or ^{3}H-cortisol) of high specific activity and upon a low concentration of endogenous F in the standard plasma. Corticosterone, cpd S and prednisolone are estimated with F as they have similar binding characteristics. Compound S can be measured separately by partition of the plasma 3 times with 4 volumes of carbon tetrachloride (CCL_4), when about 60% of the

cpd S and only 3% of F will be in the organic phase (Murphy and Pattee, 1964). Prog is a weak competitor and it can be removed from pregnancy plasma by prior extraction with light petroleum.

3.5.1.2. Gel Filtration

The substitution by gel filtration on sephadex (Murphy and Pattee, 1964) or on dextran-coated charcoal (Nugent and Mayes, 1966) for equilibrium dialysis has resulted in a reduction of the process time to 2 hours and in improved precision. Nugent and Mayes (1966) compared their method with a more specific technique involving thin-layer chromatography (TLC) and fluorescence in sulphuric acid (H_2SO_4) with internal radioisotope correction for recovery, and found the coefficient of correlation to be 0.99.

3.5.1.3. Florisil and Fuller's Earth

Following the use of equilibrium dialysis, gel filtration and dextran-coated charcoal to separate protein-bound from unbound steroid in the PB assay, the use of florisil and Fuller's earth was introduced, thus leading to a saving of time and an increase in sensitivity. Various insoluble adsorbent

materials such as florisil, Fuller's earth, Lloyd's reagent and ordinary charcoal, have long been known to have adsorbent properties and were widely used as decolorizers. The first three of these are silicates.

Murphy (1967) reported the use of several of these adsorbing agents to separate protein-bound from unbound steroids and investigated their effects on the specificity in micro and ultramicro PB assays. She concluded that Fuller's earth, coated charcoal and Lloyd's reagent were suitable for separating protein-bound and unbound F, while florisil and coated charcoal were suitable for separating protein-bound and unbound corticosterone. Such differences in the affinity of the adsorbent for various small molecules proved advantageous as in the case of Fuller's earth, where it permits the measurement of F in the virtual absence of B, cpd S and the other steroids known to bind to CBG. Earlier, Seal and Doe (1965) reported the species differences in the binding of F and B so that it is not surprising to find some variation in the binding of other steroids.

Fuller's earth and Lloyd's reagent are fine powders and require centrifugation after mixing, while florisil, a coarsely granular silicate, easily settles down to the bottom without centrifugation.

3.5.2. THYROXINE

Thyroxine binds to prealbumin and albumin as well as to TBG, and of the three or possibly more proteins which bind thyroid hormones in plasma, the TBG has the highest affinity and greatest specificity.

Until recently, it seemed unlikely that a suitable method for the measurement of free or unbound T_4 would be feasible because of the exceedingly low concentration of the free hormone in blood. The first actual measurement of free T_4 was achieved by Sterling and Hegedus (1962), and several improved variants of this method have recently been published. They are based on the determination of the dialysable fraction of serum T_4 after labelling with a tracer quantity radioactive hormone. The major obstacle in the procedure has been the fact that the dialysable fraction is so small that dialysable contaminants (chiefly iodide) in the radioactive T_4 lead to a large error unless removed from the dialysate.

In the past few years, a new type of method has been introduced to circumvent the need for iodimetry and its attendant problem — especially

iodine contamination (Murphy, 1964). These methods are analogous to radioimmunoassay procedures for polypeptide hormones. Their essential features are (1) the extraction of serum iodothyronine from proteins in the test serum, (2) measurement of the effect of the extract of a system containing labelled iodothyronine, normal serum and an adsorbent for the hormone, (3) comparison of the effects of the serum extract with the effect of pure reference compound. Alternately, the non-specific adsorbent can be replaced by a method which measures the distribution of the hormone between the bound and the free state. The specific hormone-binding protein (TBG) in the normal serum is the analogous of CBG or antibody in radioimmunoassay. The distribution ratio of labelled hormone between specific protein and non-specific adsorbent depends on the total amount of hormone in the system.

The first approach used serum only and measured hormone distribution between TBG and albumin (separated by electrophoresis), the latter acting as the non-specific adsorbent (Ekins, 1960). In later modifications dextran gel (Murphy and Pattee, 1964) or anion exchange resin (Murphy, 1965; Murphy, Pattee and Gold, 1966; Nakajima, Kuramochi, Horiguchi

and Kubo, 1966) was the adsorbent; numerous others are surely possible. At the present time, Murphy (1968) prefers to use anion exchange resin. If the extract of hormone from plasma is not complete, correction for incomplete recovery is required, but has not always been done in published methods. The method is not foolproof, since any material in blood which is extracted with the hormone in high enough quantity and which has an affinity for the T_4-binding sites will give a falsely high value. Dilantin is one such substance which is of practical importance but results indicate that it may not interfere (Murphy et al., 1966).

The procedure has been used mainly for T_4. Since tri-iodothyronine (T_3) is present in blood in much smaller amounts, and has a lower affinity for T_4-binding sites, its presence in the extract does not interfere. The opposite is true when T_3 is measured (Nauman, Nauman and Werner, 1967). In that case, the extracted T_3 must be scrupulously separated from T_4 before it is added to the test system.

3.5.3. ANDROGENS

Both ASD and testosterone exist in plasma in a directly extractable form and it is this moiety which is measured by most of the published analytical techniques. Unconjugated testosterone is transported strongly but non-specifically bound to serum albumin, and weakly but specifically bound to a β-globulin (Mercier, Alfsen and Baulieu, 1966; Pearlman and Crépy, 1966).

Various methods involving column, paper, gas-liquid and thin-layer chromatographic techniques followed by Zimmermann colour reaction, have been developed for the measurement of 17-oxosteroid sulphates in plasma (Baulieu, 1960, 1965; Eberlein, 1963; Conrad, Lindberg and Herrmann, 1965; De Moor and Heyns, 1966; Vihko, 1966). Most have yielded reasonably similar results, considering that in some instances the reported values have been corrected for losses and in others not, and that the levels of these steroids are greatly influenced by a variety of poorly defined environmental factors often classified as "stress". DHEA is the predominant plasma 17-oxosteroid and androsterone the next.

More recently, Murphy (1968) reported the development of PB-assay for measuring androgens in human plasma. The principle of the assay is similar to that of T_4 or B assay procedure.

3.5.4. PROGESTERONE

Prog in plasma is largely bound to proteins (Sandberg, Slaunwhite and Antoniades, 1957; Chen et al., 1958; Daughaday, 1958; Sandberg, Rosenthal, Schneider and Slaunwhite, 1966, and others). Although albumin appears to have the largest capacity for Prog binding and may be quantitatively most important (Eik-Nes, Schellman, Lumry and Samuels, 1954), there are other plasma protein fractions with a small capacity but a high affinity for Prog binding. Several authors have reported a high binding affinity of CBG for Prog (Murphy et al., 1963; Sandberg et al., 1966; Seal and Doe, 1966), while Prog is a weak competitor when in plasma and competing with cpds B or F (Murphy, 1967; Grad and Khalid, 1968). It has been established by now that the concentration of Prog progressively increases during pregnancy.

Several different techniques for reliable measurement of Prog in human blood under

many conceivable situations are at present available and many observations have provided estimates of Prog concentrations in body fluids.

The first physio-chemical method for the reliable determination of Prog in human blood was introduced by Zander (1954). The sensitivity of this and other spectrophotometric methods (0.3 - 0.5 µg) limited the application for reasonable blood volumes to analysis of human blood during the second half of the pregnancy (containing $>$ 5 µg/100 ml plasma). The introduction of more sensitive detection techniques (Short and Levett, 1962; Woolever, 1963) has only recently permitted accurate physio-chemical estimation of the small amounts of Prog in plasma of non-pregnant human subjects (0.01 - 3.0 µg/100 ml) and during early pregnancy (1 - 5 µg/100 ml) (Riondel, Tait, Tait, Gut and Little, 1965; van der Molen and Groen, 1965). Thus, the existing methods for the quantitative estimation of Prog in blood are spectrophotometric and fluorometric methods, isotope derivative formation and gas-liquid chromatographic methods.

Murphy (1967) reported the PB assay of Prog in pregnant human subjects using human as well as dog CBG as a source of assay protein. 17a-OH-Prog

and other petroleum ether soluble analogues were obviously interfering elements. Later, Neil, Johanssen, Datta and Knobil (1967) modified this assay by introducing a preliminary thin-layer chromatographic step and increased the sensitivity of the technique. More recently, Yoshimi and Lipsett (1968) reported a highly sensitive but more complicated method for Prog. The procedure involves the usual method of extraction with diethyl ether but the authors add a small amount of labelled Prog prior to extraction to correct for losses. Prog is then isolated by several chromatographic steps and the assaying protein (CBG) is obtained from the plasma of estrogen-treated ovariectomized woman. The assay ranges from 0 to 3 nanograms and could measure 100 picogramswith a coefficient of variation of 50%. The method awaits clinical trial and applicability.

Apart from the scientific interest, the clinical value of measurements of plasma Prog is not yet clearly defined. The lack of suitable methods has so far prevented the collection of sufficient data to indicate that measurement of Prog in blood would be better than pregnanediol estimation in the urine. On the basis of the data so far obtained, urinary

pregnanediol excretion reflects well the circulating Prog levels. Whether measurements of the plasma Prog rather than measurements of urinary pregnanediol are more useful in diagnosis in conditions associated with disturbed ovarian function remains to be investigated.

3.5.5. ESTROGENS

Although there are many methods for the detection of estrogens, none of them have been shown to be sufficiently specific for the quantitative estimation of estrogens in biological fluids without extensive preliminary purification and separation of estrogens from interfering material. Consequently, the specificity of a complete method for estrogen determination will, in general, depend not on the final detection procedure employed, but on the efficiency of previous steps taken to eliminate interfering material. Although the various detection procedures share a lack of specificity, there are considerable differences in sensitivity. Certain procedures are relatively insensitive. These include ultraviolet (UV) light absorption in the 280 mμ regions, and polarography (for estrone). Certain non-specific colour reagents

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have been used to detect and estimate estrogens in association with paper chromatography. These reagents are of particular value in the qualitative detection of estrogens in paper, or TLC. They are not much used in quantitative estimation.

Procedure for the quantitative estimation of estrogens in current use fall into the following groups: colorimetric, fluorimetric, and enzymic. The use of radioactive reagents to form radioactive derivatives from non-radioactive steroids in the measurement of these steroids in biological fluid is well established (Kliman and Peterson, 1960). However, the application of this procedure to estrogen determination has been rare, although the potential advantages are obvious.

There have been large numbers of estrogens isolated from natural sources, and several authors have observed the various physio-chemical states in which estrogens may exist in the blood. Such factors alone make the complete determination of blood estrogens a formidable problem. There are, however, further difficulties; except during pregnancy, estrogen levels in normal human blood are too low to be measured with any great degree of confidence (except

perhaps in the case of estrone). Although there is less interfering material in extracts of plasma than in extracts of urine, for instance, the presence of such material still poses a problem whenever the level of estrogen is low. Some progress has been made in the direct measurement of specific estrogen conjugates in urine, these procedures have not yet been applied to blood (except for the work of Purdy, Engel and Oncley, 1961). Difficulties in measuring protein binding of estrogens, as well as other steroid hormones, are well recognized, and methods of liberating estrogens from the binding protein, an apparently essential step in the measurement of blood estrogens, may not be always quantitative. Compared with those available for determining urinary estrogens, methods for measuring blood estrogens are in a comparatively early stage of development. Again, no methods have been proposed for the measurement of any estrogens other than the three classic estrogens in the blood of either the pregnant or the non-pregnant subject, in spite of the potential importance of the more recently discovered estrogens. Also there are no methods for the direct measurement of specific estrogen conjugates in blood.

CHAPTER V

ASSAYS OF CORTICOSTEROIDS BASED ON METHODS OTHER THAN PROTEIN BINDING

1. INTRODUCTION

The large number of functional groups present in CSs makes it impossible to find a method of determination which will be specific for a single compound. Reactions characteristic of certain groupings have been developed to estimate various classes of steroid on the microgram scale. Steroids containing the 17, 12-dihydroxy-20-oxo side-chain can be determined by reaction with phenylhydrazine and the 21-OH-20-oxo group can be estimated with tetrazolium salts. The delta 4,3-oxo group forms coloured derivatives with isonicotinic acid hydrazide and gives fluorescent products with alkali, acids, salicylic acid hydrazide and zinc acetate. Steroids can also be determined by quantitative formation of radioactive derivatives. The method of CPB can be used to estimate those steroids which are strongly bound to plasma protein. Since each method estimates a different group of steroids, values for total CSs in plasma will

differ according to the method of estimation used. The methods for determining the CSs based on PB have already been discussed. The methods based on other principles will now be described briefly.

2. FLUORESCENCE

2.1. ALKALI FLUORESCENCE

The fluorescence of certain steroids under alkaline conditions was discovered by Bush (1952). The reaction can be carried out on chromatograms by dipping the paper in 2N aqueous NaOH solution (or solutions of choline or tetraethylammonium hydroxide) and drying at 60-80°C. The fluorescence which is yellow, is excited by UV light, the optimum wave length being 365 mμ. The reaction is specific for the delta 4,3-oxo-octa hydro-naphthalene group (Bush, 1954) or groups converted to that by alkali. The technique of estimation of CSs on paper by alkali fluorescence has been discussed thoroughly by Bush (1961).

2.2. ACID FLUORESCENCE

2.2.1. INTRODUCTION

The fluorescence of steroids in concentrated acids was first applied by Sweat (1954, 1955) to the quantitative analysis of CSs in blood. It is the most sensitive of the simple methods for estimating F and B, and is the basis of several procedures.

The steroids are extracted from the plasma with dichloromethane (CH_2Cl_2) or chloroform ($CHCl_3$) of high grade purity and the extract is treated with acid reagent directly or after evaporation or after various purification procedures. The fluorescence is read at a specified time and compared with that of standards. Maximum fluorescence of CS is produced by excitation at 470 mμ. Most methods require 1-5 ml of plasma, though Glick, von Redlich and Levine (1964) have developed a micro-method which uses 20-50 μl of plasma. Internal correction for losses using radioactive F or B is included in those procedures aiming at a high degree of accuracy and specificity.

2.2.2. PRINCIPAL CORTICOSTEROIDS MEASURABLE BY THIS TECHNIQUE

The principal CSs measurable by this technique are F, B and 20-dihydrocortisol; this last steroid does not normally appear in appreciable concentration in plasma. The relative intensities of fluorescence depend upon the composition of the acid reagent and the time of development. Compound S, 21-deoxycortisol and E fluoresce slightly. The interfering fluorogens are removed by washing the plasma with light petroleum before the extraction. Evaporation of the extract and partition of the residue between 70% ethanol and petrol (Sweat, 1955) should have a similar effect. Also, washing the CH_2Cl_2 or $CHCl_3$ extract with dilute NaOH solution serves the same purpose. Therapy with triparanol (MER-29) (Mattingly, 1962) or spironolactone (Gochman and Gantt, 1962) may lead to falsely high results, and with sulphadimidine (Braunsberg and James, 1960), to falsely low values.

Estimation of fluorescence on a crude extract of plasma without further purification is adequate for routine assessment of pituitary-adrenal function (Mattingly, 1962, 1964). The results of

acid fluorescence methods correlate well with those obtained using the Porter-Silber method, though the latter are generally slightly lower (De Moor et al., 1962; Mattingly, 1962, 1964; Steenburg and Thomasson, 1964).

2.2.3. INTERFERING SUBSTANCES

Non-steroidal fluorogenic material is present in plasma, as shown by comparison of fluorescence methods with a double-isotope technique (James, Townsend and Fraser, 1967) and by the presence of fluorogens in the plasma of hypoxed or adrexed subjects (Silber, Busch and Oslapas, 1958; De Moor, Steeno, Raskin and Hendrikx, 1960). Frankel, Cook, Graber and Nalbandov (1967) have shown that in avian plasma and probably also in mammalian plasma, there are two types of interfering fluorogen, one having a fluorescence development time similar to that of CSs and the other showing a linear increase of fluorescence over longer periods of time.

The contribution of non-steroidal fluorogens can therefore be minimized in three ways; by reading the fluorescence as soon as possible after

mixing, by mathematical correction based on reading at various times, or by removal of such fluorogens before induction of fluorescence (Dixon, Booth and Butler, 1967).

Chromatography permits the separate estimation of F and B as well as the removal of interfering fluorogens. Sweat (1954) eluted separately non-steroidal material, F and B, using silica gel micro-column and with increasing concentrations of ethanol in $CHCl_3$.

Paper chromatography has been used by Peterson (1957) and by Bondy and Upton (1957). Interfering fluorogens are eluted from paper giving a high background in spite of elaborate washing techniques.

Braunsberg and James (1960) have described a highly specific system of purification, using a silica gel column for preliminary isolation of the individual steroids.

2.2.4. METHOD OF PETERSON

Peterson (1957) modified the fluorometric method of Sweat (1954) by incorporating isotope

dilution using ^{14}C-corticosterone for the quantitative estimation of B in human plasma. The method involves extensive purification of the plasma extract without concern about minor losses of steroid and 30 to 40 ml of plasma are required for each determination. The sensitivity of the assay makes it possible to detect as little as 0.2% plasma B. The author reported a range of plasma B values of 0.5 to 2.0 µg% with a mean of 1.1 µg% and a standard deviation of $\pm$ 0.3 µg% in 20 normal subjects.

2.2.5. METHOD OF SILBER, BUSCH AND OSLAPAS

Following Sweat (1954) and Peterson's (1957) publications, Silber et al., (1958) further modified the fluorometric technique, making it less specific but more practical, analytical and useful. In this procedure, F and B are not separated and inasmuch as both of these steroids fluoresce in H_2SO_4 (Sweat, 1954), the authors reported that it might be successfully applied to the plasma of man, the rat, monkey and guinea pig by merely using the appropriate steroid as the standard.

The method requires 0.5 or more plasma for each determination and the standard curve ranges from 100 to 500 ng of B.

2.2.6. METHOD OF GUILLEMIN, CLAYTON AND LIPSCOMB

Guillemin, Clayton and Lipscomb (1958) made a slight modification in the fluorometric technique by Silber et al., (1958) and determined the plasma free CS level in rat plasma. The method requires 2 ml of plasma. The authors reported basal or resting levels of plasma free CSs from 9.0 to 15.9 µg% (mean and standard error; 12.2 $\pm$ 0.30 for 128 determinations). After stress, levels increase up to 500%, while total adrex or hypox lowered the values to 3.5 - 6.5 µg%, with no change upon exposure to stress of the adrexed or hypoxed animals.

Guillemin, Clayton, Lipscomb and Smith (1959) further modified the procedure with a reduction in the volume of plasma required (0.5 ml or more) for the measurement of plasma CSs, and recovery values of 100.3% to 105.0% from plasma and water respectively were reported.

2.2.7. METHOD OF GLICK, von REDLICH AND LEVINE

Glick, von Redlich and Levine (1964) reported a micro adaptation of the fluorescence technique (Sweat, 1954; Peterson, 1957; Silber et al., 1958)

for the determination of unconjugated B and F in plasma or adrenal tissue. The method requires 20-50 µl of plasma for each determination. The authors reported values of B or F in normal subjects such as rat, monkey or human, are quite high. The reported value of B level in rat is 52-65 µg%, F levels in monkey are 21, 26 and 77 µg%, while man gives 38 µg%. The sensitivity of the method as reported by the authors is of the order of 0.5 ng of steroid with 100% recoveries reported.

3. COLOUR REACTIONS

3.1. REDUCTION OF TETRAZOLIUM SALTS

The reduction on paper chromatograms and in solution of tetrazolium salts to formazans by cpds containing an alpha-ketol group has been extensively used for detection and assay of CS containing the 21-OH-20-oxo group. There are several tetrazolium salts available of which blue (tetrazolium, 3:3'dianisolebis-4:4'-(3:4-diphenyl)-tetrazolium chloride (Rutenberg, Gofstein and Seligman, 1950) is the most sensitive.

The reaction is carried out in

alcoholic alkali. The base can be NaOH, tetramethylammonium hydroxide or choline. The solvent must be rigorously purified. The reaction is generally allowed to proceed at room temperature for 20-50 minutes, though higher temperature and shorter times may be used (Nowaczynski, Goldner and Genest, 1955; Izzo, Keutmann and Burton, 1957). The absorption maximum of the formazan derived from blue tetrazolium is 525 mμ.

Many cpds other than CSs contain alpha-ketol groups, notably sugars. The background due to interfering chromogens derived from paper can induce a serious error.

3.2. THE PHENYLHYDRAZINE REACTION (PORTER-SILBER METHOD)

The reaction of CS containing the 17,21-dihydroxy-20-oxo group with phenylhydrazine in methanolic or ethanolic H_2SO_4, to give yellow products with maximum absorption at 410 mμ, was first employed by Porter and Silber (1950) as a method for determining urinary corticosteroids. Compounds giving the reaction are frequently termed Porter-Silber chromogens. The procedure was applied by Silber and Porter (1954) to

the estimation of steroids in crude extracts of plasma. Cortisol and cpd S could be measured at this wave length but not corticosterone. The colour development requires 18 hours at room temperature. The same reaction could be carried out in 30 minutes at 60°C, but gives less reliable results (Silber and Porter, 1957).

Many cpds in blood are Porter-Silber chromogens, and the specificity of the technique depends upon the extraction and purification procedures carried out before treatment with phenylhydrazine. There are also substances in plasma which give a yellow or brown colour with H_2SO_4. To compensate for high background absorption, most workers use a blank consisting of plasma extract and reagent from which the phenylhydrazine has been omitted (Porter and Silber, 1950).

3.2.1. THE MODIFICATION OF PORTER-SILBER METHOD BY NELSON AND SAMUELS

Porter and Silber (1950) colour reaction was previously applied to crude blood extracts and on a macro scale. The authors were unable to identify any material in plasma after administration of cortisone. Nelson and Samuels (1952) improved the

procedure, primarily by employing chromatographic separation and micro cuvettes, so that they were able to measure the concentration of F in human plasma.

4. UV ABSORPTION MEASUREMENT METHOD

The steroids with alpha-B-unsaturated ketone give an absorption peak at 240 mμ. The CSs like B, F, Aldo, Prog, and testosterone, as well as other steroids may be measured by this method but require initial separation to distinguish them. Extensive purification is necessary to avoid interference by non-steroidal substances which also exhibit absorption in this region. This method is relatively insensitive so that large amounts of blood are required for reliable measurements (Weichselbaum and Margraf, 1955).

5. ISOTOPIC METHOD

The concentration of a steroid in plasma can be determined by quantitative formation of a radioactively labelled derivative. Since the specific activity of the derivative expressed as disintegrations/minute per equivalent must be the same as that of the

labelled reagent, the radioactivity of the derivative is a measure of the quantity of steroid present.

The specific activity of the reagent is determined by reaction with a pure steroid, isolation of the pure derivative and determination of its specific activity.

This method is always used in conjunction with internal correction for losses, the feasibility of the method depending upon the availability of the steroid or its derivative labelled with another radioisotope which can be counted independently from the isotope in the labelled reagent.

If the labelled steroid itself is available, it is added to the plasma; losses throughout the entire procedure are then compensated, but the amount of tracer steroid added is measured together with the endogenous steroid. This technique is termed the double isotope dilution derivative assay. If the specific activity of the tracer steroid is sufficiently high, the weight added may be negligible.

With a reagent of high specific activity the method is extremely sensitive. Since several stages of chromatography and possibly a chemical

change are used to purify the derivative, it is also highly specific. For the same reasons, it is expensive and time-consuming. The method has been employed only where accuracy and specificity are of prime importance, for example as a reference method in the evaluation of more crude procedures (James et al., 1967) or where the measurement of very small amounts is desired, as in the estimation of plasma concentrations of Aldo.

PART II

CHAPTER I

EXPERIMENTAL SECTION

1. AIMS OF THE THESIS

The literature describing the important role played by the adrenal gland and especially the GCCs, which it secretes, in the pathogenesis of LLk has been reviewed (cf Chapter III). It has been known for sometime that adrex increases the incidence of LLk in mice and conversely the 11-oxygenated corticoids decrease or delay the incidence of spontaneous or radiation-induced LLk. The mechanism whereby the GCCs suppress primitive lymphoid cells and destroy them in situ, while their lack favours lymphoid production, has also been reviewed (cf Chapter III). Therefore, it became reasonable to investigate whether adrenal hypofunction may not play an important role, though not necessarily a primary one, in the pathogenesis of Lk.

While a considerable number of papers have appeared describing attempts to assess the adrenal cortical function in human Lk (Levin, 1948; Hanlon, Mason and Stinkney, 1950; Closon and Heusghem,

1952, Dobriner, Kappas and Gallagher, 1954; Bonner, Homburger and Fishman, 1956; Zimmerman, Bloch, Williams, Hitchcock and Hoelscher, 1956; Paolino, Molinati, Pierri, Pizzini and Resegotti, 1958; Chernysheva and Arbuzoa, 1962), it is not possible from such studies to assess the importance of role of adrenal cortex in the pathogenesis of the disease, because these studies were conducted in persons already ill with the disease and not prior to the appearence of clinical symptoms. Thus, it is impossible from the studies to distinguish the role which stress due to the disease could have had on the adrenal gland from the part which changes in adrenal cortical function itself, could have played in the pathogenesis of the disease.

Therefore, studies on adrenocortical function were undertaken in the high-leukemic AKR mice, The utilization of such animals as an experimental tool was considered to be particularly suitable because about 90% of the female mice of this strain develop LLk, the percentage in males being somewhat less.

The experiments were conducted on some AKR mice while they were still apparently well and on others while they were manifestly ill with a

spontaneously appearing LLk. Determinations of plasma and urinary CS were also made in AKR mice sick with conditions other than LLk as well as in low-tumour bearing C57Bl/6J mice. In this way an attempt was made to assess the relation of adrenocortical function to the pathogenesis of LLk.

2. STUDIES DIRECTED TOWARDS THE DEVELOPMENT OF A SUITABLE ASSAY

2.1. INTRODUCTION

On undertaking to investigate the circulating levels of corticosteroids in high- and low-leukemic mice, the first problem was to find a technique with sufficient sensitivity to determine these steroids in small volumes of plasma or serum available from individual mice. Others working on the same problem worked with pooled blood samples (in *in vivo* studies) or adrenals from several animals (in *in vitro* studies) (Levine and Treiman, 1964; Triller and Birmingham, 1965; Solem, 1966). Only a paper by Glick et al., (1964) described a micro adaptation of the method of Silber et al., (1958) which appeared sufficiently sensitive for the determination of

corticoids in the blood of individual mice, but so far no reports have appeared describing such usage either by Glick et al., (1964) or by others.

All previous studies on the determination of CS in mice involved the fluorimetric estimation of the corticosteroids, a technique which is known to yield higher than true corticoid values due to a variable quantity of contaminating non-specific substances known to be included with this estimation. At the time the research on the problem of this thesis was first begun, Murphy et al., (1963) had already reported a new technique for the determination of the circulating corticoids based on the principle of competitive protein-binding. This method had the advantages over the fluorimetric techniques of greater simplicity, specificity and time-saving characteristics. However, at that time its use was intended for the human and 0.5 to 1.0 ml plasma or serum were required. Subsequently, Murphy reported a microtization of the method so that 0.1 ml of plasma or serum was required (Murphy and Pattee, 1965; Murphy, 1967). However, the micro method had up to that time been utilized only in humans, in whom the chief CS is cortisol (Grant, 1962). Therefore, the

problem was to investigate whether this method could be adapted for use in mice in whom the chief corticoid is corticosterone (Southcott et al., 1956; Wilson et al., 1958; Halberg et al., 1959; Triller and Birmingham, 1965). The studies now to be reported were designed with this in mind.

2.2. THE MICRO ASSAY

2.2.1. THE PRINCIPLE

The principle of the Murphy method involves the competitive binding of labelled and unlabelled corticoids by a globulin (CBG) normally found in the serum or plasma which specifically binds the corticoids. In the micro method first developed by Murphy for the determination of plasma corticoids in the human, the diluted test plasma or serum was boiled to denature the CBG, thus liberating the corticoids bound to the CBG. A solution (solution A) containing CBG from a standard plasma or serum and labelled cortisol was added to the unknown and an equilibration immediately occurred between the unbound corticoid and that bound to the CBG. The amount of labelled corticoid now bound to the CBG was inversely proportional to the amount of unlabelled corticoid

originally present in the unknown. Fuller's earth was then added which removed the free corticoids, both labelled and unlabelled, and the amount of labelled corticoid bound to the CBG was estimated by an appropriate radioactive-detecting device. The amount of corticoid originally present in the unknown was read off from a standard curve which had a range of from 0 to 40 ng. The details of this method are given below.

2.2.2. METHODS AND MATERIALS

2.2.2.1. Preparation of CBG-Isotope Solution (Solution A)

Add 5 ml of pooled normal human plasma or serum to 4 ml of ^{3}H-cortisol stock solution (containing 1 µc/ml). Add water to make up the volume to 100 ml. In the studies on mice, ^{3}H-corticosterone was used to make up solution A in place of the ^{3}H-cortisol.

2.2.2.2. Preparations of Standard Curve

A standard curve was prepared for each experiment. Dissolve 25 mg of cortisol in 250 ml of ethanol. This served as the stock solution for

subsequent dilutions. Prepare standard solutions of 0, 10, 20, 30 and 40 ng/ml saline and a standard curve is run for each experiment. Pipette 1 ml of each standard into a set of 3 test tubes. For studies on mice, B replaced F in the standards.

2.2.2.3. Determination of Unknowns

After boiling the unknowns for 2 minutes, and cooling to room temperature, add 1 ml solution A. Shake well. Add 17 mg of Fuller's earth and cool to 4-8°C in cold bath (or cold room). This is measured out by a specially designed spoon to speed up the procedure. Shake the samples either for 3 minutes in an automatic shaker and replace in the cold bath for 5 minutes or for a few seconds every minute for 10 minutes by hand. Centrifuge the samples for 2 minutes at 2000 - 3000 g. Pipette 1 ml of supernatant fluid and transfer to 10 ml of Bray's solution. Mix well and set up to count 4000, 5000 or 10,000 counts in a scintillation counter. The time (in minutes) was plotted as ordinates against concentration of cpd F as abscissae yielding a curve off which the unknowns are read.

2.2.2.4. Preparation of Bray's Solution

This solution is a counting medium used in connection with scintillation counters and its composition per litre is as follows: to 800 ml of dioxane in a 1 litre measuring flask add 100 ml of anhydrous methanol, 20 ml of ethylene glycol, 200 mg of POPOP (4-methyl-5-phenyloxazolyl benzene) and shake well to dissolve POPOP. Add 4 gm PPO (2,5-diphenyloxazole), 60 gm of naphthalene and make up the total volume to 1 litre by adding more dioxane.

2.2.2.5. Standard Check

As a standard check, 1 ml of solution A is added to 10 ml of Bray's solution and counts recorded. Its purpose is to check on the amount of radioactivity in solution A, and was done in each experiment.

2.2.2.6. Estimation of Precision of Assay

Normally, the index of precision (λ) is calculated for any given standard curve in order to obtain an estimate of the precision of the assay. The method of calculating this index has been given previously (Chapter IV, section 1.2.4), but

briefly it is the ratio of the standard deviation of the values obtained for the standards over the regression coefficient, which is a measure of the slope of the standard curve.

While the index of precision, λ, was calculated in the more critical experiments, a more easily calculated estimate of the slope of the standard curve was obtained in many of the preliminary experiments by calculating the ratio of the mean ordinate (the time to count a certain number of counts) for the standards containing 40 ng standard to that of the zero standard. The ratio, referred to as 'R' hereafter in the text and tables, bears an inverse relationship to λ. In the experiments to be described, the aim was to obtain values of R equal to or greater than 2, a value which indicated that there was a drop of 50% in the binding of the CS by CBG in the presence of the standard containing 40 ng steroid as compared with the zero standard. Such a drop in CBG-binding of about 2 was obtained in Murphy's original micro assay and indicates sufficient precision in estimating unknowns. Thus, the R value served as a guideline during the preliminary stages of the search for a suitable micro assay for CSs in mice.

2.2.3. EXPERIMENTS CONDUCTED

2.2.3.1. Experiments Involving the Use of Fuller's Earth (Experiments 1 to 3)

Because corticosterone is the main CS in mice, while F is the main one in humans, it was necessary to use B instead of F in preparing the standards for the micro assay. Thus, in the first experiment B replaced F both in the standards and in the preparation of solution A, while in experiment 2, B replaced cortisol only in the standards and not in solution A. However, the R value was 1.0 and 1.4 respectively (Table 1, experiments 1 and 2), indicating standard curves with slopes too shallow to be useful for the assay of corticosterone.

The third experiment compared the slope of the standard curve of the original Murphy micro assay as described in section 2.2.2., in which normal human serum (NHS) was used as the source of CBG with the slopes of the 2 other standard curves in which mouse serum was used as the CBG source. One of the latter standard curves was obtained by utilizing F to prepare the standards and solution A, while B was utilized in the same way in place of F to provide

Table 1. SUMMARY TABLE OF EXPERIMENTS DESIGNED TO TEST CONDITIONS NECESSARY TO PRODUCE SLOPE OF SATISFACTORY STEEPNESS

Expt. No.	Source of CBG	Tritiated Corticoid	Standards	Resin	$\frac{T\ 40}{T\ 0}$
1	Human Serum	B	B	Fuller's Earth	1.0
2	" "	F	B	" "	1.4
3	" "	F	F	" "	2.0
	Mouse Serum	F	F	" "	1.5
	" "	B	B	" "	1.0
4	Human Serum	B	B	Florisil	2.3
5	" "	B	B	"	2.2
6	" "	B	B	"	2.3
7	" "	B	B	"	2.2
	" "	B	B	"	1.9

the third curve. While the R value for the original Murphy assay was satisfactory at 2.0, the remaining 2 curves had unsatisfactory values of 1.5 and 1.0 respectively. (Table 1, experiment 3).

2.2.3.2. Experiments Involving the Use of Florisil (Experiments 4 to 7)

Having attempted to vary the source of CBG without notable success, it was decided to change the resin from Fuller's earth to florisil on Dr. Murphy's suggestion. Accordingly, 89 mg florisil was used, together with solution A prepared from NHS and ^{3}H-B. The remainder of the procedure remained unchanged. The R value was 2.3. (Table 1, Experiment 4). Thus, this method yielded a curve of useful slope for the determination of B in the micro range. The standards of the next 3 experiments were prepared in the same way and the R value range from 1.9 to 2.3 (Table 1, Experiments 5 to 7).

In Experiment 5, plasma B levels were determined in 9 CF_1 female mice, 13 months' old and adapted to 26-27°C. The blood samples were collected between 10 and 10:30 a.m. by orbital bleeding (Riley, 1960). Whatever volume of mouse plasma obtained,

a final volume of 1 ml was made up with normal saline. The procedure described in section 2.2.2.3 was then followed.

Table 2 presents the data both for the standards and the unknowns. The standard curve was satisfactory, but only 5 of the 9 unknowns fell within the range of the standards, and these gave an acceptable mean of 13.0 µg% with a range from 8.5 to 18.0 (Southcott et al., 1956; Wilson, 1958). Three of the 4 samples which gave values below that of the lowest standard had an initial volume of less than 0.10 ml. Inasmuch as this may have been due to the small initial volume of plasma of these samples, it was decided in the next 2 experiments to test the possibility that boiling for 2 minutes was insufficient to liberate B from the CBG expecially in volumes of plasma smaller than 0.10 ml.

Accordingly, in Experiment 6, blood samples were obtained by decapitation from CF_1 female mice, 13 months' old and adapted to 26-27°C. Three samples each of 0.05 ml serum and 0.10 ml serum, were autoclaved and a similar 6 samples extracted with methylene chloride, to test alternative means of liberating the bound B from the CBG.

Table 2. THE DETERMINATION OF CORTICOSTERONE IN FEMALE MOUSE PLASMA UTILIZING BOILING TO FREE THE BOUND STEROID

Standard Cpd B (ng)	Mouse	Plasma Volume (ml)	Average Time Taken for 5000 Counts (Minutes)	Cpd B (µg/100 ml)
0			1.64	
10			2.21	
20			2.84	
30			3.15	
40			3.58	
	A	0.04	1.66	-
	B	0.05	1.25	-
	C	0.05	1.47	-
	D	0.10	1.45	-
	E	0.10	2.24	10.0
	F	0.10	2.14	8.5
	G	0.10	2.31	11.2
	H	0.10	2.66	17.4
	I	0.10	2.71	18.0

The extraction procedure was conducted as follows:

The samples were made up to 1 ml by the addition of water. For the extraction of the serum corticoids, 1 ml of methylene chloride was added to each sample in glass-stoppered centrifuge tubes, shaken vigorously and centrifuged. The organic layer containing cpd B was removed by means of a long needle and syringe. The extraction was then repeated. Methylene chloride was evaporated off by a gentle steam of air. To each dried tube 1 ml of 2% ethanol in saline was added, shaken and placed in a waterbath at 45°C for a minute to dissolve the corticoids. Then, the procedure as described in section 2.2.2.3. was followed.

Autoclavation was conducted for 10 minutes in a pressure cooker at 10 - 12 lbs. pressure. The samples were cooled and the procedure described in section 2.2.2.3. was then followed.

The results are shown in Table 3, and indicate that either method of liberating cpd B from CBG was satisfactory at both serum volume levels.

Table 3. DETERMINATION OF CORTICOSTERONE IN MOUSE SERUM UTILIZING METHYLENE CHLORIDE EXTRACTION OR AUTOCLAVATION TO FREE THE BOUND CORTICOSTERONE FROM CBG

Treatment	Serum Volume (ml)	Average Time Taken for 5000 Counts (Minutes)	Cpd B (µg/100 ml)
Methylene	0.05	2.61	5.4
Chloride	0.1	3.09	10.4
Auto-	0.05	2.62	5.4
clavation	0.1	3.05	9.5

In Experiment 8, the percentage recovery of added B was tested in the mouse serum samples obtained as described in Experiment 7. To this end, 9 ng cpd B was added to 0.1 ml serum in the form of 0.9 ml of the 10 ng standard, while control samples of 0.1 ml serum were made up to 1 ml with 2% ethanol in saline, the solution in which the standards were made up. All samples and standards were autoclaved for 10 minutes at 10-12 lbs. pressure. The mean and standard error of the 5 unknowns was 14.5 ± 0.2 µg%, while that of the 5 recoveries was 22.2 ± 1.3 µg%, an 86% recovery (Table 4).

2.2.4. CONCLUSIONS

Murphy's micro method for the determination of plasma or serum F levels in humans can be adapted to determine the circulating B levels in mice by using florisil in the place of Fuller's earth to bind the free steroid. Satisfactory separation of bound B from CBG was achieved by either extraction with CH_2Cl_2 or autoclavation for 10 minutes at 10-12 lbs. pressure. Recoveries at 86% were also satisfactory.

Table 4. THE DETERMINATION OF CORTICOSTERONE IN POOLED MOUSE SERUM, USING AUTOCLAVATION TO FREE THE BOUND STEROID

	Sample	Average Time Taken for 5000 Counts (Minutes)	Cpd B (µg%)	Mean + Standard Error (µg%)
Un-known	1	296	15.0	14.5 ± 0.2
	2	301	15.8	
	3	299	15.5	
	4	283	12.2	
	5	291	14.0	
Re-covery	6	322	19.4	22.2 ± 1.3
	7	329	20.6	
	8	338	22.5	
	9	335	21.7	
	10	364	27.0	

2.3. THE ULTRAMICRO ASSAY

2.3.1. INTRODUCTION

The adaptation of Murphy's micro assay for the determination of F to one for the determination of B proved to be possible. This assay required at least 0.05 ml plasma or serum and preferably double that amount. While this volume could be obtained by orbital bleeding of the one year old or older CF_1 female mouse and more could be obtained by decapitation, the problem of obtaining this volume by orbital bleeding from considerably smaller mouse strains such as the high-leukemia AKR and their proposed controls, the inbred low-leukemia C57Bl/6J that are about 25-30% smaller in one year old mice, proved much more difficult. Indeed, preliminary trials revealed that this was not possible, and we found it difficult to obtain 0.2 - 0.3 ml blood from the decapitation of AKR and C57 mice younger than 3 months of age. Yet, in our studies, the plan was to include such young animals as in the case of AKR strain which represented the pre-leukemic stage. Moreover, it was proposed to include mice from whom it was planned to take several blood samples by orbital bleeding within a period of 24 hours. This obviously

emphasized the utilization of small volumes of plasma to avoid any trauma to the animals. The pooling of blood from several mice was an unsatisfactory solution as this would tend to conceal individual differences and therefore it was decided to attempt to develop an ultramicro assay for the determination of corticosterone.

2.3.2. STUDIES DESIGNED TO DISCOVER AN ACCEPTABLE CBG

In the attempt to develop an ultramicro assay, the main effort was directed towards trying to discover a plasma which would contain a CBG that would permit an approximately 50% drop in the binding of B by CBG in the presence of the 4 ng standard as compared with the binding of the steroid in the presence of the zero standard. Towards this end, experiments were conducted with human, rat, monkey, fish and dog plasma. Where only R values were calculated, only 0 and 4 ng standards of B were set up; where the index of precision was set up, 0, 1, 2, 3 and 4 ng standards were set up.

2.3.2.1. Human Serum as the Source of CBG

In the first experiments involving human serum as the source of the CBG, it was decided to use the serum of a patient treated with estrogen for carcinoma of the prostate. In the micro method, the NHS is used at the 5% level in preparing solution A. Therefore, an ultramicro method of one-tenth of the range of the micro method would have required 0.5% NHS, assuming that proportionality existed. However, inasmuch as the serum used was obtained from a human treated with estrogen which is known to raise the circulating CBG level to about 3 times the normal level (Peterson, et al., 1960), one-third of 0.5% or about 0.2% of the serum from the carcinomatous patient was prepared for use in the ultramicro assay. The estrogenized serum was tried because it was hoped to provide a CBG with a greater than normal affinity for corticosterone. Accordingly, in the first experiment, a series of standards were set up in triplicate for zero and 4 ng cpd B, and taken through the procedure as described under sections 2.2.2.1., 2.2.2.2., 2.2.2.4 and 2.2.2.5., with the following changes: (a) solution A was made up by adding 4 µc% ^{3}H-B to 0.2% estrogenized human serum (^{3}H-B was kept at the 4 µc% concentration in all experiments reported here)

and (b) 60 mg florisil was used as the resin instead of Fuller's earth. Florisil was used in all the subsequent assays involving corticosterone. In Experiment 1, R value of 1.2 obtained (7.17 minutes for 4 ng B over 6.17 minutes for the zero standard) was an unsatisfactory value. Hence the serum from an estrogenized carcinomatous patient was not satisfactory for use in the ultramicro method.

In Experiment 2, serum from an estrogenized patient, from a normal human and from a normal Sprague-Dawley female rat were each used to prepare a solution A at 0.2% concentration. The R value for each serum from both humans were 1.1 while in the case of the rat it was 1.3. These values were still too low.

In Experiment 3, the serum from a woman in late pregnancy was used. It has been reported that the serum CBG increases markedly under these conditions (De Moor et al., 1962). Solution A was made up with this serum in 0.2, 1, 0.5 and 2% concentrations, but this yielded unsatisfactory R values of 1.1, 1.2, 1.3 and 1.1. Because of the poor slopes obtained with NHS, it was decided to begin testing other sera as a source of CBG.

In the experiments so far described, the ^{3}H-B, which was dissolved in 10% alcohol was added directly to the serum used for preparing solution A. Indeed, the addition of 1 ml of 10% alcohol to 0.125 ml of human serum caused a slight milkiness suggesting damage to the protein. Therefore, in Experiment 4, NHS was used at the 0.5% level in solution A which was prepared in two ways (a) ^{3}H-B in 10% alcohol was added directly to the NHS without previously diluting the NHS and (b) ^{3}H-B was added to NHS which was previously diluted to half the desired final volume. Another variable in this experiment was the amount of florisil added. In the experiments reported so far, about 60 mg of florisil was added to each test tube but in this study the effect of 60, 120, 180 and 240 mg florisil was tested. Zero and 4 ng standards were prepared for each of the conditions and the R ratios are given in Table 5.

It is clear from the table that diluting of the serum before addition of ^{3}H-B in preparation of solution A gives a somewhat better result than when the serum is not diluted beforehand and that 60 mg florisil gives somewhat better results than the other amounts tested.

Table 5. THE EFFECT OF DILUTING SOLUTION A BEFORE ADDING ^{3}H-B AND OF VARIABLE AMOUNTS OF FLORISIL ON THE BINDING OF ADDED COMPOUND ^{3}H-B BY CBG. (Experiment 3)

Corticosterone Standards (ng)	Dilution of Solution A before Addition of ^{3}H-B							
	No				Yes			
	Florisil (mg)							
	60	120	180	240	60	120	180	240
0	-	5.28[1]	13.25	9.95	3.05	5.64	7.41	11.00
4	-	7.26	15.45	13.40	5.08	7.94	11.34	15.46
$\frac{\text{Standard 4}}{\text{Standard 0}}$ = R	-	1.4	1.2	1.3	1.7	1.4	1.5	1.4

[1] Mean time (minutes) to count 5000 counts in 1 ml supernatant solution following removal of free labelled and unlabelled cpd B by Florisil.

In Experiment 5, comparison was made between (a) the effect of 2 concentrations of human serum in the making up of solution A and (b) the presence of 2 different amounts of added florisil (30 and 60 mg amounts). The results shown in Table 6 indicate that 60 mg of florisil is to be preferred to 30 mg and the 0.5% solution to the 0.2% one. Therefore, in all further experiments with NHS, florisil was fixed at 60 mg.

In Experiment 6, solution A was prepared with NHS at the 0.5% and 1% levels, and these were compared with the 0.5% serum of a female rat of the Wistar strain. The R ratios for 0.5% and 1% NHS and 0.5% rat serum were 1.5, 1.5 and 1.1, respectively. Thus solution A made up with 0.5% or 1% NHS gave the same results and either seemed to be preferable to that of the solution A prepared with rat serum at 0.5% level.

In Experiment 7, comparisons were made between 4 different kinds of solution A containing NHS at the 0.5%, 1%, 2% and 5% levels, for which the R values obtained were 1.6, 1.5, 1.4 and 1.1 respectively. Thus, the 0.5% and 1% seemed to be best in that they produced lines of greater slope than the other concentrations.

Table 6. THE EFFECT OF 0.2% AND 0.5% NORMAL HUMAN SERUM IN SOLUTION A AND OF 30 mg AND 60 mg FLORISIL ON THE BINDING OF ADDED ^{3}H-B BY CBG

Corticosterone Standards (ng)	Concentration of Human Serum in Solution A			
	0.2%		0.5%	
	Florisil (mg)			
	30	60	30	60
0	14.36[1]	24.91	9.29	11.99
4	17.58	34.48	14.41	20.39
$\frac{\text{Standard 4}}{\text{Standard 0}} = R$	1.2	1.4	1.5	1.7

[1] Mean time (minutes) to count 5000 counts in 1 ml supernatant solution following removal of free labelled and unlabelled cpd B by Florisil.

In Experiment 8, in which NHS was kept at the 0.5% level, one set of standards was dissolved in 2% ethanol in 0.9% NaCl and the other set was dissolved in saline containing very little ethanol (0.02 - 0.03%). The purpose of this experiment was to find out whether 2% alcohol in the standards had any effect on the binding capacity of the CBG in the way that 10% alcohol had in Experiment 3. However, the R ratios for the alcoholic series were 1.6 and for the non-alcoholic standards 1.5, hardly any difference.

In the next 5 experiments (Experiments 9 to 13), a complete set of 0, 1, 2, 3 and 4 ng standards was prepared in triplicate. In all 5 experiments, the concentration of NHS in solution A was 1% except in Experiment 9, where 0.5% was compared with 1%. Florisil was utilized at the 60 mg level in each. For these 5 experiments, slopes (b), standard deviations (s), and indices of precision (λ), were calculated and the results are shown in Table 7.

In Experiment 9, 0.5% and 1% concentration of NHS serum in solution A gave lamda which were close to each other (Table 7). This experiment was a repetition of Experiment 5 but with a whole set of standards.

In Experiment 12, there were two sets of standards, one set boiled and the other was autoclaved before adding solution A. The indices of precision for these two were 1.084 for boiled standards and 0.445 for the autoclaved ones (Table 7). The smaller λ value was due both to a steeper slope and a smaller standard deviation (Table 7).

Inasmuch as a λ with values greater than 0.300 are unsatisfactory (Dorfman, 1950), the λ values of 0.431 to 9.931 obtained in Experiments 9 to 13 inclusive were unacceptable.

Before abandoning NHS as a source of CBG, it was decided to test the effect of T_4 on the affinity of CBG for cpd B in view of the report by Labrie et al., (1965, 1968) who claimed that T_4 enhanced the binding affinity of CBG for B. In this experiment, 5 µg of T_4 was added <u>in vitro</u> to 25 ml of solution A to give a final concentration of 20 µg%, a level that was equivalent to that found in the serum of hyperthyroid persons. Four different types of solution A were prepared, each containing T_4 as indicated above, but with 0.2, 0.5, 1 and 2% respectively. The R values for these 4 samples

Table 7. THE SLOPES, STANDARD DEVIATIONS AND INDICES OF PRECISION OF STANDARD CURVES OBTAINED WHEN USING HUMAN SERUM AS SOURCE OF CBG

Experiment Number	Slope (b)	Standard Deviation (s)	Index of Precision $\lambda = s/b$
8	36.7	19.3	0.527
	37.5	16.2	0.431
9	20.0	18.2	0.909
10	18.7	9.8	0.523
11	19.0	20.6	1.084
	24.7	11.0	0.445
12	11.5	114.2	9.931

varied from 1.1 to 1.4, unsatisfactory values in all cases. Thus, the addition of T_4 *in vitro* did not increase the binding affinity of transcortin for corticosterone.

2.3.2.2. Rat Serum as the Source of CBG

Following the failure to develop a suitable assay for the determination of corticoids using NHS as a source for CBG, it was decided to test sera from rats.

Accordingly, in Experiment 15, both male and female rats of the Brown Norwegian, Wistar and the Sprague-Dawley strain were bled from the tail vein. Six solutions A were made up with their sera, all at a concentration of 0.2%. Florisil in all experiments with rat sera was used in the amount of 60 mg. The R values showed no remarkable difference due to strain or sex, all the values being at a low unsatisfactory level (Table 8).

Experiment 16: Seal and Doe (1965) reported that castration increases the CBG level in the rat. Therefore, blood was taken from a male Sprague-Dawley and a male Wistar rat 6 weeks after

Table 8. R VALUES USING RAT SERUM AS THE SOURCE OF CBG

Strain	Male	Female
Brown Norwegian	0.8	1.1
Wistar	1.1	1.2
Sprague-Dawley	1.2	1.1

castration. Moreover, Seal and Doe (1965) claimed that the porcupine fish (Diodon hystrix) had a CBG in the serum with a very high specific affinity for corticosterone. Accordingly, this material was obtained from the Bellairs Research Institute of McGill University in Barbados, West Indies, and compared in this experiment along with the rat sera, each comprising 1% of its own tracer solution. The results showed R values of 1.1 for both rats and a value of 1.0 for the porcupine fish, again unsatisfactory values.

Experiment 17: Solutions A, containing 0.1, 0.2 and 0.5% castrated male Sprague-Dawley rat serum (same serum as in Experiment 16) yielded R values of 1.8, 1.9 and 1.5. Thus, the 0.1 and 0.2% concentrations of male castrated rat serum was better than the 0.5% and according to Experiment 16, better also than the 1% level at which point an R value of only 1.1 was obtained. It was previously shown that the best R values obtained with NHS were at the 1% concentration.

Experiment 18: Solutions A containing 0.025, 0.05 and 0.2% serum from male castrated Sprague-Dawley rat were compared with that containing

0.2% serum from a castrated Wistar rat. R values of 1.0, 1.2 and 1.8 were obtained for solutions A containing 0.025, 0.05 and 0.2% serum respectively for the Sprague-Dawley rat while the R value for the Wistar rat was 1.4. Thus, it would appear that the 0.2% level of serum of the Sprague-Dawley rat gave the best results.

Experiment 19: The sera of 2 castrated male rats, one of the Sprague-Dawley strain and the other of the Wistar strain, were used to prepare Solution A at the 0.2% level in each case and a complete standard curve with 0, 1, 2, 3 and 4 ng% of B were set up. The slopes, standard deviations and indices of precision were calculated and are shown in Table 9. The indices of precision of 0.286 and 0.316 were in the acceptable range.

Experiment 20: Two sets of standards (0, 1, 2, 3, 4 ng) were set up, and each solution A made up with 0.2% serum from a male castrated Sprague-Dawley rat. One set of standards was autoclaved for 10 minutes at 10 lbs. pressure and the other was not heated. The slopes, standard deviations and indices of precision for the 2 sets of standards were shown in Table 9. For unknown reasons, the indices of precision were markedly increased.

Table 9. THE SLOPES, STANDARD DEVIATIONS AND INDICES OF PRECISION OF STANDARD CURVES OBTAINED WHEN USING SERUM FROM CASTRATED MALE RATS AS SOURCE OF CBG

Experiment Number	Remarks	Slope (b)	Standard Deviation (s)	Index of Precision $\gamma = s/b$
19	Sprague-Dawley	74.2	21.2	0.286
	Wistar	45.9	14.5	0.316
20	Autoclaved Standards	55.8	70.3	1.259
	Non-heated Standards	29.3	46.2	1.577
21	Autoclaved Standards	47.7	49.8	1.045
	Non-heated Standards	63.7	38.1	0.598

Experiment 21: This experiment was a repetition of the previous one and gave an unacceptable high indices of precision.

Experiment 22: Because there was a considerable decline in the index of precision in the course of these studies, it was felt that perhaps one reason may be that there was some deterioration of the CBG on standing. Therefore, in this experiment, a comparison was made between CBG which was prepared on the day of the experiment with that prepared 2 days before the experiment. Serum from a castrated male Sprague-Dawley rat at the 0.2% level was used. However, poor results were obtained with both sera which yielded R values of 1.2 and 1.4 for the 2-day old and the fresh sample respectively. With this failure it was decided to investigate monkey serum as a source of CBG.

2.3.2.3. Rhesus Monkey Serum as a Source of CBG

While work was proceeding in our lab on the suitability for ultramicro assay using human, rat or porcupine-fish sera as a source of CBG, Dr. B.P. Murphy of the Department of Investigative

Medicine, McGill University, was examining a variety of other sera with the same object in mind. In the course of her investigations, she obtained results which indicated that monkey serum could be used as a source for CBG in a 0 to 4 ng assay for B and when notified of this, we proceeded to conduct experiments utilizing monkey serum as the CBG source. She also suggested that 50 mg florisil be used, that ethanol precipitation be the method of freeing bound corticoid from CBG in the unknowns and that the total volume of solution A and standard or unknown be 1 ml. These recommendations were followed in all experiments involving monkey serum except in Experiment 35 where 2 ml was the total volume used.

Experiments 23 and 24: These 2 preliminary experiments were designed to see whether the assay developed so far in Murphy's lab would yield acceptable results in our lab. One other suggestion made by Dr. Murphy, viz. that monkey serum should constitute 0.5% of solution A, was also tested in these 2 experiments. The results shown in Table 10 indicate rather poor standard curves with high indices of precision.

Table 10. DATA OBTAINED USING MONKEY (RHESUS) SERUM AS THE SOURCE OF CBG

Experiment Number	State of Serum	Slope (b)	Standard Deviation (s)	Index of Precision ($\lambda = s/b$)	R Value
23	Frozen 0.5%	27.1	42.1	1.552	1.2
24	" "	45.5	39.2	0.861	1.3
25	" "	26.5	43.3	1.632	1.7
26	Refrig. 1%	15.7	11.4	0.725	1.8
27	Fresh	21.0	4.4	0.210	1.9
28	Frozen	18.9	8.2	0.432	1.9
29	Frozen	28.7	14.3	0.500	2.1
30	Frozen	31.1	6.0	0.192	2.4
31	Fresh	19.1	24.7	0.291	2.0
32	Refrig.	19.1	3.2	0.167	1.9
33	Refrig.	33.0	15.9	0.482	2.1
34	Frozen		3.8	0.206	1.8
35	Refrig.	16.5	3.8	0.233	1.8

I - Sample from individual mouse; P-Pooled sample

Mouse Strain	Sex	Age (mos.)	Serum/ Plasma Test Volume (λ)	Serum/ Plasma Corticoid (μg%)	Amount Added for Recovery (ng)	% Recovery
AKR	M	10	10	1.5	1	80
AKR	M	10	20	4.3	2	100
AKR	M	10	10	2.0	1	58
	M	10	20	4.0	2	65
			30	3.3	3	58
AKR	M	7	20	2.0	2	80
			30	2.3	3	98
C57Bl/6J	M	9	10	0	1	80
			20	1.0	2	101
C57Bl/6J	M	9	10	2.0	1	110
			20	2.7	2	100
			20	2.7	2	84
			30	2.8	3	60
C57Bl/6J	M	12	10	0	1	30
			10	0	3	37
			20	0	1	31
			20	0	2	55
			30	0	3	47
C57Bl/6J	M	12	10	0	1	35
			10	0	2	100
			10	0	3	75
			20	0	2	40
			30	0	3	37
C57Bl/6J	F	10	10	2.5	1	65
			10	2.5	2	72
			20	1.3	2	50
			30	1.5	3	25

Experiments 25 and 26: In these experiments, the concentration of monkey serum was changed to 1% in tracer solution and not 0.5% as in the previous set of experiments. This did not result in any marked change in the index of precision (Table 10).

In all subsequent experiments, monkey serum constituted 1% of solution A.

Experiment 27: This experiment differed from the earlier ones in that in Experiments 23-26 inclusive, 0.5 ml of solution A was mixed with 0.5 ml of standard whereas beginning with Experiment 27 the ethanol of the added standard was evaporated before 1 ml of solution A was added. The results showed a marked improvement in the index of precision (Table 10).

Experiment 28: It was a repetition of Experiment 27 and in addition unknown samples of 10 and 20 lamda plasma obtained by orbital bleeding from a 10-month old male AKR mouse were employed for B determination. For recovery experiment, 1 and 2 ng of B were added to 10 and 20 lamda plasma samples. 1.5 and 4.3 µg% cpd B was detected in the 10 and 20 lamda samples respectively, and the respective recoveries of added B were 80 and 100%.

Experiment 29: In this experiment the amount of plasma for unknown determinations was increased to 30 lamda sample in addition to the 10 and 20 taken previously and also for recovery 3 ng B was added to unknown samples. The index of precision was slightly higher in this experiment and the per cent recoveries were lower as seen in Table 10. This was the only experiment in this series in which blood was obtained by decapitation; in all other cases it was obtained by orbital bleeding.

Experiment 30: This was a repetition of the previous experiment except that the plasma was taken from a 7-month old AKR male and 20 and 30 lamda samples were taken for testing. Two and 3 ng of B were added to the 20 and 30 lamda samples respectively. The results showed the best index of precision obtained so far and the per cent recovery was 80 and 90% for the 2 and 3 ng added (Table 10).

Experiment 31: Ten and 20 lamdas of pooled serum were obtained from 9 months' old C57Bl/6J male mice. It was planned to use this strain of mice (hereafter referred to as C57 mice) as control for the AKR mice and therefore it was felt that tests of recoverability should be conducted also in

this strain. Accordingly, to 10 and 20 lamdas test samples, 1 and 2 ng of B were added. The serum corticoids value obtained for the 10 lamda sample was zero, with 80% of the added B recovered. In the case of the 20 lamda sample, the plasma corticoid value gave a reading of 1.0 µg% and a 101% recovery (Table 10).

Experiment 32: Duplicate test samples of 20 lamda were employed in this experiment and in addition a 30 lamda sample was also taken. The serum used was a pooled sample from 9 months' old C57 mice as in the previous experiment, and 1, 2 and 3 ng of B were added to the 10, 20 and 30 lamdas of the test samples respectively. The values obtained for the serum corticoids were between 2.0 and 2.8 µg% and the recoveries were from 60 to 110%, the more cpd B the less recovered. The index of precision of the standard curve was good.

Experiments 33, 34 and 35 were essentially the same as Experiment 32, the only difference being that 12-month old males of the C57 strain were used in Experiments 33 and 34 and a 10-month old female of the same strain was used in Experiment 35 (Table 10). Examination of the results show a failure to detect B in the plasma of the samples in Experiments 33 and 34

and except in 2 instances, low recoveries of added corticosterone. In Experiment 35, corticoids could be detected in the plasma but the recoveries still tended to be too low in at least 2 instances (Table 10).

Thus, the monkey serum as the source of CBG generally yielded curves of adequate indices of precision but there was some problem remaining with the determination of the circulating corticoids (CCS)in C57 mice as well as the recovery of added material.

2.3.2.4. Dog Plasma as a Source of CBG

Because of the problems encountered with rhesus monkey serum, it was decided to investigate other sources of CBG and when Murphy suggested the use of dog plasma, this was taken up. This source proved satisfactory for the determination of corticosterone in ultramicro amounts in mice as was seen from the tests of precision, accuracy and specificity. The better results obtained with dog plasma as compared with serum fromhumans, monkeys or rats was due to the difference in CBG present in these various sources and not due to the fact that plasma was used in one case and sera in the others for dog

serum has also been utilized with equally good effect. However, it is preferable to use plasma or serum from males rather than females because the latter may be pregnant which would then raise the circulating levels of CBG and corticoids. Even with males, experience has shown that plasma from several different animals may prove unsatisfactory before a satisfactory sample is obtained and therefore each new batch of male dog plasma should be tested by running a standard curve and determining its index of precision. If too low, the test should be repeated with plasma from another dog.

2.3.2.4.1. Preparation of Standards and Solution A

Standards containing 0, 1, 2, 3, 4, 6 and 8 ng of B in absolute ethanol were set up in triplicate and the ethanol evaporated under a gentle stream of filtered air.

Tracer or solution A was prepared by adding 2.5 ml/dog's plasma or serum to about 15-20 ml distilled water. ^{3}H-B (specific activity 158 µc/µg) was dissolved in absolute ethanol to yield a solution of 10 µc/ml, 0.4 ml of which was added to the diluted dog's serum or plasma. Distilled water was added to yield a final volume of 100 ml.

To each of the dried standards, 1 ml solution A was added. An immediate equilibration between the unbound and bound corticoid resulted, and the amount of ^{3}H-B bound to the CBG was inversely proportional to the amount of unlabelled B originally present. To ensure complete solution of the dried material, it was incubated at 45°C for 5 minutes and then cooled to 8°C in a bath. To each tube 40 mg florisil (measured with a specially-designed plastic spoon) was added to remove any free B, both labelled and unlabelled. After standing for 10-15 minutes at 8°C, 0.5 ml of the supernatant, which contained the bound ^{3}H-B was pipetted into Bray's solution, and twice counted in a liquid scintillation spectrometer to 5,000 counts. The time (in minutes) to count 5,000 was plotted as ordinates against concentration of unlabelled B as abscissae yielding a standard curve off which the unknowns were read.

In the course of the various studies to be reported later, 29 assays for corticosterone in the ultramicro range were carried out utilizing dog plasma yielding an acceptable mean and standard error of 0.195 $\pm$ 0.022 for the index of precision. This information is included in Table 11 together with

Table 11. THE INDEX OF PRECISION OF AN ASSAY FOR THE ESTIMATION OF CORTICOSTERONE IN MICE UTILIZING DIFFERENT SERA AND PLASMA AS THE SOURCE OF CBG

Source of CBG	Index of Precision (Lamda = s/b)
Normal Human Serum	1.978 ± 2.055 (7)
Castrated Male Rat Serum	0.847 ± 0.910 (6)
Rhesus Monkey Serum	0.576 ± 0.138 (13)
Dog Plasma or Serum	0.195 ± 0.022 (29)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were determined.

similar data for the NHS, castrated male rat serum and rhesus monkey serum previously reported in sections 2.3.2.1. and 2.3.2.2. A representative standard curve utilizing dog plasma as the source of CBG in the preparation of solution A is given in Figure 1.

2.3.2.4.2. Tests of Specificity

Tests of specificity were conducted as follows: 1 ng of a variety of steroids was dried, each in a separate test tube, after which the procedure followed was as described in the third paragraph in section 2.3.2.4.1. The time (in minutes) to count 5,000 was obtained for each sample and means and standard errors were calculated for the replicates of each steroid investigated. In this way the competition between ^{3}H-B and each unlabelled steroids was investigated. The results are presented in Table 12.

The results showed that cortisol, 17a-hydroxy-11-deoxy-corticosterone and 17a-hydroxy-Prog were more effective in competing for the ^{3}H-B than was B. However, Triller and Birmingham (1965a, 1965b) and Ertel and Ungar (1968) have found no

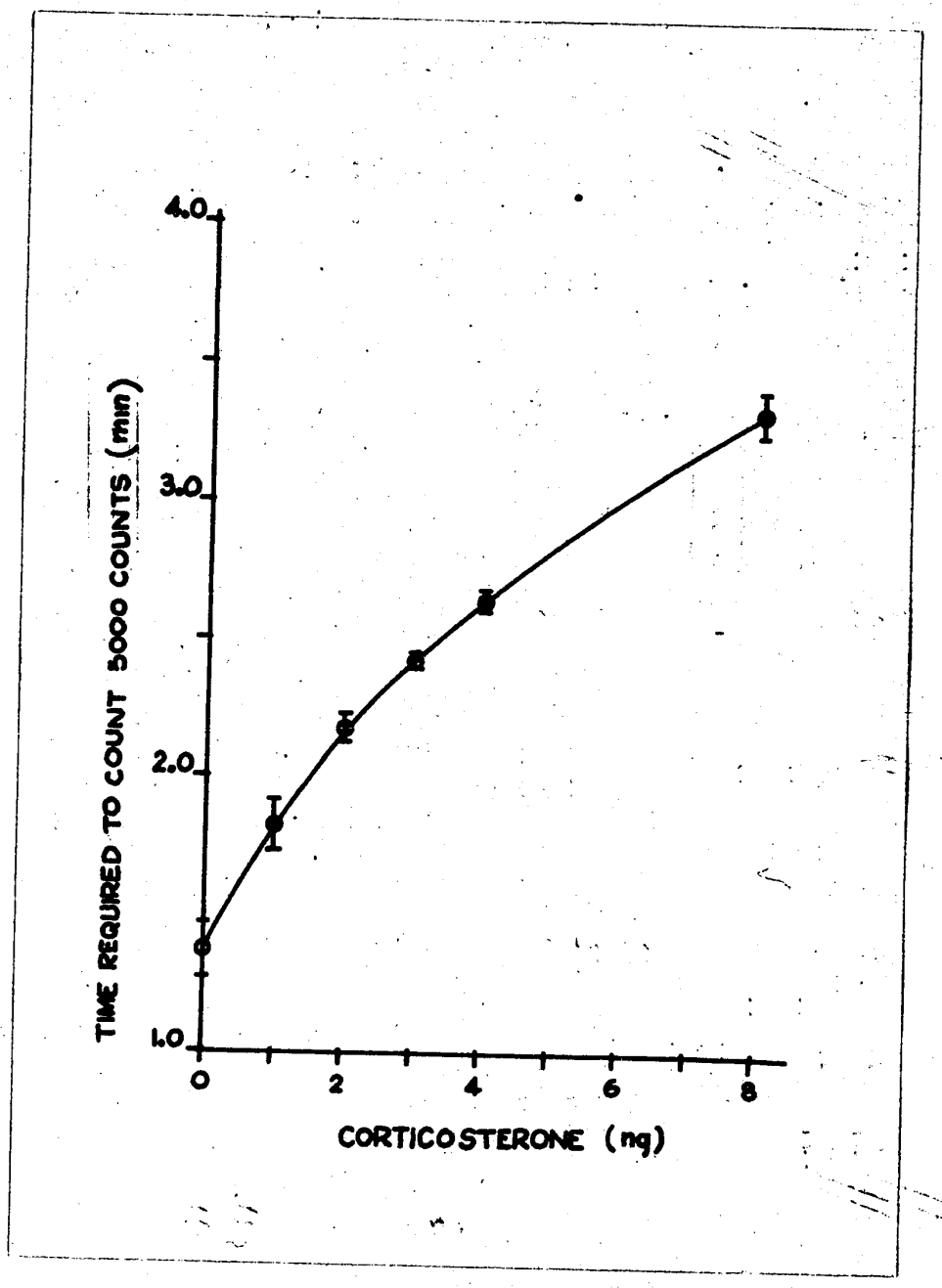

Figure 1. A representative standard curve for the determination of corticosterone in the 0 to 8 ng range utilizing the corticosteroid-binding globulin present in male dog plasma. The length of the lines drawn through each point on the curve equals two standard deviations. The index of precision of this curve is 0.205.

Table 12. THE COMPETITION OF VARIOUS STEROIDS FOR THE CBG IN DOG PLASMA AS DETERMINED BY THE TIME TAKEN (Min.) TO COUNT 5000 COUNTS OF TRITIATED CORTICOSTERONE BOUND TO CBG FOLLOWING EXPOSURE TO 1 NANOGRAM OF VARIOUS UNLABELLED STEROIDS

Compound	Experiment 1	Experiment 2	Experiments 1 and 2
None	1.38 ± 0 (3)	1.13 ± 0.02 (3)	1.26 ± 0.05 (6)
Corticosterone	1.95 ± 0.02 (7)	1.64 ± 0.03 (7)	1.79 ± 0.04 (14)
Cortisol	2.44 ± 0.02 (4)	1.92 ± 0.02 (4)	2.18 ± 0.09 (8)
17a-Hydroxy-11-Deoxy-Corticosterone	2.17 ± 0.12 (4)	1.70 ± 0.01 (4)	1.93 ± 0.09 (8)
17a-Hydroxy-Progesterone	2.12 ± 0.03 (4)	1.84 ± 0.03 (4)	1.98 ± 0.06 (8)
Cortisone	1.68 ± 0.03 (4)	-	1.68 ± 0.03 (4)
Progesterone	1.55 ± 0.01 (4)	-	1.55 ± 0.01 (4)
Estriol	1.53 ± 0.02 (4)	1.18 ± 0.02 (4)	1.36 ± 0.07 (8)
Estrone	1.42 ± 0.02 (4)	1.14 ± 0.01 (4)	1.28 ± 0.01 (8)
Estradiol-17β	1.37 ± 0.04 (4)	1.06 ± 0.02 (4)	1.20 ± 0.06 (8)
Testosterone	1.39 ± 0.02 (4)	-	1.39 ± 0.02 (4)
Aldosterone	1.37 ± 0.02 (4)	-	1.37 ± 0.02 (4)

Note: The number in the brackets gives the number of replications for which the adjacent mean and standard error were calculated.

evidence for the formation of 17-hydroxylated steroids by mouse adrenals, thus indicating an absence of biosynthetic pathways for F, E and their precursors in this species. Also, testosterone, estriol, estradiol and estrone all had negligible activities in this assay. Murphy (1967) conducted similar tests on the affinity of dog plasma for a variety of steroids including cpds B, F, S, E, Prog, Aldo, 17-hydroxy-4-pregnene-3,20-dione with essentially the same results. Thus, this method of determining CCS concentration in the mouse showed B, the main circulating corticosteroid in the mouse (Southcott et al., 1956).

2.3.2.4.3. Preparation of Unknowns

The unknowns were handled in the same way as described for the standards in the third paragraph of section 2.3.2.4.1. In order to separate the bound hormone (B), absolute ethanol was used to precipitate the binding protein (CBG) followed by drying of the samples with a gentle stream of air. To verify this, two experiments were set up: in the first, 10, 20 and 30 λ of sera from both AKR and C57 mice, 1 ml absolute ethanol was added to precipitate the corticoid-binding proteins, thus liberating the corticoids. The precipitate was retained with the

supernatant during evaporation and then the procedure was followed as described for the standards in section 2.3.2.4.1. The results showed that 20 λ sera yielded significantly higher CC values than those yielded by 10 λ of the same serum ($0.05 > P > 0.025$ by analysis of variance, Table 13). Thus, in 4 out of 6 animals, the 20 λ values were higher than the 10 λ ones. Similarly, when comparisons were made between 20 λ and 30 λ volumes, the latter gave higher readings than the former in 4 out of 4 animals, the overall difference being significant (Table 13: $0.025 > P > 0.01$).

In the second experiment 1 ml ethanol was added to 10 and 20 λ sera from AKR and C57 mice. The samples were then centrifuged and the supernatant removed for processing as before. The result showed no significant difference in the different volumes employed for the determination of B level in serum ($P > 0.70$, Table 14). Subsequently, the ethanol extraction procedure became the standard process for the test samples prior to evaporation.

2.3.2.4.4. The Recovery of Added Corticosterone (Test of Accuracy)

The accuracy of the method was assessed by determining the recovery of 1 ng of B

Table 13. THE DETERMINATION OF CORTICOSTERONE (µg%) IN 10, 20 AND 30 LAMDA SERUM OF AKR AND C57Bl/6J MICE UTILIZING THE ALCOHOL PRECIPITATION PROCEDURE

Strain	Sex	Age (mos.)	10 Lamda	20 Lamda	30 Lamda
AKR	M	10	2.2 ± 0.6 (4)	3.5 ± 0.3 (4)	
AKR	M	10	3.0 ± 1.7 (3)	8.1 ± 1.7 (3)	
AKR	M	7	6.5 ± 2.1 (2)	3.9 ± 0.3 (3)	
AKR	M	10		8.1 ± 1.7 (3)	9.5 ± 1.7 (3)
AKR	M	7		3.9 ± 0.3 (3)	6.7 ± 1.0 (3)
C57	M	7	1.1 ± 0.7 (2)	2.8 ± 0.9 (3)	
C57	M	9	1.6 ± 1.1 (3)	5.6 ± 0.8 (3)	
C57	F	10	2.8 ± 1.3 (3)	1.8 ± 1.3 (3)	
C57	M	9		5.6 ± 0.8 (3)	8.1 ± 1.9 (3)
C57	F	10		1.8 ± 1.3 (3)	4.4 ± 0.9 (3)

Note: The number in the brackets gives the number of replications for which the adjacent mean and standard error were calculated.

Table 14. THE DETERMINATION OF CORTICOSTERONE (μg%) IN 10 LAMDA AND 20 LAMDA SERUM OF AKR AND C57Bl/6J MICE UTILIZING THE ALCOHOL EXTRACTION PROCEDURE

Strain	10 Lamda	20 Lamda
AKR	4.3 ± 2.1 (7)	4.0 ± 1.5 (7)
C57Bl/6J	9.6 ± 3.6 (6)	9.6 ± 3.8 (6)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were calculated.

added to the plasma or serum of male and female C57 and AKR mice. The results varied from a low of 89 in the male C57 animals to 103% in male AKR mice (Table 15). There was no statistically significant difference in this respect either due to strain ($0.30 > P > 0.20$) or sex ($0.50 > P > 0.30$).

2.3.2.4.5. Test of Precision in the Determination of Test Samples

This test was carried out by determining the standard deviation of 77 duplicate B determinations (44 in C57 mice and 33 in AKR animals) over a reasonable range of concentrations (Snedecor, 1952; Brown, Bulbrook and Greenwood, 1957). The standard deviations varied from 0.6 µg at the lowest range to 2.8 µg at the highest (Table 16).

2.3.3. DISCUSSION

In the ultra-micro assay for the determination of cpd B in the mouse, human and rat sera were found to be unsuitable for use as a source of CBG. Inasmuch as B is the major CS in the rat and in the mouse, rat and mouse sera might have been more suitable than human serum as a source for suitable

Table 15. THE PERCENT RECOVERY OF 1 NANOGRAM OF CORTICOSTERONE ADDED TO THE SERA OR PLASMAS OF AKR AND C57Bl/6J MALE AND FEMALE MICE

Strain	Male	Female
AKR	103 ± 5 (18)	101 ± 6 (17)
C57Bl/6J	89 ± 7 (19)	100 ± 6 (15)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were calculated.

Table 16. STANDARD DEVIATION (S.D.) OF DUPLICATE DETERMINATIONS OF CIRCULATING CORTICOSTERONE FROM AKR AND C57B1/6J MICE

	Circulating Corticosterone (µg/100 ml)			
	0-2.5	2.5-5.0	5.1-10	>10
S.D. (µg)	0.6	0.7	1.4	2.8
No. of Pairs	31	15	13	18

CBG; yet, this was not so. Indeed, mouse serum was also less suitable as a source for CBG than human serum in the assay for B in the 0-40 ng range in the mouse. Monkey serum, on the other hand, seemed more satisfactory than either of the other two sera and yielded indices of precision which, once the method had been developed, were close to those reported for other less sensitive assays. However, there was still the problem of the determination of CC in the plasma or serum of C57 mice where the recovery of added B was poor.

Several attempts were made to obtain serum or plasma which according to published reports should contain elevated CBG. Thus, serum was obtained from a patient receiving estrogens for a carcinoma of the prostate and also from a woman in the late stages of pregnancy. Both of these individuals should have had elevated CBG's, although these were not determined in the present study. However, neither of those sera proved suitable for use in the assay for corticosterone. Moreover, the sera from the female rat of 3 species were no more suitable than that of the male rat for this purpose, even though the CBG in the former has been shown to be twice as high as that

of males (Gala and Westphal, 1965). Castration of the male rat which also leads to an increase in CBG (Gala and Westphal, 1965), also failed to improve the microassay. Finally, the selection of the serum from porcupine fish which has been reported to have a more than 20 times greater affinity for corticosterone-binding than cortisol-binding, also failed to yield a satisfactory assay.

The problem centred not on the discovering of a CBG with a relative high capacity but one with a high affinity for corticosterone. The problem turned out to be one of finding a CBG with a sufficiently high affinity for corticosterone which would permit an approximately 50% drop in the binding of B by CBG in the presence of the 4 ng standard as compared to that in the presence of the zero standard. Interestingly enough, dog plasma turned out to have a CBG with the required affinity even though the levels of CBG and circulating corticosteroids (CCS) are known to be low in this species. Murphy (1967) described other conditions affecting the binding of CSs by CBG.

The suitability of dog plasma or serum for the ultramicro assay was shown by the

acceptable values for tests of precision conducted on the standards (Table 11) and unknowns (Table 16), and tests of accuracy and specificity in Tables 15 and 12 respectively. A value of less than 0.300 for the index of precision for the assay for cpd B in mouse plasma or serum was especially satisfactory in view of its ultramicro range. This can be seen when compared with the assays so far reported in the literature.

Dorfman (1950) reviewed some 14 bioassays for adrenocortical hormones developed before 1950 and of these the most sensitive was that by Eggleston, Johnston and Dobriner (1946). However, this assay would not detect amounts less than 5 μg E and was obviously inadequate for our purposes as its more sensitive detection point was still 500 times too high for our requirements. In 1953, Grad, Sher and Symchowicz investigated an eosinopenic test developed by Spears and Meyer and showed that it was more sensitive than earlier tests, but still the smallest amount of B that could be assayed by this method was about 4 μg, again it was inadequate for our studies. In 1961, Braunsberg and James reviewed the methods developed between 1954 and 1960 along with

some earlier studies and examination of their tables revealed the assay requiring the least volume of blood still required 2 ml. A further problem posed by these methods was that they were oriented to the study of human blood in which the major CS is F, whereas the major CS in the mouse is corticosterone. Moreover, the method requiring 2 ml plasma is a complicated procedure involving extraction, purification and separation of the corticoid (Hollander and Vinecour, 1958).

Since that time, several other methods for determining the adrenocorticosteroids have been published. Thus, in 1962, Braunsberg and James described a simplified fluorimetric method for cortisol which required 8 ml plasma and therefore was unsuitable for our purpose. Again, Kornel in 1962 described a procedure based on that of Porter and Silber which required 10 ml of plasma. In 1964, Stachenko, Laplante and Giroud described a double isotope-derivative assay for B which, although reliable, could not detect B below the 100 μg level and was again a complex procedure involving purification of steroids through 4 paper chromatographies. In 1965, Spencer-Peet, Daly and Smith described another

fluorimetric method for determination of CSs in plasma and it requires 1-2 ml of plasma sample. Finally, an assay that appeared closest to the needs of our study was the one published by Guillemin et al., (1959). This one was developed specifically for the determination of B in the rat, but still requires 0.5 to 2 ml plasma — too much for our needs.

Therefore, all of these procedures are unsuitable for our purpose, except the ultramicro assay. Indeed, this assay is more sensitive than all known assays on adrenocortical function so far reported in the literature, and the only assay of any hormone including those other than the CSs which are more sensitive than the present ones are those used to detect certain protein hormones and involving the use of radioimmune procedures (Berson, Yalow, Glick and Roth, 1964). Thus, Berson et al., (1964) who reviewed these methods and reported that insulin and parathyroid hormone can be detected in as little as 0.50 ng/ml, while HGH can be detected in as little as 0.20 ng/ml.

This followed our studies on the plasma and urinary CSs in leukemic and healthy mice.

2.3.4. SUMMARY

In the attempt to develop an ultramicro assay for the determination of cpd B in young and old, male and female, high-leukemia AKR and low-leukemia C57, a series of experiments were conducted utilizing sera from humans (non-pregnant, pregnant and estrogenized), rats (male and female normals and castrated males), and rhesus monkey. The human and rat sera were found to be unacceptable but there were indications that the monkey serum might be useful. However in our hands some problems presented themselves while attempting B determinations in AKR and especially in C57 mice. On the other hand, dog plasma proved satisfactory for the ultramicro assay. This was shown by tests of precision, accuracy and specificity.

CHAPTER II

CIRCULATING CORTICOSTERONE IN RESTING AKR AND C57 MICE

1. INTRODUCTION

The studies described in the previous Chapter showed that dog plasma provided a CBG with the requisite affinity for B on which to base an ultra-micro assay which would meet accepted standards of precision, accuracy and specificity. Therefore, it was then possible to proceed with main aim of this thesis, viz., the estimation of adrenocortical function in the ALLk of AKR mice. To this end, the plasma and urinary corticoids were determined in resting and ACTH-stimulated mice, both of the high-leukemia AKR and low-leukemia C57 strains, the latter serving as controls. In this Chapter, details regarding the investigations on the resting levels of plasma B in the various groups of mice will be described.

2. MATERIALS AND METHODS

2.1. ANIMALS

Both AKR and C57Bl/6J mice were used in this study and were obtained from the Jackson Lab in Bar Harbor, Maine; some of these were also bred in our laboratory. In all cases, the dates of birth were known and recorded. All animals were housed in a room at 26-27°C with a humidity of 45-55% and lit up from 7:15 a.m. to 8:30 p.m. EST. Shades were placed on the windows leading to the outside and the corridors to prevent the entry of light into the room from the outside when lights inside the room were off. The animals were housed in metal cages with wire-mesh bottoms and fed Purina Fox Chow and water ad libitum.

The plasma or serum corticoid level was determined in male and female mice of both strains, from 2 to over 14 months in AKR mice and from 2 to 33 months in C57 mice. Both healthy and sick mice were investigated: in the C57 mice sickness did not involve lymphatic leukemia, while in the AKR strain most of the sick were suffering from lymphatic leukemia though some were ill from other causes.

All animals were observed carefully when alive for signs of disease. Mice were considered ill when they manifested disturbances in respiration, had a mucous or bloody discharge from the nares, enlargement of lymph nodes in the cervical, axillary or inguinal regions, had diarrhea or diuresis, a lusterless or dirty fur, with or without bald patches, were hunched, tended to remain motionless, held the head on one side (a sign of middle ear disease) or lost considerable weight. All animals, apparently sick and healthy, were also examined at autopsy for further evidence of disease.

At autopsy, 35 AKR mice with a very marked enlargement of thymus, spleen and lymph nodes were considered to have ALLk. Fifteen AKR mice appeared healthy while alive and yet at autopsy presented a moderate enlargement of the lymphatic organs which as will be demonstrated later were significantly larger than normal but smaller than those with ALLk. These mice were considered to be sick with "incipient lymphatic leukemia" (ILLk). Another 17 AKR mice were judged to be ill, either while alive and/or at autopsy, but did not show moderate or marked enlargement of the lymphatic tissue at autopsy. There were

146 AKR and 127 C57 mice judged healthy and 20 C57 judged ill, but not with leukemia. The thymus, spleen and adrenals were weighed in 55 healthy AKR, 16 with ALLk, 7 with ILLk and 10 AKR mice ill but not with leukemia (SNLk). Spleens and adrenals were also weighed in 20 male C57 mice, and thymic, splenic and adrenal weights were determined in 26 female C57 mice. All C57/mice/healthy.

Corticosterone was determined in blood collected from individual mice that were isolated in plastic cages 2 days before decapitation. The blood was collected between 10:30 a.m. and 11:00 a.m. in a group of about 20 animals, consisting of animals of either sex and strain, several ages, sick and well. Initial determinations were made mostly on serum, but later plasma was utilized because centrifugation produced a sharper separation of liquid from cells in the small volumes of blood samples collected from individual mice. Murphy and Pattee (1964) found that serum and plasma gave the same results with this assay. The term "circulating corticosterone" (CC) denotes corticosterone determined in either plasma or serum, disregarding what small amounts may be found in the red blood cells (Braunsberg and James, 1961).

2.2. CHEMICAL METHODS

The details of the method have been described earlier in the thesis (section 2.3.2.4.) and have also been published elsewhere (Grad and Khalid, 1968).

2.3. STATISTICAL ANALYSES

t tests and several types of analyses of variance were also carried out. In experiments involving several factors with disproportionate subclass numbers, two types of analyses of variance were conducted: one as described by Snedecor (1952) and the other by Ferguson (1959).

3. RESULTS

The CC levels in 127 healthy male and female C57 mice from 2 to 33 months of age are shown in Table 17. An analysis of variance showed that there was a significant difference due to sex, the females having the higher values (Tables 17 and 18, $P < 0.0005$). There was also a highly significant effect due to age (Tables 17 and 18, $P < 0.0005$). The CC level was highest at 2 months of age, significantly lower at 4 months ($P < 0.001$), and higher at 6 months, and still higher at 8 months (the difference

Table 17. THE CIRCULATING CORTICOSTERONE LEVELS (μg%) IN NORMAL C57Bl/6J MALE AND FEMALE MICE OF DIFFERENT AGE

Age (months)	Male	Female
2	10.7 ± 1.1 (8)	7.9 ± 1.4 (8)
4	1.2 ± 0.4 (10)	3.3 ± 1.0 (10)
6	3.1.± 0.6 (6)	7.4 ± 3.1 (6)
8	3.2 ± 0.8 (9)	11.1 ± 2.7 (8)
11	2.1 ± 1.4 (3)	6.7 ± 2.7 (7)
12-13	3.0 ± 0.5 (8)	6.3 ± 0.9 (10)
23-24	2.6 ± 1.2 (9)	7.2 ± 2.4 (6)
25-26	1.6 ± 0.6 (7)	2.6 ± 0.8 (9)
31-33	3.9 (1)	14.1 ±13.3 (2)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were determined.

Table 18. ANALYSIS OF VARIANCE OF DATA IN TABLE 17

Source of Variation	Preliminary Analysis Degrees of Freedom	Mean Square
Subclass means	17	84.34
Sex	1	321.42
Age	8	124.72
Individuals (Error)	100	21.40

Source of Variation	Completed Analysis Degrees of Freedom	Mean Square
Sex (A)	1	309.52[a]
Age (S)	8	123.23[a]
A x S	8	39.09
Individuals (Error)	100	21.40

[a]Significant at the 0.05% level.

between 4 and 8 months values was significant, $0.01 > P > 0.001$). Thereafter, it declined gradually till 25-26 months ($0.01 > P > 0.001$), the decline occurring mainly in females. At 31-33 months there were only 3 animals, 2 of which had relatively low values and one a high value. The age x sex interaction was of borderline significance ($0.10 > P > 0.05$).

CC levels were also determined in 20 sick C57 mice (5 males and 15 females). These animals were judged to be sick on the basis of observation while they were alive and also by the findings made at autopsy. Of the 20 sick C57 animals, 11 had enlarged spleens, but in these only one had a slightly enlarged thymus, 4 had a small one and in 6 it was not visible. Eight of the 20 mice had involvement of the liver and 7 of the kidney. Three had enlarged adrenals and 2 had hyperemic ones. Two others showed no pathology at the autopsy, but appeared ill while alive.

The CC levels of sick C57 mice and of healthy animals of corresponding age are shown in Table 19. The values of 2 sick animals are excluded from Table 19 because there were no values for healthy

Table 19. THE CIRCULATING CORTICOSTERONE LEVELS (μg%) OF HEALTHY AND SICK C57B1/6J MICE

Age (months)	Healthy		Sick	
	Male	Female	Male	Female
11	2.1 ± 1.4 (3)	6.7 ± 2.7 (7)	7.3 ± 6.3 (2)	9.7 ± 0.5 (6)
23-24	2.6 ± 1.2 (9)	7.2 ± 2.4 (6)	7.3 ± 1.5 (2)	5.0 ± 2.6 (3)
25-26	1.6 ± 0.6 (7)	2.6 ± 0.8 (9)	0.3 (1)	4.5 ± 1.5 (4)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were determined.

C57 mice of the same age. However, they are included later in Table 27. Of 6 possible comparisons in the CC values between healthy and sick in Table 19, the values were higher in the sick mice in 4 cases, lower in the 2 others. Inasmuch as females had significantly higher values than males in the normal C57 mice (Table 17), it was not possible to pool the male and female values and compare them by t test. Therefore, the values of healthy males were compared with sick males and similarly in the case of the females. Thus, the overall mean and standard error for the 19 healthy males in Table 19 were 2.1 ± 0.8 µg% while for the 5 sick males they were 3.9 ± 2.5 µg%, a non-significant difference ($0.60 > P > 0.50$). A comparison of the difference in the CC level due to sex will be made later in healthy and sick animals (Table 27) where more data are available than in Table 19.

The comparison of the CC level of healthy with that of the sick C57 females showed no significant difference, using an analysis of variance for an R x 2 table with disproportionate sub-class numbers (Tables 19 and 20, $0.50 > P > 0.30$). Nor were the differences due to disease at different ages statistically significant from one another (Tables 19 and 20, $0.50 > P > 0.30$).

Table 20. ANALYSIS OF VARIANCE OF DATA OF FEMALE C57Bl/6J MICE IN TABLE 19: Comparison of Healthy and Sick

Source of Variation	Degrees of Freedom	Mean Square
Age (A)	2	26.23[a]
Disease (D)	1	11.05[a]
A x D	2	25.76[a]
Error	29	21.86

[a]Not significant ($0.50 > P > 0.30$)

The statistical analysis conducted on the CC level of the 146 healthy AKR mice revealed that just as with the C57 animals, the females had the significantly higher values (Tables 21 and 22, $P < 0.0005$). Moreover, there were significant changes due to age (Tables 21 and 22, $0.05 > P > 0.025$). Thus, just as in the C57 mice, the values of AKR animals were high at 2 months of age and declined significantly by 4 months ($0.05 > P > 0.02$). Thereafter, the values increased and remained above the 4 month old level up until 13 months of age, the oldest age at which the CC values were studied in healthy AKR mice. During this interval the value at 8 and 12 months of age were significantly above those of the 4 months age level ($0.01 > P > 0.001$). There was no significant age x sex interaction (Tables 21 and 22, $0.20 > P > 0.10$).

In addition to the means and standard errors of healthy AKR mice from 2 to 13 months old, Table 20 contains also the data for the comparable ages of healthy C57 mice.

Because differences between males and females were statistically significant in both AKR and C57 mice, it was not possible to pool the male

Table 21. THE CIRCULATING CORTICOSTERONE LEVELS (μg%) IN HEALTHY AKR AND C57Bl/6J MICE OF BOTH SEXES AND OF DIFFERENT AGE

Age (months)	AKR Male	AKR Female
2	6.0 ± 0.7 (8)	5.9 ± 0.7 (8)
4	2.1 ± 0.7 (11)	2.3 ± 0.4 (11)
6	3.3 ± 0.8 (8)	4.3 + 0.7 (7)
7	-	2.0 ± 0.3 (3)
8	3.0 ± 0.5 (27)	9.9 ± 2.3 (17)
9	3.5 ± 1.2 (4)	3.9 ± 1.6 (5)
10	5.6 ± 2.2 (4)	7.2 ± 3.8 (2)
11	2.7 ± 4.5 (2)	6.4 ± 3.1 (2)
12	4.6 ± 1.0 (12)	8.6 ± 1.6 (13)
13	5.5 (1)	1.1 (1)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were determined.

C57Bl/6J	
Male	Female
10.7 ± 1.1 (8)	7.9 ± 1.4 (8)
1.2 ± 0.4 (10)	3.3 ± 1.0 (10)
3.1 ± 0.6 (6)	7.4 ± 3.1 (6)
3.2 ± 0.8 (8)	11.1 ± 2.7 (8)
2.1 ± 1.4 (3)	6.7 ± 2.7 (7)
3.0 ± 0.5 (8)	7.2 ± 0.8 (8)
-	3.1 ± 2.9 (2)

Table 22. ANALYSIS OF VARIANCE OF DATE OF AKR MICE IN TABLE 21: Comparison of Males versus Females

Source of Variation	Degree of Freedom	Mean Square
Age (A)	9	45.85[a]
Sex (S)	1	340.50[b]
A x S	9	35.07
Error	126	22.68

[a]Significant at the 2½% level

[b]Significant at the 0.05% level

and female data for each strain, and then test the difference between the strains in this regard. Nor was it possible to do a complete 3-way analysis of variance with disproportionate sub-class numbers because of the unavailability of a suitable statistical method to deal with this problem. Therefore, the CC values of male AKR mice were compared with those of male C57 animals, and similar comparisons were made for females of both strains.

The results in the males showed that there was a statistically significant effect due to age (Tables 21 and 23, $P < 0.0005$), the chief reason for which was the high values at 2 months of age with very low values at 4 months. There were no reliable differences due to strain when age was not considered (Tables 21 and 23, $P > 0.50$). However, there was a significant age x strain interaction (Tables 21 and 23, $0.01 > P > 0.005$). The reason for this was that the decline between 2 and 4 months was more marked in the C57 than in the AKR males.

In female AKR and C57 mice, there was also a very significant difference due to age when the strain effect was ignored (Tables 21 and 24, $0.005 > P > 0.001$). Again, the chief reason for this

Table 23. ANALYSIS OF VARIANCE OF DATA OF MALE MICE IN TABLE 21: Comparison of AKR versus C57Bl/6J

Source of Variation	Degree of Freedom	Mean Square
Age (A)	7	52.21[a]
Strain (S)	1	0.02
A x S	7	18.98[b]
Error	105	6.42
Total	120	9.77

[a]Significant at the 0.05% level

[b]Significant at the 2% level

Table 24. ANALYSIS OF VARIANCE OF DATA OF FEMALE MICE IN TABLE 21: Comparison of AKR versus C57Bl/6J

Source of Variation	Degree of Freedom	Mean Square
Age (A)	8	112.01[a]
Strain (S)	1	4.42
A x S	8	15.12
Error	100	31.12

[a]Significant at the 0.5% level

was the marked decline in the CC values between 2 and 4 months of age just as occurred in the males of both strains. However, in the females, there was in addition a peak at 8 months in both strains which in fact exceeded the values at 2 months (Table 21).

There was no significant difference between the female C57 and female AKR, nor was there a significant age x strain interaction (Tables 21 and 24, $P > 0.50$).

Thirty-five AKR mice (14 males and 21 females) were considered to be sick with ALLk because they had tumours of the lymphatic tissue. An analysis of variance on their CC data showed that there was no significant effect due to age, sex or the age x sex interaction (Tables 25 and 26, $0.50 > P > 0.30$).

The data of the 15 AKR mice with ILLk and the 17 AKR mice with SNLk were also presented in Table 25 to make possible comparisons between animals with varying degrees or types of illness as well as comparisons between the sexes and the various age groups. Inspection of the table showed that the CC values of the female mice with ILLk or SNLk did not appear to be higher than those of the males, as was true in the healthy C57 and AKR animals.

Table 25. THE CIRCULATING CORTICOSTERONE LEVELS (µg%) IN SICK AKR MICE OF BOTH SEXES AND OF DIFFERENT AGES

Age (months)	Incipient Lymphatic Leukemia	
	Male	Female
6	3.4 ± 0.7 (2)	2.5 (1)
7	-	-
8	4.4 ± 2.1 (4)	1.8 (1)
9	2.1 ± 0.8 (3)	1.1 (1)
10	1.0 (1)	
11	-	2.3 (1)
12	2.7 (1)	
14		

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were determined.

Acute Lymphatic Leukemia		Non-Leukemic Illness	
Male	Female	Male	Female
3.9 (1)	26.0 (1)		
-	8.3 (1)		
10.1 ± 2.2 (3)	17.7 ± 5.5 (8)	2.5 ± 1.3 (3)	1.0 ± 0.0 (2)
11.1 ± 0.4 (2)	-	2.0 ± 0.9 (4)	
9.1 (1)	21.8 ±10.6 (2)	1.0 ± 0.3 (3)	0.6 (1)
13.8 ±10.3 (2)	9.9 ± 2.0 (2)		15.9 (1)
12.8 ± 5.4 (3)	5.6 ± 2.8 (2)	4.6 (2)	
13.2 ± 3.7 (2)	-	8.4 (1)	

Table 26. ANALYSIS OF VARIANCE OF DATE OF ACUTE LEUKEMIC AKR MICE IN TABLE 25

Source of Variation	Degree of Freedom	Mean Square
Age (A)	7	37.10[a]
Sex (S)	1	62.00[a]
A x S	7	72.74[a]
Error	19	124.31

[a]Not significant ($0.50 > P > 0.30$)

Inasmuch as there were too few data in each age group with ILLk or SNLk with which to conduct an analysis of variance, the data of these mice were grouped together ignoring differences due to age and presented in Table 27. The data of healthy and sick C57 mice and healthy AKR mice as well as those with ALLk were included to make comparisons between the various groups easy. $\underline{t}$ tests were utilized to test the significance of differences in this table.

Healthy female mice, whether C57 or AKR, had significantly higher values than males (Table 27, $0.01 > P > 0.001$). This was also shown in earlier analyses where the age effect and the age x sex interaction had been eliminated, and for this reason the probability of the difference there was less than that described here. There was no corresponding difference due to sex either in animals with ILLk (Table 27, $0.20 > P > 0.10$), or with ALLk ($0.40 > P > 0.10$) or with SNLk ($0.70 > P > 0.60$).

Table 27 also showed that AKR mice with ALLk had significantly higher CC values than those of all the other groups. This was true for males alone, for females alone and for both males and

Table 27. COMPARISON OF CIRCULATING CORTICOSTERONE (µg%) IN C57Bl/6J AND AKR MICE, HEALTHY AND SICK

	C57Bl/6J	
	Healthy	Sick
Total	5.1 ± 0.5 (127)	4.1 ± 0.6 (20)
Male	3.4 ± 0.5 (61)	3.9 ± 2.5 (5)
Female	6.6 ± 0.8 (66)	4.2 ± 0.4 (15)

Note: The number in the brackets gives the number of animals for which the adjacent mean and standard error were determined.

	A K R Leukemia		
Healthy	Incipient	Acute	Non-Leukemic Sick
4.9 ± 0.4 (146)	2.4 ± 0.4 (15)	13.1 ± 1.7 (35)	3.2 ± 0.9 (17)
3.6 ± 0.5 (77)	2.5 ± 0.3 (11)	11.3 ± 1.7 (14)	2.8 ± 0.7 (13)
6.3 ± 0.6 (69)	1.9 ± 0.3 (4)	14.3 ± 2.6 (21)	4.6 ± 3.3 (4)

females combined (Table 27, $P < 0.05$). On the other hand, there were no significant differences in this respect between healthy AKR mice and healthy C57 animals, males and females, separately or together (Table 27, $0.80 > P > 0.70$). Furthermore, animals with ILLk had significantly lower values not only than AKR mice with ALLk (Table 27, $P < 0.001$), but also reliably less than those of healthy AKR mice (Table 27, $P < 0.001$). Examination of these differences in males and females separately showed that in the females it was very highly significant (Table 27, $P < 0.001$), whereas in the males it was of borderline significance (Table 27, $0.10 > P > 0.05$).

On the other hand, AKR mice with SNLk did not have any reliably different CC values than did healthy AKR mice, whether comparisons were made for males and females separately or together (Table 27, $P > 0.10$).

Of the 17 mice in the SNLk category, 7 had hyperemic adrenals, 2 had enlarged adrenals, 4 had involvement of the liver, 1 of the kidney, 1 of the gastrointestinal tract, 1 of the bladder and 4 had slight enlargement of isolated lymph nodes, without involvement of thymus and spleen. Several animals had several of these conditions.

Table 28 lists the weights of the thymus, spleen and adrenal as well as the body weight and the CC of only those AKR mice of which the above organs were weighed.

The weights of the thymus and spleen were significantly higher in the AKR mice with ALLk than in those of all the other groups (Table 28, $P < 0.01$). The second highest weights of thymus were found in the ILLk group; these were significantly higher than those of the healthy AKR as well as those of the SNLk (Table 28, $0.01 > P > 0.001$). The spleens of the ILLk mice also weighed more than those of the healthy AKR and SNLk animals but only in the healthy did this difference approach statistical significance (Table 28, $0.10 > P > 0.05$).

The adrenal weights of the animals with ILLk had significantly higher values than those of the healthy AKR animals (Table 28, $0.01 > P > 0.001$), but there were no significant differences between the adrenal weights of the ILLk, ALLk or SNLk groups (Table 28, $P > 0.40$). Neither the organ weights, nor the body weights, nor the CC levels were significantly different in the SNLk group from those of the healthy group (Table 28, $P > 0.10$).

Table 28: THYMUS, SPLEEN AND ADRENAL WEIGHTS AND CIRCULATING CORTICOSTERONE OF HEALTHY, INCIPIENT LEUKEMIC, ACUTE LEUKEMIC AND NON-LEUKEMIC SICK AKR MICE

	Healthy N = 55	Incipient Leukemia N = 7	Acute Leukemia N = 16	Sick: Non-Leukemic N = 10
Body Weight (gm)	29[1] ± 1[2]	31 ± 1	27 ± 1	29 ± 1
Thymus Weight (mgm)	38 ± 4	106 ± 19	443 ± 79	29 ± 8
Spleen Weight (mgm)	71 ± 3	146 ± 42	574 ± 75	87 ± 14
Adrenal Weight (mgm)	4.1 ± 0.2	5.1 ± 0.3	4.6 ± 0.6	5.2 ± 0.7
Circulating Corticosterone (µg%)	3.3 ± 0.4	1.8 ± 0.4	12.9 ± 2.6	4.0 ± 1.5

[1]mean

[2]standard error

Finally, the CC level was significantly higher in the group with ALLk than that of all the other groups (Table 28, $P < 0.01$). Moreover, the ILLk group had significantly lower values than those of the healthy AKR mice (Table 28, $0.01 > P > 0.001$), but not significantly lower than those of the SNLk mice (Table 28, $0.20 > P > 0.10$). Although the mean CC value in the SNLk group was higher than that of the healthy, the lack of significance between the ILLk and SNLk was due to the fact that there was a higher standard error in the SNLk which raised the value in the denominator in the calculation of $\underline{t}$ in the $\underline{t}$ test.

The highest mean body weight was found in the animals with ILLk but the differences were significant only when compared with the mean body weight of the ALLk animals (Table 28, $0.01 > P > 0.001$). There were no significant differences in this respect between the ILLk, SNLk or healthy AKR mice (Table 28, $0.20 > P > 0.10$).

Table 29 lists the morphological data of male and female C57 mice as well as their CC levels. There was no significant difference in body weight ($0.70 > P > 0.60$) but the weight of the spleen

Table 29. BODY, THYMUS, SPLEEN AND ADRENAL WEIGHTS AND CIRCULATING CORTICOSTERONE VALUES OF HEALTHY MALE AND FEMALE C57Bl/6J MICE

	Male	Female
Body Weight (gm)	27[1] ± 1[2]	25 ± 4
Thymus Weight (mgm)	-	15.5 ± 5
Spleen Weight (mgm)	70 ± 7	100 ± 7
Adrenal Weight (mgm)	4.3 ± 0.4	5.6 ± 0.3
Circulating Corticosterone (µg%)	2.9 ± 0.4	4.9 ± 0.8

[1]mean

[2]standard error

The body weight and circulating corticosterone values were obtained from 29 males and 35 females, while the remaining data were obtained from 20 males and 26 females.

and the adrenal as well as CC levels were all reliably higher in females ($P < 0.05$).

A comparison of the morphological data of Table 29 with those of healthy AKR mice in Table 28 revealed the following: although the C57 mice weighed less than the AKR animals, the differences were not statistically different nor was there any reliable difference in the adrenal weight between the two groups ($P > 0.10$). On the other hand, the weight of the thymus of C57 females was significantly below that of healthy AKR animals ($P < 0.001$) while the reverse was true in regard to their splenic weight ($P < 0.001$). The splenic weights of healthy C57 males were almost identical with those of the healthy AKR mice.

Although a number of reports have appeared in the literature on the determination of the CC level in mice, meaningful comparisons with the data reported here are not possible because most of the papers did not indicate the time of day the blood sample was collected or the age and sex of the animals, variables known to influence the CC level. Moreover, all reports involved the determination of the CC level in pooled samples of mouse blood, some of which was

derived from both sexes, whereas the present report provides for the first time data obtained from individual mice. CC levels determined by the fluorometric technique generally gave values well above those reported in this paper (Halberg, Albrecht and Bittner, 1959; Eleftheriou, 1964; Oyama and Platt, 1964) due to a variable quantity of contaminating non-specific substance known to be included with this estimation, and not determined by the radioassay described in this paper (Murphy, 1967). However, two reports on the determination of the CC level in mice make some comparison possible.

The first reported values between 8 and 9 µg% for 10-15-week-old AKR males and slightly but not significantly higher values for C57Bl/10 mice of the same age and sex (Levine and Treiman, 1964). However, as the time of day the blood was collected was not reported, and as the diurnal variation in the CC level had been known to involve about a 100% change in the values within the same day, a more precise comparison with our data is not possible.

The second paper reported values of 6.3 ± 0.6 and 12.1 ± 1.1 µg% for male and female WLO

mice (Solem, 1966). The blood was collected at noon when it would be expected that the CC level would be somewhat higher than that of samples collected 1-1½ hours earlier as in the present study (Halberg et al., 1959; Solem, 1966). Indeed, the CC levels of 2- and 4-month-old C57 mice were lower (Table 4) than those reported for 3-month-old WLO mice (Solem, 1966). Moreover, as there was a marked change in the CC level between 2- and 4-month-old C57 mice (Table 4), a change which may also occur in WLO animals, the differences in the values may be due to differences in the age of the mice in the two studies. Strain differences may also be a factor, but probably most important is the fact that the data of both the above reports were determined fluorimetrically, with its higher corticoid values.

The concentration of a hormone in the circulation is the resultant of its production by the adrenal cortex, of the rate of its release from the gland, the space in which it is distributed, its binding by CBG, its metabolism into other cpds and its rate of excretion. Thus, the same CC level in two or more groups might be due to different causes which balance each other, while similar differences might

also be due to different mechanisms. Thus, further work is indicated before definitive statements can be made about the change in adrenal cortical function during aging in C57Bl/6J mice. However, an examination of the pertinent reports in the literature may provide some indication of the significance of the findings reported here.

4. DISCUSSION

4.1. DATA OF C57 MICE

The CC concentration in C57 mice showed a peak at 2 months, a low point at 4 months, with an increase thereafter until 8 months, and then a decline again until 25 or 26 months. Of the three values (one male and two female) obtained from animals 31-33 months of age, two were low and one was very high. The latter can be considered exceptional and does not invalidate the over-all significance of a decline in the CC level due to aging.

The borderline significanbe of the age x sex interaction was due to a sharper decline in the CC concentration between 2 and 4 months in males than in females and in the more marked decline

with increasing age in females after 8 months of age. Thus, the correlation coefficients (r) between CC value and age for male and female mice between 8 and 31-33 months old gave a non-significant value for 37 males ($r = -0.1422$, $P > 0.10$), and a significant one for the 42 females ($r = +0.3247$, $0.05 > P > 0.02$). When the data of both males and females were combined, an $r = -0.2558$ was obtained, significant at the 5% level.

Relatively few studies have been carried out on the age changes in adrenocortical activity in mice. One group investigated the <u>in vitro</u> CS production by BALB/c female mice and found no reliable change from 3 weeks to 3 months of age, but a decline thereafter to $2\frac{1}{2}$ years of age (Nandi, Bern, Biglieri and Pieprzyk, 1967). The same authors reported a decline in <u>in vitro</u> CS production in male mice between 3 weeks and 3 months and in the CC level between 4-6 weeks and 14-16 weeks. ACTH injected into male mice produced a similar response in plasma CS in 3- and 12-week-old animals, but a greater response in the correspondingly older female (Solem, 1966). Halberg et al., (1959) reported higher serum B levels in 4-week- than in 15-month-old animals

from 6:00 a.m. to 6:00 p.m. with a tendency to a reversal of values the following 12 hours. Young persons with a mean age of 24-35 years did not have a significantly different plasma corticoid level than did old persons with a mean age of 71 years (Grad, Kral, Payne and Berenson, 1967). If 3 months in the life of the mouse are considered equivalent to 1 year in the human, then the failure to observe age differences in the human is in contrast to that observed in 8-month- and 25-26-month-old mice. The reason for the decline in the CC level between 2 and 4 months of age is not known at the present time, but there are similar declines in the basal metabolic rate and heart rate in rats during this period (Grad, 1953).

Structural changes in the adrenal glands with advancing age have been reported by Bourne and Jayne (1961) and Korenchevsky (1961). The amount of connective tissue present in the adrenal cortex increases with age and the ZR becomes wider and more vascularized during senescence (Cooper, 1925; Dribben and Wolf, 1947). Structural changes in the cortical and medullary cells may also occur with age (Jayne, 1953; Meyers and Charipper, 1956). However,

the most prominent structural change in the adrenal gland is an accumulation of lipid pigment associated with some degree of cellular degeneration in the inner zone of the cortex (Bourne and Jayne, 1961). Samorajski and Ordy (1967) showed that there was an age-related increase in the number of pigment-containing cells in the adrenal glands of C57 mice between 4 and 30 months of age. However, they demonstrated that there were no histochemical or ultrastructural features of the adrenal lipid pigment that could be associated with senescence.

The data of this study also showed that reliably higher values were observed in females than in males at all age levels with the exception of 2 months of age when the males had the higher values. Solem (1966) showed that female WLO mice had higher values than male mice at rest as well as following injections with ACTH or formalin. Twelve-weeks-old male mice housed in groups of 20 were more responsive to ACTH than males of the same age housed singly; however, there was no difference in the responsiveness of 2 similarly housed groups of 12-weeks-old females. There was no evidence that fighting in males accounted for this difference. The

PC level was very high in female mice during pregnancy near to parturition and fell rapidly following the first day after parturition (Solem, 1966).

Gonadectomy resulted in an increased response to ACTH in males and a decrease in females when compared with intact controls (Solem, 1966). Replacement doses of testosterone injected in castrated males and of estradiol in spayed females restored the normal responsiveness to ACTH. Large doses of either hormone caused a decreased ACTH responsiveness in both intact males and females. Small doses of testosterone had no effect on the ACTH responsiveness in intact males, but decreased the response in normal females. On the other hand, the administration of small doses of estrogens had no effect on the response of PC to ACTH in intact females, but had a stimulating effect in normal males (Solem, 1966). This parallels the findings of Kitay in rats (1961, 1963a, 1963b, 1963c).

In this regard, the amount of B in the adrenal determined at the time of peak physiologic secretion was significantly higher in female C Bagg albino mice and in hybrids than in males (Halberg and Haus, 1960). The same authors found higher adrenal

B content in female than in male C57Bl subline 1 mice. Ovariectomy has been shown to lower adrenal B in C mice (Halberg, Bittner, Cole, Haus and Kaiser, 1961). The female has the heavier adrenal glands - a well-known fact first reported by Deansley (1938), and also by us (Table 29) - but the sex difference in adrenal B is not due to this because the higher adrenal B in females was also apparent when the results were expressed per milligram of adrenal tissue.

In this connection, the mature female characteristically has a lower eosinophil count and a higher adrenal ascorbic acid concentration than do males in C57Bl subline 1 mice (Halberg, Hamerston and Bittner, 1957). There is evidence that the lower eosinophil count in females was brought about by a higher level of circulating C-11 oxygenated corticoids (Halberg, 1952) which cause the number of these cells in the circulation to decline (Haus, 1959).

Small doses of estrogens administered to hypoxed, unilaterally adrexed mice caused a marked inhibition in the size and metabolism of the remaining adrenal (McKerns and Bell, 1960). The latter authors and Berliner and Dougherty (1958)

also demonstrated a decreased in vitro biosynthesis of CSs by adrenals from estrogen-treated mice. On the other hand, the work of Kitay in rats (1961, 1963a, 1963b, 1963c) and of Solem in mice (1966) demonstrated a stimulatory effect for estrogens in animals with intact pituitaries. Therefore, the stimulatory effect of the estrogens on the adrenal cortex is indirect and indeed Tepperman, Engel and Long (1943) suggested that it acted by stimulating ACTH output by the pituitary. The action of estrogen on the pituitary may be direct as suggested by Kitay's work in the rat, and/or direct as suggested by the work of Glenister and Yates (1961) who found that the liver - the main site of CS inactivation - has a greater capacity for B inactivation in female rats than in males. They also found that the biological half-life of cpd B is significantly less in females than in males which by negative feedback would result in a greater ACTH output by the pituitary of the female.

Moreover, the estrogens are known to increase the CBG level in the blood (Peterson et al., 1960; Seal and Doe, 1965; Daughaday, 1967) and this would have the effect of reducing the relative amounts of unbound B, whose circulating level controls ACTH

output, while that of bound B has no such effect. Glenister and Yates (1961) earlier reported that the adrenal cortex is much more active *in vitro* in females than in male rats. Further studies are required in this regard.

4.2. DATA OF AKR MICE

The only report in the literature on the CC level of AKR mice has already been mentioned in this discussion (Levine and Treiman, 1964) and while the reported values of 8-9 µg% for 10-15-week-old mice are comparable to those obtained in the present study, an appropriate comparison cannot be made for reasons already given.

There were no significant differences between the CC level of healthy AKR and C57 mice and, in the former as in the latter, healthy females had the higher CC levels than males. Moreover, AKR animals with ALLk had very significantly higher CC values than those of all other groups. That the animals judged to be ill with ALLk were in fact ill with this disease was proved by the presence at autopsy of very markedly enlarged thymuses, spleen and lymph nodes. Similarly, animals considered to have

incipient leukemia were placed in this category because their lymphatic organs were enlarged relative to those of the controls but were still relatively smaller than those with ALLk. The ILLk did not show an increase in the CC level and in fact had lower than normal levels. It should be emphasized that these mice gave every appearence of being normal while they were still alive.

In both C57 and AKR mice, there was a marked drop in the CC level between 2 and 4 months of age. In AKR mice, 4 months of age can be considered a preleukemic period inasmuch as the first signs of leukemic illness in representative groups of AKR mice generally appeared between 150 and 200 days. That the adrenal gland may play an important role in the pathogenesis of ALLk has already been discussed earlier, but the discussion as to whether the CC levels decline in 4-month-old AKR is related to the development of Lk in AKR mice will be discussed in the next Chapter. The same applies to a discussion of the significance of the depressed CC values in AKR mice with ILLk.

The high CC level in mice with ALLk may be a non-specific response to the stress of

the disease. However, the CSs are known to depress lymphoid production, and indeed, synthetic derivatives of the natural corticoids are used in therapy of this disease. Therefore, the increased CC level may help combat the disease and help to prolong the survival of the sick mice. However, our studies showed that when the abnormal growth of the lymphatic organs had just begun, as in the AKR mice with ILLk, the CC level was not immediately increased and was, as mentioned earlier, even less than in the normal. Indeed, the below normal CC level may have paved the way for the abnormal proliferation of the lymphatic tissue. This will be discussed more fully later.

Despite the fact that animals with ALLk showed increased CC level, the SNLk animals which presumably were also stressed by their disease, did not show any significant change in CC level relative to the controls. This was true not only in the AKR mice but also in the C57 animals.

Eleven of 20 C57 sick mice had enlarged spleen; of these 1 had a moderately enlarged thymus as well. Possibly, this animal had ILLk but in view of the normally low incidence (less than 1%)

of lymphatic neoplasms in this strain and the higher incidence (15-20%) of reticulum cell tumours type A (Murphy, 1966), this animal may have had an incipient tumour of this type. The remaining 10 mice with enlarged spleens may also have had reticulum cell neoplasms but further study is required to establish this definitely. Certainly, they did not suffer from ALLk inasmuch as their thymuses were either small or non-existent, and involvement of this organ is primary in ALLk (Furth, 1946; Grad, Berenson and Kaplan, 1958). Moreover, the CC level in the C57 sick mice, with enlarged spleens, was 5.7 ± 1.2 µg%, significantly below those of ALLk ($P < 0.001$) but significantly above the CC level of C57 sick mice without enlargement of the spleen, the mean and standard error here being 1.8 ± 0.5 µg% ($0.01 > P > 0.001$). Further study is indicated here.

The higher CC values found in healthy females than males in both the AKR and C57 strain was not apparent in sick AKR mice, whether sick with Lk or other disease, and not apparent also in sick C57 rodents. On the other hand, the mean CC level of 6-7-week-old AKR mice bearing the transplanted BW5147 for 1 week was 5.8 µg% (the standard error was 0.9 µg%)

of lymphatic neoplasms in this strain and the higher incidence (15-20%) of reticulum cell tumours type A (Murphy, 1966), this animal may have had an incipient tumour of this type. The remaining 10 mice with enlarged spleens may also have had reticulum cell neoplasms but further study is required to establish this definitely. Certainly, they did not suffer from ALLk inasmuch as their thymuses were either small or non-existent, and involvement of this organ is primary in ALLk (Furth, 1946; Grad, Berenson and Kaplan, 1958). Moreover, the CC level in the C57 sick mice, with enlarged spleens, was 5.7 ± 1.2 µg%, significantly below those of ALLk ($P < 0.001$) but significantly above the CC level of C57 sick mice without enlargement of the spleen, the mean and standard error here being 1.8 ± 0.5 µg% ($0.01 > P > 0.001$). Further study is indicated here.

The higher CC values found in healthy females than males in both the AKR and C57 strain was not apparent in sick AKR mice, whether sick with Lk or other disease, and not apparent also in sick C57 rodents. On the other hand, the mean CC level of 6-7-week-old AKR mice bearing the transplanted BW5147 for 1 week was 5.8 µg% (the standard error was 0.9 µg%)

in 16 males while the corresponding value in 14 females was 15.9 ± 3.4 µg%, a significant difference ($0.01 > P > 0.001$). Thus, with the transplanted tumour as in healthy mice but in the spontaneous disease, the females had the higher values.

5. SUMMARY

The CC level was determined in 213 mice of high leukemia AKR strain, and 147 in low leukemia C57Bl/6J mice. One hundred and twenty-seven C57 mice were healthy and 20 were ill, but not with ALLk; 146 AKR mice were healthy, 15 had incipient leukemia; 35 had ALLk and 17 were ill but not with ALLk or ILLk. Illk mice which appeared healthy while alive had a moderate but significant enlargement of the lymphatic organs. However, mice with ALLk were obviously ill while alive and showed a very marked enlargement of these tissues at autopsy.

The mean CC of healthy AKR mice was not significantly different from that of healthy C57 animals, but healthy females had a significantly higher mean CC than healthy males. However, no significant differences in CC between the sexes were observed

in mice sick from any cause. Four-month-old mice of both strains had significantly lower CC than 2-month-old animals and lower also than those 6 months or older. After 4 months of age, healthy females showed a greater increase in CC than males. The CC of mice with ALLk was very significantly higher than that of all other groups, but the ILLk mice had lowest values relative to all other groups.

CHAPTER III

PLASMA AND URINARY CORTICOSTEROIDS IN ACTH-STIMULATED AKR AND C57 MICE

1. INTRODUCTION

Having investigated the resting CC levels in healthy and sick AKR and C57 mice, the next step was to extend the findings in the direction of determining CC levels in mice whose adrenals were stimulated by ACTH. Moreover, the urinary corticosteroids (UCS) were also determined so as to better assess the level of adrenocortical function in leukemic and other groups of mice.

2. MATERIALS AND METHODS

2.1. ANIMALS

Both AKR and C57 mice were utilized. These were procured, housed and fed as described in the previous Chapter. The plasma CC and UCS were determined in 12 healthy AKR mice (7 males and 5 females), 19 AKR mice sick with ALLk (6 males and 13 females), 3 AKR mice sick with ILLk (1 male and 2 females), 3 AKR mice sick from causes other than Lk (2 males and 1 female) and 11 healthy C57 animals

(6 males and 5 females). The mice were 7 to 12 months of age and their condition was determined while alive and after death as described in the previous Chapter.

The mice were investigated as follows: first, the mice were isolated for 48 hours in plastic cages following which each animal was housed separately in a metabolism cage and its urine collected for 24 hours. Urine present in the bladder at the start of the urine collection was discarded by applying pressure in the region of the bladder. Twenty-four hours later similar pressure was applied so that any urine remaining in the bladder would be ejected and collected. Then, 0.04 I.U. ACTH (Duracton from Nordic Biochemicals) was injected subcutaneously, and a 24-hour urine sample was collected for the next 4 consecutive days. The metabolism cages were carefully washed down with distilled water to ensure that dried urine was washed into the collection bottles. Following the urine collection, the animals were allowed to rest for 1 week in regular cages after which a blood sample was obtained by orbital bleeding between 10:00 a.m. and 10:30 a.m. ACTH was then again injected as previously described, and a second and third sample were obtained by orbital

bleeding 4 and 24 hours later. The animals were then sacrificed by decapitation and an autopsy performed. Thymus, spleen and adrenals were removed for weighing; body weights were determined at appropriate intervals.

2.2. CHEMICAL METHODS

The method for determining plasma CC was identical with that described in the previous Chapter. However, the urine was handled as follows: 150 to 250 λ were placed in each of two test tubes, extracted twice with 1 to 2 ml CH_2Cl_2, and the organic phase transferred to other test tubes and evaporated. The procedure for determining the UCS from this point on was as described for the CC.

2.3. STATISTICAL ANALYSES

Analyses of variance, *t* tests, chi-square tests and correlation analyses were the types of statistical analyses to which the data reported in this Chapter were submitted. A probability of less than 0.05 was considered significant. In those instances when a mouse died after providing

one or more values after ACTH administration, but still not a full set of 3 PC and 4 UCS values, missing data were obtained by a method described by Snedecor (1966). Analyses of variance and t tests were then conducted on the data including the estimates, but one degree of freedom was removed for each estimated value. These estimates were also included in reporting the data in Tables 30 and 31, as they gave a more correct picture of the relative change of the CC and UCS on consecutive days following ACTH administration than would have been the case if such values had been omitted, especially if earlier data obtained from the same animal for which estimates were obtained had values which deviated considerably from the mean.

3. RESULTS

3.1. PLASMA CORTICOSTERONE DATA (Tables 30 and 32)

Four hours after ACTH administration, 37 of the 39 mice showed an increase in the PC level, 1 decrease occurring in healthy AKR male and another in an SNLk male. Twenty-four hours after ACTH administration, the values had declined from

the 4 hour values (26 of the 39 mice showing such a decline, 13 a further increase).

The distribution of increases and decreases between the various groups were not significantly different, according to chi-square test ($0.70 > P > 0.50$). The 24-hour values were still generally above the pre-ACTH levels.

Prior to ACTH administration, mice with ALLk had significantly higher values than apparently normal AKR mice of both AKR and C57 mice ($0.01 > P > 0.001$). ALLk mice also had the higher values after ACTH administration but the corresponding differences were less marked. Inasmuch as there were only 3 mice with ILLk and 3 that were ill but not with Lk (SNLk mice), it was not possible to conduct statistical analyses between these and the other groups. However, both the ILLk and SNLk mice yielded values which were well below those of ALLk mice, and SNLk mice presented values which on the whole were higher than those of healthy AKR and C57 mice. This was true both before and after treatment with ACTH. Also, female mice of all groups had consistently higher values than males both before and after ACTH

Table 30. PLASMA CORTICOSTERONE (µg%) IN AKR AND C57Bl/6J MICE BEFORE AND AFTER ACTH ADMINISTRATION

Animals	No. of Mice	Pre-ACTH	Post-ACTH	
			4 Hours	24 Hours
AKR STRAIN				
Healthy Males	7	2.9[1] ± 0.7[2]	5.1 ± 0.7	5.8 ± 1.9
" Females	4	4.8 ± 1.9	13.2 ± 6.3	11.0 ± 3.0
Acute Leukemic Males (ALk)	2	6.9 ± 0.7	12.3 ± 2.0	7.0 ± 0.7
" " Females (ALk)	9	24.4 ± 6.0	36.7 ± 12.3	28.1 ± 9.9[3]
Incipient Leukemic Mice (ILk)[4]	3	3.6 ± 0.5	7.4 ± 1.2	8.9 ± 2.5
Sick but not Lk Mice (SNLk)[5]	3	7.8 ± 2.4	10.3 ± 3.2	11.8 ± 1.8[3]
C57 STRAIN				
Healthy Males	6	1.6 ± 1.0	4.4 ± 1.7	3.0 ± 0.9
" Females	5	2.7 ± 0.6	8.7 ± 2.5	5.6 ± 1.2

[1] mean
[2] standard error
[3] the data of the ALk mice and of the sick but not Lk mice each included 1 estimated value for 24 hours calculated as described in section 2.3.
[4] 1 male, 2 females
[5] 2 males, 1 female

administration, but the differences were not statistically significant. Similarly, AKR mice had higher values than C57 animals, but again the differences were not statistically significant.

Following ACTH administration, mice with ALLk responded with a higher maximum PC level than healthy AKR mice who in turn showed a higher maximum than healthy C57 animals. The maximum response of the ILLk and SNLk mice was of a similar order as that of healthy AKR and C57 animals. The maximum increase in PC level as a result of ACTH administration was greater in female mice than in males in all groups. The over-all difference between the various groups achieved statistical significance, according to analysis of variance ($0.025 > P > 0.01$).

3.2. URINARY CORTICOSTEROID DATA (Tables 31 and 32)

One day following ACTH administration, 44 of the 47 mice showed an increase in the UCS, all 3 decreases occurring in mice with ALLk (1 male and 2 females). On day 2, 24 of the mice showed a further increase in UCS, as compared with day 1, two-thirds of the decreases occurring in ALLk females, C57 males and SNLk mice. The declines

Table 31. URINARY CORTICOSTEROIDS (ng/24 hours) IN AKR AND C57Bl/6J MICE BEFORE AND AFTER ACTH ADMINISTRATION

Animals	No. of Mice	Pre-ACTH	Post ACTH Day 1	Day 2	Day 3	Day 4
AKR STRAIN						
Healthy Males	7	34[1] ± 17[2]	43 ± 17	68 ± 25	71 ± 37	27 ± 4
" Females	5	117 ± 21	215 ± 29	221 ± 50	113 ± 32	63 ± 2
Acute Leukemic Males (ALk)	6	91 ± 25	164 ± 47	171 ± 52	124 ± 37[3]	90 ± 29[3]
" " Females (ALk)	12	179 ± 34	222 ± 41	197 ± 31[3]	195 ± 30[3]	156 ± 28[3]
Incipient " Mice (ILLk)[4]	3	71 ± 35	125 ± 47	168 ± 37	144 ± 31	81 ± 28
Sick but not Lk (SNLk)[5]	3	105 ± 59	198 ± 92	108 ± 61	68 ± 36	54 ± 29[3]
C57 STRAIN						
Healthy Males	6	48 ± 6	113 ± 31	85 ± 11	51 ± 7	32 ± 7
" Females	5	112 ± 32	176 ± 42	196 ± 41	134 ± 13	86 ± 21

[1] mean
[2] standard error
[3] The data of ALk male mice included 1 estimated value for Day 3 and 2 for Day 4 calculated as described in section 2.3. Similarly, the data of ALk female mice had 2, 3 and 5 estimates on Days 2, 3 and 4 respectively while the data of the sick but not Lk mice had 1 missing value on Day 4.
[4] 1 male, 2 females
[5] 2 males, 1 female

were observed by the third day in 41 of the 47 animals and the same number showed a further decline on the fourth and last day. The values on day 4 were either below those of the pre-ACTH values or at about the same level.

AKR mice with ALLk yielded higher values than healthy AKR mice prior to ACTH administration and generally also afterwards, near significant differences being achieved in males on days 2 and 3 ($0.10 > P > 0.05$) and significant differences on day 4 for both males ($0.05 > P > 0.02$) and females ($0.01 > P > 0.001$). ILLk and SNLk mice gave values which were generally below those with ALLk, and when compared with the values of healthy animals of either strain, SNLk gave values which were above normal while ILLk gave essentially normal values before and after ACTH injection.

Both before and after ACTH, AKR female mice of all groups presented consistently higher values than males, with significant differences on the first ($0.05 > P > 0.02$) and second ($P = 0.02$) day post hormonal treatment in healthy mice, and pre-ACTH ($0.05 > P > 0.02$) and day 4 in ALLk animals ($0.02 > P > 0.01$). In C57 mice, females also had the

Table 32. THE MAXIMUM INCREASE IN THE PLASMA CORTICOSTERONE AND URINARY CORTICOSTEROIDS AFTER ACTH ADMINISTRATION IN AKR AND C57 MICE

Animals	No. of Mice	Plasma Corticosterone (µg%)	Urinary Corticosteroids (ng/24 hours)
AKR STRAIN			
Healthy Males	7	4.6[1] ± 1.2[2]	46 ± 19
" Females	4	10.3 ± 5.5	126 ± 38
ALk Males	2	6.0 ± 2.7	124 ± 40
" Females	9	15.9 ± 4.5	118 ± 27
ILk Mice[3]	3	5.8 ± 2.8	101 ± 27
SNLk Mice[4]	3	6.2 ± 2.1	93 ± 45
C57 STRAIN			
Healthy Males	6	3.0 ± 0.7	74 ± 23
" Females	5	6.8 ± 2.7	127 ± 30

[1] mean

[2] standard error

[3] 1 male, 2 females

[4] 2 males, 1 female

higher values, but the differences did not exceed borderline significance (days 3 and 4).

Differences in UCS due to strain were not significant either before or after ACTH treatment.

Following ACTH treatment, the maximum increase in UCS was greater in ALLk males than in healthy males of either strain, but the same was not true in the case of the females. In fact, the only 2 mice which showed no increase in UCS in the 4 days following injection of the hormone were ALLk females. Healthy AKR and C57 females showed a higher maximum than males of the corresponding strain, but such a difference was not apparent in ALLk mice.

Prior to injection with ACTH, there was no remarkable difference between the C57 and AKR healthy mice. After ACTH, C57 males had generally higher values than AKR healthy mice, while in females, the C57 mice had the lower values for days 1 and 2 and the higher ones for days 3 and 4.

3.3. MORPHOLOGICAL DATA (Table 33)

Differences due to Lk: Healthy AKR mice weighed more than AKR mice with Lk, but differences were reliable only in males ($0.05 > P > 0.02$). Lk mice had heavier thymuses than healthy AKR mice, but the differences were reliable only in females ($P < 0.001$). However, the mice with Lk had significantly heavier spleens than healthy mice in both sexes ($0.05 > P > 0.02$). Lk mice had smaller adrenals than healthy AKR mice, and here also the difference was significant only in females ($0.01 > P > 0.001$).

Differences due to Sex: Males weighed more than females but achieved significance or approached it only in healthy C57 ($0.05 > P > 0.02$) and AKR ($0.10 > P > 0.05$) mice.

Thymus weights were heavier in females in all groups, but achieved significance only in mice with ALLk ($P < 0.01$). Female AKR mice, healthy or ill, had smaller spleens than males but the differences were not significant. On the other hand, C57 females did have significantly heavier spleens than males ($0.01 > P > 0.001$). Adrenals were heavier in females than males, but the differences were significant or approached it only in healthy AKR ($0.02 > P > 0.01$) and C57 mice ($0.10 > P > 0.05$).

Table 33. BODY WEIGHTS AND THE WEIGHTS OF THYMUS, SPLEEN AND ADRENALS IN AKR AND C57 MICE

	No. of Mice	Body (gm)	Thymus (mgm)	Spleen (mgm)	Adrenal (mgm)
AKR STRAIN					
Healthy Males	7	28[1] ± 1[2]	43 ± 7	61 ± 9	4.6 ± 0.7
" Females	5	24 ± 1	48 ± 15	54 ± 4	7.2 ± 0.5
Acute Leukemic Males (ALk)	6	23 ± 2	227 ± 96	408 ± 163	4.2 ± 0.5
" " Females (ALk)	13	22 ± 1	696 ± 105	389 ± 115	4.6 ± 0.3
Incipient Leukemic Mice (ILk)[3]	3	24 ± 2	87 ± 32	144 ± 33	4.7 ± 2.4
Sick but not Lk (SNLk)[4]	3	18 ± 2	24 ± 4	63 ± 7	4.5 ± 0.6
C57 STRAIN					
Healthy Males	6	29 ± 1	21 ± 6	71 ± 4	4.1 ± 0.1
" Females	5	24 ± 1	30 ± 7	122 ± 14	5.9 ± 0.8

[1] mean

[2] standard error

[3] 1 male, 2 females

[4] 2 males, 1 female

Differences due to Strain: There was no significant difference in body weight between the AKR and C57 strains. AKR mice had the heavier thymuses but the differences were significant only in males ($0.05 > P > 0.02$). On the other hand, C57 mice had the heavier spleens with differences being significant only in females ($0.01 > P > 0.001$). C57 mice also had heavier adrenals than AKR mice but the differences were not significant.

Correlations between PC, UCS and Morphological Data: In AKR females, both healthy and ALLk, and in C57 males, there were significant positive correlations between the pre-ACTH PC levels and the 4 hour and 24 hour post-ACTH PC levels. There were also significant positive correlations between the pre-ACTH UCS values and one or more of the post-ACTH readings in all groups except in AKR healthy females in which there was nevertheless significant positive correlations between the UCS data obtained on the several days after ACTH injection. There were no significant correlations between the PC data on the one hand and the UCS on the other hand, nor between adrenal weight on the one hand and either pre- or post-ACTH PC values on the other.

However, there was a significant negative correlation between adrenal weight and the third day post-ACTH UCS values in ALLk males and significant positive correlations in C57 males between adrenal weights on the one hand and the pre-ACTH UCS and the first day post-ACTH UCS on the other. The remaining 27 correlation coefficients between these 2 variables in all the groups were not significant. There was also no significant difference between the degree of hypertrophy of the thymus or spleen of ALLk mice and their PC or UCS level, either before or after ACTH treatment. There was no significant correlation between thymus and spleen in healthy AKR or C57 mice or in mice with ALLk.

4. DISCUSSION

4.1. PLASMA CORTICOSTERONE (PC) DATA

Although a number of reports have appeared in the literature on the determination of the CC level in mice, only one was conducted in AKR mice (Levine and Treiman, 1964). The authors reported values of 8-0 µg% for 10-15 week old AKR males and slightly but not significantly higher values for C57Bl/10 mice of the same age and sex. However, as

the time of day the blood was collected was not reported, and as the diurnal variation may result in about 100% difference between the maximum and minimum values within a 24-hour period (Halberg, Paterson and Silber, 1959; Solem, 1966), a more precise comparison with our data was not possible.

Comparisons of the values reported in this study with those of still other reports would be even less meaningful because either the age or sex of the animals - variables known to influence the CC level - was not reported. Furthermore, all previous reports of the determination of the CC level in mice involved the use of pooled samples of blood, some of which was derived from both sexes, whereas the present report provides for the first time data obtained from individual mice. Moreover, the CC level determined in mice prior to the present study were determined fluorimetrically which is known to yield higher corticoid values due to a variable quantity of contaminating non-specific substances known to be included with this estimation, and not determined by radioassay utilized in the present study (Murphy, 1967).

Recently, the PC levels were determined in 59 unstimulated C3H mice of the mammary tumour-bearing HeJ substrain and the non-mammary tumour-bearing FeJ substrain utilizing the same technique as in the present series of AKR and C57 mice (Sancho, Grad and Murphy, 1969). Mean values of 17.2 µg% for 22 healthy FeJ mice and 14.1 µg% for 22 healthy HeJ mice were obtained. The results were higher than those observed in AKR and C57 mice and this difference may be due to strain differences, but cannot be due to age differences as the ages of the mice in the 2 studies were comparable. The mean PC levels of mice with mammary tumour (MT) (25.9 µg%) were also definitely higher than those of all other non-MT-bearing mice of either sex or substrain, and in this respect the result is similar to that observed in AKR mice with ALLk, as compared with healthy AKR or C57 mice (Table 30). However, unlike the sex difference in PC level observed in AKR and C57 mice, there was no sex difference in C3H mice.

The fact that 95% of the mice showed an increase in the PC level 4 hours after the administration of ACTH might have been due not only to the injection of the exogenous ACTH but may also have been

produced by increased stimulation of endogenous ACTH brought on by the stress of the orbital bleeding, stress itself being an activator of the anterior pituitary-adrenocortical system, with maximum effects on circulating eosinophils at 4 hours and with a return to normal within 8 hours (Speirs and Meyer, 1946, 1947). However, 24 hours after ACTH administration, 80% of mice still had higher PC levels and this cannot be ascribed to any stress imposed on the mice the previous day but must be due to the stimulating effect of the exogenous ACTH on adrenal cortex function.

Moreover, the fact that following ACTH administration higher PC values were obtained by the competitive protein-binding technique is further evidence that this technique is in fact determining corticosteroids which in very many experiments have been shown to be secreted in increased amounts into the circulation by the adrenal cortex upon stimulation by ACTH. Taken together with the tests for accuracy, precision and specificity reported earlier, this is further evidence of the reliability of the competitive protein-binding technique for the determination of the CC levels in mice.

An examination of the pre-ACTH data showed that the PC values for mice with ALLk were significantly above those of healthy AKR and C57 mice. Also, females had consistently higher values than males. These results confirm the findings based on much larger numbers and reported in the previous Chapter. What differences there are between the findings there and here can be reasonably ascribed to the relatively fewer mice investigated in this part of the study. Thus, there were only 2 male mice with ALLk, 3 with ILLk, 3 with SNLk, and 22 healthy AKR and C57 mice in this study as compared with the previous 146 healthy AKR, 127 healthy C57 and 35 mice with ALLk.

After ACTH treatment, the PC values of all groups were higher than those obtained prior to hormonal treatment (Table 30), but the ALLk females showed a greater increase than healthy AKR and C57 mice or mice with ILLk or SNLk (Table 32). There have been no comparable reports in the literature describing the effect on the PC level of ACTH administration on patients or animals with ALLk as compared with normal subjects, and therefore further critical comment in this regard is not possible.

Also, the finding that both the ILLk and SNLk mice yielded PC values which were well below those of the ALLk mice both before and after ACTH cannot be compared with similar studies conducted by others as none have been reported in the literature.

Our experiments also demonstrated that although the PC response to ACTH was greater in healthy AKR mice than in healthy C57 animals, the differences were not significant (Table 30 and 32). In this connection, Levine and Treiman (1964) showed that 10-15 week old pre-leukemic AKR mice were much less responsive to stress than C57Bl/10 and A/JAX mice although the resting corticoid values of the 3 strains were not very different from each other. Also, Solem (1966) showed that the adrenals of 12-week old pre-leukemic mice of the high CS strain produced significantly less corticosteroids than did the low-leukemic WLO mice of the same age in response to ACTH both <u>in vivo</u> and <u>in vitro</u>. The difference between these results and our own findings may be due to the difference in the age of the mice investigated: our mice were 7 to 12 months' old and theirs between 2 and 4 months' old when there is a marked drop in CC level, as described in the previous Chapter. Further studies are indicated here.

Our results also showed that females of both strains responded with greater increases in PC level to administered ACTH than did males, and this is in agreement with previous reports on this subject (Kitay, 1961; Solem, 1966). The reason for the higher PC level in female mice under resting conditions was discussed in the previous Chapter.

4.2. URINARY CORTICOSTEROIDS (UCS) DATA

The ultramicro assay utilized in the present study was sufficiently sensitive for the determination of the UCS in individual mice and as only 150 λ were utilized for each determination of a 24-hour urine volume which generally exceeded 1 ml, sufficient material was always available for study from each animal.

Tests of precision and specificity described earlier apply here also, although it is not possible at the present time to be certain that the method determines corticosterone specifically. However, the methylene chloride extraction limits the cpds to corticosteroids with structures very closely similar to those of cpd B.

Tests of specificity described earlier in the thesis showed that cortisol is a major competitor to corticosterone for positions on the CBG, but inasmuch as the mouse lacks the 17-hydroxylation pathway in the biosynthesis of cortisol (Triller and Birmingham, 1965, 1965a, 1965b; Nandi et al., 1967; and others), the possibility of determining cortisol in mouse urine does not arise.

Tests of accuracy were not conducted by the author, but 85-90% of corticosterone added to C3H urine was recovered by others in our laboratory (Sancho et al., 1969).

No reports have appeared in the literature describing the amount of corticosteroids in urine of mice, either in individual animals or in pooled samples, and therefore, it is not possible to make comparisons with work conducted in other laboratories. However, the UCS were determined in C3H/HeJ and C3H/FeJ mice in our laboratory (Sancho et al., 1969), and the results for 10 males and 12 females in each of FeJ and HeJ mice were 58 ± 10, 160 ± 15, 84 ± 23 and 170 ± 19 ng/24 hours respectively. Thus, the data of AKR and C57 mice were somewhat lower than those of C3H mice. Also, mice bearing MT had higher

UCS amounts than before the appearance of the tumour, a situation comparable to that observed in AKR mice with ALLk when compared with healthy controls (Table 31). Finally, females had higher UCS values than males in C3H mice just as in AKR and C57 animals (Table 31).

One study conducted in Strain 2 guinea pigs in the late stages of a transplantable L_2C/NB leukemia and a liposarcoma revealed that the urinary exbretion of F, 2-a-hydroxycortisol and 6β-hydroxycortisol was increased nearly 3-fold (Nadel and Burnstein, 1956). Later the same authors showed that there was a statistically significant higher adrenal production rate of the same 3 CSs in Strain 2 guinea pigs bearing the L_2C/NB lymphoblastic leukemia (Burnstein and Nadel, 1967).

Ninety-four per cent of AKR and C57 mice showed an increase in UCS on the first day after injection with ACTH, and 84, 63 and 49% on the second, third and fourth day respectively. The high percentage of mice showing such a response on second day in particular could not be due to the stress of the injection because the effect of such a stress does not extend beyond 6 or 7 hours as previously mentioned.

Therefore, the effect on the Day 2 UCS must have been due to the specific pharmacodynamic effect of the ACTH itself. Inasmuch as the ACTH-inducted increase in UCS in other species is well established, the finding that the competitive protein-binding technique will register such increases in mouse urine also provides further evidence that the use of this technique under the new conditions is acceptable.

A comparison of the post-ACTH UCS data with that reported by other groups is not possible as no studies of this nature were reported in the literature up to the present time.

4.3. MORPHOLOGICAL DATA

The main reason in presenting the morphological data was to demonstrate that the mice claimed to have had ALLk did in fact have abnormally enlarged lymphatic tissue as exemplified by thymic and splenic weights and that those claimed to have had ILLk also had enlarged lymphatic tissue as seen by the weights of the same 2 organs, which were heavier than normal tissues but lighter than those of mice with ALLk (Table 33). Moreover, the ILLk

mice were considered to be sick with this condition because their lymph nodes were also observed to be larger than normal, and yet smaller than in mice with ALLk. Finally, there was the fact that when the ILLk mice were alive, they appeared quite well in striking contrast to the ALLk animals. Only autopsy revealed them to be at the beginning of their leukemic illness.

The failure to observe a sex difference in the weights of thymus or spleen in healthy AKR mice was verified in a larger group of 35 males and 20 females where the mean weights of thymus were 31 ± 5 and 26 ± 8 mgm, and the mean weights of spleen were 71 ± 3 and 73 ± 5 mgm. Adrenal weights were larger in the females of this group than in males (4.5 ± 0.3 as against 3.9 ± 0.3 mgm) but the differences were less marked than recorded in Table 33.

Prior to the more recent attempts to assess adrenocortical function in Lk by the direct determination of the CS within the adrenal cortex as well as in the circulation and the urine, a considerable number of studies were published with the same aim but involving less direct ways of assessing adrenocortical activity.

Thus Dalton (1944) demonstrated a marked lipid loss in adrenals of mice bearing the generalized and infiltrating Y103 stem cell transplanted Lk and a considerably lesser fat depletion in mice with the localized lymphoma 72942. Similarly, Arnesen found in a series of studies that a lipid depletion occurred in adrenals of AKR/0 mice at the time of sexual maturation before active Lk appeared and that it could be reversed by chronic treatment with high doses of ACTH (Arnesen, 1955, 1956) or cortisone acetate (Arnesen, 1964). This lipid depletion was more marked in the zona fasciculata than in the zona granulosa. Levine (1948) reported a marked decrease in adrenal cholesterol in preleukemic AKR mice and during the acute stages of spontaneous and transplanted Lk in the same strain. Several authors observed a thymic hyperplasia in apparently healthy AKR mice relative to several low-leukemic strains (Birmingham and Grad, 1954; Metcalf, 1960; Schmid and Hutchison, 1968). Metcalf cited this as evidence of adrenocortical hypofunction in AKR mice when considered together with the relative unresponsiveness of the lymphoid organs of AKR mice to adrenalectomy and ACTH, a refractoriness which did not extend to the corticosteroids themselves (Metcalf, 1960).

The finding that ACTH can restore the normal lipid level in pre-leukemic AKR adrenal cortex also supports the hypothesis that the pre-leukemic adrenal is hypofunctional, although the lipid depletion per se can be found in both the hypo- and hyperfunctional adrenal cortex. Indeed, the observation by Molbert and Arnesen (1960) and Arnesen (1963) that lipid depletion was accompanied not only by loss of lipid vacuoles but also a very marked increase in the number of mitochondria indicates that the adrenal cortex is hyperfunctional. Arnesen attempted to resolve this dilemma by suggesting that adrenal metabolism as a whole might be hyperactive but that aspect of metabolism involved specifically with the synthesis of the CSs was hypoactive (Arnesen, 1963).

While such studies provide interesting data, they are secondary to studies involving direct assessment of adrenocortical function by determining CS concentrations in the circulation and urine. However, this was not possible in the mouse-- the experimental animals most investigated in cancer research -- due to the lack of a sufficiently sensitive assay. The ultramicro assay described in the present report fills this gap.

4.4. CORRELATIONAL DATA

While there were significant positive correlations between the pre- and post-ACTH PC values and also between the pre- and post-ACTH UCS data, there were no significant correlations between the PC and UCS data. Nevertheless, the following was observed with respect to these data: in both PC and UCS data, mice with ALLk had higher values than healthy AKR and C57 animals and higher also than mice with ILLk or SNLk, and in both instances, did females have higher values than males. While it was not possible to make statements about the level of adrenocortical function from the PC data only, it is possible to make such assessments when the UCS data are also available. Therefore, it can now be stated that the adrenal cortex is hyperactive when AKR mice suffer from ALLk and that in general females have more active adrenal cortices than males. As for the ILLk animals, it can be stated tentatively that their level of adrenocortical function is below that of mice with ALLk, but whether it is also below that of healthy AKR mice as indicated by the below normal PC levels reported in the previous Chapter cannot be stated with more certainty until the UCS

of a larger number of ILLk mice are available. SNLk mice despite the stress of their illness did not have higher UCS values, but had instead values which were more or less at the normal level and well below those suffering from ALLk. Finally, only about 10% of the possible correlations between adrenal weights on the one hand and PC or UCS data on the other reported in this and previous Chapter were significant.

5. SUMMARY

The PC level and UCS amounts were investigated before and after injection of ACTH in 12 healthy AKR mice, 19 sick with ALLk, 3 sick with ILLk, and 3 sick with SNLk. Into which category of illness the mice were placed depended on their appearance while alive and the size of the lymphatic tissue at death. Eleven healthy C57 mice were also investigated.

The PC and UCS data were elevated in mice with ALLk relative to that of healthy AKR and C57 mice as well as to ILLk and SNLk animals. All groups of mice other than the ALLk appeared to have a similar level of adrenocortical function, but additional data is required from the ILLk and SNLk

groups especially. Females of both strains and whether sick or well appeared to have a higher level of adrenocortical function than males. In general, the degree of response of the PC and UCS to ACTH depended on their pre-ACTH values, and while there was some evidence that PC of females with ALLk reacted with the greatest maximum response to ACTH, this was not so in the case of the UCS. Indeed, in this case, other groups showed at least a similar maximum response.

PART III

GENERAL DISCUSSION

The studies reported in this thesis developed as follows: as it was the aim of the thesis to assess adrenocortical function in the Lk of AKR mice, it was first necessary to develop a suitable assay for determining CSs in small volumes of plasma or serum. Murphy's competitive protein-binding technique for determining the CSs was first tested for its use in mice in its micro form in the 0 to 40 µg range. This was found to be satisfactory when strains of fairly large 1 year old mice such as those of the CF_1 strain were utilized, or when used on pooled plasma or serum obtained from several animals. However, its requirement of plasma or serum was too high for the study of the PC of individual mice of the smaller AKR and C57 strains particularly at the younger age levels, and therefore an attempt was made to develop an ultra-micro form of the assay in the 0 to 8 µg range. The problem became one of finding a CBG with the requisite affinity for corticosterone, the main CS

in the circulation of the mouse. Experiments were conducted with serum and plasma from the human, rat, monkey and finally on Murphy's suggestion from the dog. The latter proved satisfactory for the purposes cited above as shown by tests of precision, accuracy and specificity. Later experiments involving ACTH administration showed that the ultramicro technique yielded elevated PC and UCS values as would be expected from the vast amount of evidence that ACTH specifically stimulates increased CS production by the adrenal cortex, thus providing further evidence of the suitability of the technique.

Subsequently, the PC levels and UCS amounts were determined in healthy and sick high-Lk AKR mice and low-Lk C57 animals before and after stimulation with ACTH. While it was not possible to assess the level of adrenocortical function from the PC determinations alone, it was possible to do this when the UCS data became available. Therefore, it is now possible to state that the level of adrenocortical function in apparently healthy AKR mice is not significantly different from that of healthy C57 animals. Also, for the same reasons, healthy females of either strain can be said to have a

significantly higher level of adrenocortical function than healthy males.

Both strains of mice showed a decline in the PC level between 2 and 4 months of age which was similar in the females of both strains but steeper in C57 males than in AKR males. In males of both strains, the PC level rose till 6 months of age and remained more or less at the same level until a year old whereas in females the peak occurred at 8 months remaining more or less at the same level thereafter until a year old. Because there were no UCS values in this aspect of the study, it is not possible to make meaningful statements about the relative state of adrenocortical function at different ages in the 2 strains of mice.

Mice sick with ALLk had higher PC levels and UCS amounts than healthy animals of either strain and higher also than animals sick from causes other than Lk. Therefore, ALLk mice can be said to have a significantly higher level of adrenocortical function than the other mice of this study. The same assay revealed a similar elevation of adrenocortical function in C3H mice bearing mammary tumours (Sancho et al., 1969).

Another group of AKR was considered to be ill with Lk because at autopsy they presented significantly enlarged lymphatic tissue when compared with controls, and yet the enlargement was not as marked as in mice considered to have ALLk. Another difference between these 2 groups of mice was the fact that the ILLk group appeared quite well while alive, but the ALLk mice gave every appearance of being sick: irregular respiration, hunched back, unkempt fur lacking the normal lustre, visibly enlarged cervical and axillary lymph nodes, etc. Therefore, the apparently well AKR mice with enlarged lymphatic tissue at autopsy were considered to be ill with incipient lymphatic leukemia (ILLk). Because their PC levels and UCS amounts were less than those of the ALLk mice, their adrenocortical function can be considered to be below that of ALLk animals. ILLk mice also had PC levels which were generally below those of healthy AKR mice, but this was more apparent in the data of the 15 ILLk mice reported in Chapter II than in those of the 3 ILLk mice of Chapter III. However, UCS values were available only for the smaller number of ILLk mice and these were not below those of the healthy AKR mice.

Further UCS studies are required before it is possible to make any definitive statement, about the level of adrenocortical function in ILLk mice.

In both strains there were mice that were sick but from causes other than Lk. These showed no significant difference in PC level as compared with healthy controls and the same was true in the case of the 3 UCS values available for the SNLk mice. This would indicate that the stress of the illness did not increase their level of adrenocortical function as compared with controls.

Inasmuch as the data of the ACTH series indicated that there was a significant positive correlation between the pre-ACTH PC values on the one hand and the post-ACTH PC values on the other, and similarly between the pre- and post-ACTH UCS data, differences in these variables existing between groups before ACTH injection tended to persist afterwards. Thus, ALLk mice had the highest pre-ACTH PC values and had the highest post-ACTH PC values also. The same was true of the UCS readings but here there was some indication that female ALLk mice that had the highest pre-ACTH readings had lesser increases

than did mice of other groups receiving ACTH. This would indicate that the adrenal cortex of female ALLk mice was operating closer to its maximum than that of the other groups.

In conclusion, the problem of adrenocortical function in the spontaneous lymphatic leukemia of AKR mice is presently at the following point: when AKR mice are healthy, their resting level of adrenocortical function and their responsiveness to ACTH does not appear to be markedly different from that of healthy C57 animals. However, when AKR mice have full-blown acute lymphatic leukemia, their level is well above not only of healthy AKR and C57 mice, but also higher than that of AKR and C57 mice sick from causes other than Lk. The question as to what is the level of adrenocortical function in mice just prior to the development of Lk or in its first stages is still open, because although the PC level of such animals are below those of comparable controls, further UCS studies in these groups are required.

Finally, these studies indicate that it is now possible to investigate adrenocortical function serially in individual mice utilizing the

ultramicro competitive protein-binding radioassay. Inasmuch as there are more inbred strains of mice than of any other mammalian series currently under investigation, this should make possible a more accurate assessment of adrenocortical function in a wide variety of conditions than was possible hitherto.

PART IV

SUMMARY AND CONCLUSIONS

The competitive protein-binding radioassay for the determination of the CS as developed by Murphy (1967) was adapted for use in determining corticosterone in the circulation and urine of mice. Tests of precision, accuracy, specificity and of stimulation with ACTH revealed that the technique was suitable for this purpose.

The PC level was determined in 146 healthy AKR mice, 50 AKR mice with LLk (15 in the incipient stage and 35 in the acute stage), and 17 with SNLk. The PC was also determined in 127 healthy C57 mice and 20 C57 mice sick with a disease other than LLk. Each group contained animals of both sexes and a wide range of ages, and were studied under non-stimulated conditions.

Another set of PC and UCS values were obtained from 12 healthy AKR mice, 19 sick with ALLk, 3 sick with ILLk, 3 with SNLk and 11 healthy C57 mice, all of which received 0.04 I.U. ACTH

subcutaneously in 2 injections administered a week apart. A maximum of 5 consecutive twenty-four hour urine samples were collected from each animal under study, one before and four after the first ACTH injection. A week later, 3 blood samples were obtained by orbital bleeding for the PC determinations, one before and the others 4 and 24 hours after the second ACTH injection. Thymuses, spleens and adrenals were removed for weighing from representative numbers of mice from each group of each series. The PC levels were also determined in 16 male and 14 female AKR mice, 6-7 weeks old, and bearing the BW5147 transplantable tumour for 1 week.

There was no significant difference in PC level and UCS amounts between the healthy mice of the high-Lk AKR strain and healthy mice of the low-Lk C57 strain, while healthy females of either strain had significantly higher PC and UCS values than males. Female AKR mice bearing the transplantable BW5147 tumour for one week also had higher PC levels than the corresponding males. However, no significant difference due to sex was observed in the PC levels of mice sick with the spontaneous ALLk. Mice with ALLk had significantly higher PC and UCS

values than the other groups of mice investigated including ILLk and SNLk mice. Mice with ILLk had PC levels which were below normal and although their UCS were at the normal level, they were too few on which to base any firm conclusions. Mice with SNLk had PC and UCS values which were not remarkably different from healthy mice. In general, differences in PC and UCS levels between groups existing prior to stimulation with ACTH remained afterwards also.

Both mouse strains showed a decline in PC levels between 2 and 4 months of age which was similar in degree in the females of both strains, but which was steeper in C57 than in AKR males. In males of both strains the PC level rose till 6 months of age whereas in females the peak occurred at 8 months. After the maximum was reached, the values remained more or less at the same level in C57 males and in both sexes of the AKR strain but declined further in C57 females.

Thymic and splenic weights were significantly higher in mice with ALLk than in all other groups including mice with ILLk that in turn had significantly higher lymphatic tissue weights than non-leukemic AKR controls.

In conclusion, the level of adrenocortical function was the same in healthy AKR and C57 mice, higher in healthy females than healthy males, higher in mice with ALLk than in all other groups, including ILLk and SNLk mice. Adrenocortical function of SNLk mice did not appear to be reliably different from that of healthy mice. The below normal PC levels in ILLk mice suggest that their adrenocortical function may be depressed below that of healthy mice, but further UCS studies are required.

PART V

CLAIMS TO ORIGINAL RESEARCH

1. Utilizing a competitive protein-binding radioassay, it was possible to determine serially the plasma corticosterone and urinary corticosteroids in individual mice.

2. Both AKR and C57 mice showed a decline in PC levels between 2 and 4 months of age which was similar in degree in the females of both strains, but which was steeper in C57 than in AKR males. In males of both strains, the PC level rose until 6 months of age whereas in females the peak occurred at 8 months. After the maximum was reached, the values remained more or less at the same level in C57 males and in AKR males and females, but the values decline further in C57 females.

3. Healthy female AKR and C57 mice had significantly higher PC and UCS values than males. Female AKR mice with spontaneous ALLk had higher UCS values than male AKR mice with ALLk, but there was no significant difference in PC between the 2 groups, nor was there a difference due to sex in the PC levels

of ILLk or SNLk mice. On the other hand, female AKR bearing the transplantable BW5147 for one week had significantly higher PC values than the corresponding males.

4. Mice with ALLk had significantly higher PC and UCS values than those of all other groups investigated, while SNLk mice had PC and UCS values which did not appear to be reliably different from those of healthy animals.

5. In general, differences in PC and UCS values between groups of mice existing prior to stimulation with ACTH persisted after stimulation also.

6. There are mice with incipient lymphatic leukemic in the AKR strain that appear outwardly healthy but have significantly larger lymphatic tissue than controls. However, such enlarged lymphatic tissue is still significantly smaller than that of AKR mice with acute lymphatic leukemia. Moreover, the latter have a significantly higher level of adrenocortical function than mice with incipient leukemia and there was a suggestion from the significantly lower PC level in such incipiently sick mice that their level of adrenocortical function was below normal.

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