ADRENOCORTICOTROPHIC HORMONE AND INTERMEDIN

by

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INTRODUCTION

The following work was originally undertaken in December 1951, with the hope of studying the ACTH-like activity of human blood in different physiological and pathological conditions. The acid acetone extraction of blood and the adrenal ascorbic acid depletion in hypophysectomized rats (Sayers' test) were selected as the most suitable means of approach at this time, without realizing the difficulties attendant upon the setting up of this test in the absence of extra technical assistance. When Sulman, in the beginning of 1952, proposed the melanophore expansion in the frog as a simple test for ACTH, the preceding difficulties seemed partly solved, and it was decided to investigate this new method of assay. However, at the same time, Sulman postulated an identity of ACTH and intermedin, necessitating an examination of his claim and the repetition of that part of his work on which this conception was based. Very soon a controversy arose in the literature and the question of the identity of ACTH and intermedin was much debated. Although this question does not yet seem definitely settled, it now appears that if the two hormones are not identical, they seem in certain conditions to bear an obscure relationship to each other. In spite of this controversy, the investigation of the Sulman test in clinical conditions was continued, with the hope of showing, as Sulman had, that a melanophore expanding factor was present in the blood of those patients in whom an increase of

circulatory ACTH would be expected.

The present work reviews the literature dealing with ACTH-like activity in blood, gives an account of the controversy surrounding Sulman's work and attempts to illuminate the claims of the latter in relation to what actually is known of intermedin in man and of the adrenal cortex in pigmentation. Details of the tests used in the clinical investigations and their results are presented.

REVIEW OF THE LITERATURE

PART ONE : ACTH-LIKE ACTIVITY IN BLOOD

The increasing emphasis on the importance of the pituitary-adrenal axis has made the study of the presence and behaviour of ACTH in the blood a subject worthy of investigation. Such a study could be hoped to enlighten the relationship of the adrenal cortex with its specific pituitary hormone. The demonstration of a change in pituitary corticotrophin output in connection with varying rates of adrenal cortical hormone secretion is of more than academic interest. Knowledge at this point would presumably help greatly in the differential diagnosis between primary and secondary adrenal hyperfunction, elicit further information concerning the mechanism of response to stress and permit the more direct study of the conditions which induce a release of corticotrophin.

The first part of this work will present a review of the literature at present available on the determination of ACTH activity in the blood. The methods of blood extraction and the assay used for this specific purpose will be considered.

1. FATE OF ACTH IN THE BLOOD

Enzymatic inactivation

Pincus (1) showed that certain highly purified ACTH preparations maintained their full potency over a period of several hours when

added to bovine whole blood or plasma, but lost 40-70% of their activity after one hour when added to rat or human blood or plasma. Heating the rat or human blood to 50°C. for three to four hours prevented this inactivation. The same ACTH preparations when added to a mixture of beef blood and heated rat blood again lost 40-70% of their potency in one hour. The author suggested the presence of an ACTH inactivating system in blood consisting of a heat-labile and a heat-stable substance. Both factors would be present in rat and human blood.

Another possible interpretation of these results may be that destruction of the ACTH is due to its heterogenicity with respect to the host blood, particularly in the case of human blood. In either event, the above observations raise the questions as to whether ACTH injected in the human subject by any route is not partially destroyed before reaching the adrenal cortex and whether the ACTH inactivating system does not play a part in the physiological regulation of hypophyseal-adrenal function (35). Finally, they may explain why it is difficult to find an appreciable level of circulating pituitary corticotrophin in the blood in normal conditions.

Biological half-life.

The few studies concerned with the actual rate of disappearance of injected ACTH in animals or man are in general agreement that the hormone disappears rapidly from the blood stream. When rats were

injected with 5 mgm of a Li's ACTH preparation, one minute after the injection the level of ACTH in the plasma varied from 18 to 26 γ per cc, and thereafter fell in logarithmic fashion over a period of thirty minutes of observation, at which time the concentration was less than 0.5 γ per cc. The half-time of disappearance in this study was $5\frac{1}{2}$ minutes (2). These results were recently confirmed by Grenzell et al (3), who also reported that adrenalectomy in rats did not affect significantly the rate of disappearance of ACTH from the blood.

Sonenberg (4,5), using an ACTH preparation labelled with radioactive I^{131} observed a very rapid entrance into and disappearance of radioactivity from the adrenal gland of the rat. The quantities of the ACTH preparation used, labelled with 100 microcuries of I^{131} varied between 200 and 3,200 γ. The degree of localization of radioactivity in the adrenals was only 0.1% of the injected dose and diminished to one-half of the maximum value recorded in approximately five minutes, which provides an interesting comparison with the biological half time in blood recorded by the previous experimenters.

ACTH was administered intravenously by Sayers (16) over a period of thirty minutes to two normal male subjects (50 and 100 LAIA units respectively), and the level of the hormone in the blood was observed. At the end of the period of infusion, the concentration of the hormone in the plasma was 500 γ (250-1000) per 100 ml. in the subject receiving the higher dose; one and one-half hours later it was 50 γ per 100 ml., and after two hours the concentration was indistinguishable

from pre-infusion levels (less than 10 γ per 100 ml.). The rate of disappearance was the same in the second subject.

Samples of urine were obtained at various times during the course of the experiment. The concentration of the hormone was never found to be greater than 10 γ per 100 ml. of urine. It was estimated that in each subject less than 200 γ of ACTH appeared in the urine during the 24 hours following administration of the hormone. There was no enhancement of ACTH content after acidification of the urine at pH 1, which minimized the possibility of the hormone's being excreted in conjugated form. The author remarks that, in contrast to ACTH, gonadotrophin is excreted in the urine in appreciable amounts after a single injection in the human. It is pointed out that the very rapid disappearance of the hormone from the circulating plasma may be due to inactivation, or to rapid diffusion into the tissues. There is the further possibility that the exogenously administered ACTH may behave differently from the endogenous hormone (6).

2. METHODS OF ASSAY OF ACTH-LIKE ACTIVITY IN BLOOD

Method based on adrenal weight and structure

Among the first methods of assay selected to evaluate the adrenocorticotrophic potency of blood extracts, the increase in weight and histological change of the adrenals of intact infantile mice were used as an index of activity. Several German investigators (7,8,9) appeared to have been the first to be interested by the presence in the

blood extract of a "factor" which possessed the same properties as the corticotrophic factors studied by Anselmino and Hoffman. In the method used by Jores (9), mice weighing six to eleven grams were injected twice a day with an extract of serum equal to 0.05 cc per gram of body weight, and were then killed 24 hours after the last injection. The weight of the adrenal glands was recorded, and expressed in terms of 100 grams of body weight, according to the formula :-

$$\frac{\text{weight of adrenal glands} \times 100}{\text{body weight}}$$

It was claimed that the value obtained was never greater than 20 in the case of control animals. Values greater than 25 were interpreted as positive responses and were considered to be due to the corticotrophic activity of the blood extracts. The increase in thickness of the cortex and the distribution of lipids were studied histologically and were found to increase where the test was positive.

The assay proposed by Bates (10), using as a guide the increase in adrenal weight of the two-day old chick, has proven to be useful in testing the ACTH activity of pregnant mare's serum. The injections of the extract to be tested were given three times daily during a period of five days, after which the adrenal weights of the injected chicks were compared with a control group. The unit of adrenotropic activity was defined as the amount of material which produces a 25% increase in weight of the glands. This test has since been criticized for its lack of sensitivity.

The test involving the size and histological appearance of the

hypophysectomized rat's adrenal, extensively used by Collip, Anderson and Thomson (11), has also been used by other investigators (12). Adult rats unilaterally adrenalectomized ten to fourteen days after hypophysectomy received twice daily injections of the extract to be tested for six days. The weight and histological appearance of the second adrenal was then compared with the first.

The weight maintenance test proposed by Simpson, Evans and Li (13) was based on an appreciation of the amount of corticotrophin necessary to maintain the adrenal weight of 40 day-old hypophysectomized rats. They were injected twice daily during a period of fifteen days.

The tests using hypophysectomized rats are far more reliable than those practised on intact animals since the action of the animal's own pituitary is eliminated during the assay. Nevertheless, assays based on adrenal weight are of very limited use in dealing with human blood because they are time-consuming and require too great a quantity of blood. If one considers that the minimal amount of the hormone which would restore the lipids in the sudanophobic zone of hypophysectomized rats has recently been stated to be equivalent to 30 to 75 Y of Armour LAIA Standard (14), the above conclusion seems amply justified. The tests have, however, been mentioned here because they were sometimes employed in the work on the assay of ACTH in animal blood and in human blood.

Method based on adrenal-ascorbic acid depletion (the Sayers' Assay)

The most reliable and practical test applicable to the assay of

ACTH activity in body fluids is the Sayers test (15). Early studies by this author showed that the injection of ACTH induced a rapid decrease in the cholesterol and ascorbic acid content of the adrenal glands of rats. This fact, together with the known very high concentration of ascorbic acid in normal adrenocortical tissue, and the observation that the level of the vitamin present in the adrenal cortex after hypophysectomy was not affected by stressing situations (and, indeed, was approximately the same in the two glands of an animal 24 hours after the operation) constitutes the basis of the assay. The ascorbic acid depletion is expressed as the difference between its concentration in the left adrenal gland removed just before the intravenous injection of the substance to be tested, and the concentration in the right adrenal, removed one hour after the injection. A rectilinear relationship exists between the depletion and the logarithm of the dose. The test is very sensitive, amounts as small as 0.20 ϒ of the hormone (and, in the hands of the author, 0.15 ϒ) being detectable. Intact rats are of little or no value for the assay since the responses obtained in these animals are highly variable and non-specific (15).

Since the publication of Sayer's work, practically all the measurements of ACTH activity in body fluid are based upon his test (16, 17,18,19,20).

3. METHODS OF EXTRACTION OF ACTH-LIKE ACTIVITY FROM BLOOD

Acid acetone precipitation

Until recently the most widely used method for the extraction of ACTH in the blood was based upon the classical acid acetone precipitation procedure introduced by Lyons for the extraction of the hormone from the pituitary gland (21). A certain amount of blood is precipitated, as soon as possible after collection, with a corresponding volume of acid acetone, and filtered. To the filtrate is added sufficient pure acetone to bring the concentration to 92% and, after standing preferably overnight, the precipitate formed is collected, dried and dissolved in distilled water or saline, and then injected into the test animal. Minor modifications are recommended by different authors. Sometimes the whole blood is treated (22) and sometimes only the serum (16). Recently Reiss, preferring not to separate the serum, has mixed the whole blood directly with acid acetone, or has dry-frozen it without centrifugation (22). Although there is an absence of positive proof, the importance of rapid acid acetone precipitation has been stressed, to avoid the enzymatic destruction of the hormone. A second extraction of the protein precipitate after resuspension in water and retreatment with acetone has been advocated with apparent success. The last procedure is claimed to have given 100% recovery when a known quantity of ACTH was added to serum after separation of the blood cells (3).

It is hardly necessary to point out that these procedures lack specificity. To obviate the use of what they called empirical methods of concentration and purification, certain experimenters (17) abandoned the pretreatment of blood, and tried to inject fresh untreated serum into the test animal - a technique which is of limited use because of the toxicity of the serum itself when more than a small quantity is injected, especially by the intravenous route. Sydnor (18) in a recent publication, has criticized the acid acetone extraction on the ground of the toxicity of the blood concentrates, and in his early work preferred to lyophilize the serum subsequently to be tested in hypophysectomized rats (6).

Oxycellulose adsorption

Since the publication of Astwood's new method of ACTH preparation (23,31), the problem of extraction of the hormonal activity from blood was studied on what was hoped to be a more rational basis. Astwood's technique, which leads to a high degree of purification of corticotrophin, involves the extraction of acetone-dried pituitary powder with glacial acetic acid at 70°C. The "crude" corticotrophin is then obtained by acetone precipitation. When this precipitate is dried, it is dissolved in a weak solution of acetic acid and allowed to be adsorbed on oxycellulose powder by stirring during 24 hours. The subsequent elution of the oxycellulose with dilute hydrochloric acid yields the active principle in a form representing a great increase in purity and potency per unit of weight.

Without any modification this method has been applied to animal blood and, in a few instances, to human blood by Sydnor and Sayers (18). In their assay the eluate of oxycellulose was directly injected into the test animal and was found to be non-toxic. According to them, the oxycellulose technique appears to be specific for ACTH as demonstrated by the fact that when the blood of hypophysectomized rats is so treated, the eluate induces no depletion of adrenal ascorbic acid in the Sayers test. It was shown that 82 ±12% of the hormone added to the hypophysectomized rat's blood could be recovered.

The above method, in spite of its "specificity" by comparison with the acid acetone extraction, presents some disadvantages. First, as used by Sayers the preparation of the blood extract requires as long as four days and, in the hands of a single experimenter, does not permit the preparation of more than one or two extractions at the same time. Moreover, the blood sample has to be rather large in order to avoid the injection of too great a volume of eluate into the test animal. It would be impractical, for instance, to have to inject 5 cc of the 0.1N HCl eluate intravenously into an hypophysectomized rat, which is the quantity probably necessary when dealing with the blood of normal subjects. One way of avoiding this difficulty would be by elimination of the hydrochloric acid of the eluate on an anion exchange resin, followed by lyophilization. It must be concluded, therefore, that the method requires considerable simplification if it is to prove of practical use in the investigation of ACTH activity, particularly in human blood.

4. ACTH LEVEL IN BLOOD OF MAN AND EXPERIMENTAL ANIMALS

Jores reported in 1935 (7) the presence of a corticotrophic factor in the blood of two patients suffering from Cushing's Disease, and he was perhaps the first to consider that the pituitary basophilic adenoma might produce an increased output of the hormone in the circulation. His method has already been described in the preceding pages..

Bartelheimer (1942) using the same test (adrenal weight increase in mice), and studying different types of diabetes, reported an increase of corticotrophin in the blood of patients suffering from "diabetes due to extra-pancreatic causes" (8). Although it is not the purpose of this study to discuss the pathological classifications of this author, it must be mentioned that although the ACTH content of the blood of pancreatic diabetic patients did not differ from the blood of the control subjects, the patients classified as cushingoid type and acromegalic type of diabetes presented an abnormal elevation of the corticotrophic factor in the blood.

Bartelheimer (24) also studied the hyperglycemia of insulin shock and found an inverse relationship between the glucose level and the corticotrophic activity of the blood. It will be remembered that these two investigators stressed the importance of calculating adrenal weight on the basis of 100 gm. body weight per mouse. Blood from normal subjects gave a factor of 20, but in the patients suffering from Cushing's disease, "extra-pancreatic diabetes" or induced insulin

hypoglycemia this was increased to 30.

Paschkio (1940), using the method described by Jores, reported ACTH activity in the serum of a case of Cushing's disease (25). Two months following the initiation of oestrogen therapy in the hope of influencing the course of the disease, the value of blood corticotrophin was only slightly elevated. In another article (26), the same investigator stated that in cases of hypertension and adrenal tumour the results were negative.

Golla and Reiss (1942) showed that the weight of the adrenal gland in hypophysectomized rats and in intact nine-day old chicks was increased by injections of pregnant mare's serum (12). In the rat the right adrenal was removed before the injection of an acetone-dried pregnant mare's serum preparation and its weight was compared with the weight of the left adrenal removed seven days after the first injection. The animal received a 35-37 milligram equivalent of dried serum twice daily during this period. However, it was remarked that the preparation, although it increased the adrenal weight, did not affect the cortical sudanophobe zone, as did a pituitary corticotrophin studied in a parallel experiment. This adrenal weight factor was claimed to be stable after boiling. The sera of some other animals (rabbits, rats etc.) have occasionally shown a similar activity in the hands of the same authors (12).

Using the Sayers assay procedure, Cooke (1948), working apparently with an acetone precipitate of blood from normal subjects,

found that the preparation was able to cause a decrease of 50-100 mgm of ascorbic acid per 100 gm of fresh adrenal tissue. Approximately the same results were obtained by this experimenter when an extract equivalent to 250 ml. of urine obtained from normal individuals was injected into hypophysectomized rats. The details of this experiment are unfortunately not given (20).

Taylor et al (1949), using blood subjected to no extraction procedure, and injecting 2-3 ml. of serum per rat, obtained the following results by the Sayers assay. They were unable conclusively to demonstrate the presence of ACTH in the blood of normal subjects, or in patients suffering from adrenal cortical hyperfunction. A weak activity was shown in certain cases of untreated Addison's disease. Interestingly enough, the two highest values (expressed in terms of ascorbic acid depletion) were given by two patients from whom the blood had been removed via the external jugular vein. It will be noted that the values of ascorbic acid depletion obtained even in the cases of Addison's disease were very low, except in the last two cases just mentioned (17).

Sayers et al (1949), after injecting a lyophilized preparation of serum corresponding to 2 ml. of plasma per test animal, did not find ACTH in detectable amounts in the blood of normal subjects, and concluded that the blood of normal subjects contained less than 10 Υ of the hormone per 100 cc of plasma. The test was equally negative when the urine of normal subjects was studied under the same conditions (6).

The most recent attempts to demonstrate ACTH activity in the blood have given, as we shall see, strikingly different results.

Working with acid acetone extracts of serum assayed by the adrenal ascorbic acid depletion method in hypophysectomized rats, Bornstein et al (1950) found in a series of 12 normal subjects a mean ACTH-like activity equivalent to 197 Υ of the hormone per 100 ml. of plasma. By comparison with this value, increased activities were found in Cushing's Syndrome (mean, 451 Υ); in the days following a surgical operation (mean, 337 Υ); and in uncontrolled heart failure (412 Υ). Normal values were obtained in diabetes mellitus without ketosis, and in controlled congestive heart failure. An extract corresponding roughly to 1.2 cc of serum was injected into each rat, from 4 to 6 animals being used per test. With this quantity of serum, a fall of ascorbic acid of 88-100 mgm (expressed per 100 gm of adrenal tissue) was obtained with the serum of normal subjects (16). This result would tend to show that the hormone is present at an amazingly high concentration in human subjects, reaching approximately the equivalent of 2 Υ of ACTH in little more than 1 ml. of serum. If this should actually be so, the determination of ACTH activity in the blood would surely be quite a simple matter, hardly necessitating the extraction of the serum since the test animals can support easily 1 to 3 cc of serum injected intravenously, as Taylor showed.

These results were confirmed by Parrott (19), who found the highest ACTH activity in the blood of Cushing's disease, in a diabetic

woman during the 31st week of pregnancy, and in a case of "adipose gynism".

Using the adrenal weight maintenance test, Bornstein et al (27) were able to demonstrate ACTH-like activity in plasma extracts. The twice daily injection of the equivalent of 9 ml. of plasma (extracted by the acid acetone method) increased the adrenal weight 20%, in comparison with the control, after 14 days.

The findings of the preceding investigations using acid acetone extraction were not reproducible in the hands of Sydnor and Sayers (18), nor were quantitative yields of ACTH recovered from the blood.

Genzell et al (1951), investigating the increased formation and secretion of ACTH in adrenalectomized rats, have produced, in one of the most stimulating studies of this question, some very pertinent facts concerning the presence of ACTH activity in the blood (3).

The authors showed that it was impossible to find any ACTH activity in the plasma of normal rats, even when amounts of the extract as large as 64 cc of serum were injected into each test animal, but that, in a very short time after adrenalectomy, the ACTH appeared in the blood in a measurable amount (0.122 Υ per cc of serum). They further showed that the amount of ACTH increased progressively in the blood during the first week after the operation and remained at a constant level (0.28 Υ per cc) during five weeks of observation. In these studies a double extraction of the blood was performed with acid acetone; the

Sayers bioassay was used; the extract equivalent of serum injected per animal was never less than 5 cc. When ACTH was added to the serum sample, a recovery of almost 100% was obtained.

Sydnor and Sayers (1952) in a preliminary experiment (18-28) using the oxycellulose adsorption technique of Astwood, found an activity of ACTH corresponding to 2-3 γ of ACTH per 100 ml. of total blood in normal animals, and 10-12 γ per 100 ml. one week after adrenalectomy. If the latter value is to be multiplied by two to express the ACTH activity per 100 ml. of serum, then the values found here agree well with the results obtained by Genzell et al. The equivalent of 46 and 12 ml. of total blood from normal and adrenalectomized animals respectively must be injected per test animal to obtain a significant ascorbic acid depletion.

In a few instances human subjects were studied and the indication is that the blood of patients with Addison's disease has a higher than normal concentration of ACTH. (Blood from normal adult males contained less than 1 γ per 100 ml. of blood, whereas blood from male patients with Addison's disease contained 2-4 γ per 100 ml.) Two normal and three Addisonian subjects were studied (18).

The method of assay, the preparation and quantity of blood or serum extract injected per test animal, and the results of the different authors reviewed are presented in Table ~~16~~ 9.

5. CONCLUSIONS

Although it is sometimes difficult to compare the data obtained from different laboratories (see Table I), a number of conclusions may be drawn from the review of the literature presented concerning the problem of assaying ACTH in blood.

i) Results suggest the necessity of using hypophysectomized animals for the assay of ACTH. The more sensitive the bioassay, the more evident is this fact, especially when the animal is submitted to stressing procedures (such as anaesthesia and unilateral adrenalectomy, as in the Sayers test) prior to the intravenous injection of the test material. Even if manipulation of the animal were minimal and limited to the injection of the test substance only, the unspecific effect of the latter, related or not to its possible toxicity, may elicit an uncontrolled pituitary secretion of corticotrophin.

There may be exceptions, however, to this prerequisite of hypophysectomy. Recent studies have indicated that the adrenals of the young chick or the infant rat do not respond to stress by a decrease in ascofbic acid, suggesting that tests on very young animals may not be influenced by participation of the intact hypophysis (29,30).

ii) The extraction of serum by the acid acetone method has proven, in spite of being a rather crude procedure, to give very reliable results in the hands of certain investigators (3) and can be fairly well compared with the preliminary data obtained by the oxycellulose technique (18),

at least as far as the ACTH activity in the blood of the adrenalectomized rat is concerned.

iii) The equivalent of blood to be injected in the test animal varies considerably depending on the different authors, and seems worthwhile considering. A brief look at Table I will show that the amount of serum which had to be injected per animal is in most cases quite large. In general, no positive results were obtained with the equivalent of 2-3 cc except in Addison's disease, reported by Taylor et al (17). In the light of these data, the results obtained by Bornstein with 1.2 cc of serum (16) are quite surprising. They were confirmed by Parrott (19), although she does not state the quantity of serum extract injected into each test animal. Both these authors suggest that the discrepancy between their results and those of previous workers lies in the very prompt enzymatic destruction of ACTH in the blood.

In support of their view it must be admitted that others using the Sayers test do not mention the time lapse between obtaining of the blood and its extraction.

Time-relationship studies performed by Pincus (1) on "in vitro" blood (see the beginning of this chapter) suggest that considerable destruction of ACTH activity might occur in the animal experiments if the blood were not extracted until after all the collections had been completed (100 cc to 1000 cc being collected from a large group of animals) (3,18). When centrifugation is performed in order to obtain serum the time lag is even longer. On the other hand, when ACTH is added

to the blood and immediately extracted, a good recovery is obtained. It has also been observed (19) that the extract of blood, when dried, retains its maximum activity for only about 24 hours. According to Parrott, this instability is one of the main difficulties of the technique. On the other hand, the authors who found respectively 197 Y and 80 Y of ACTH in the blood of normal individuals have not published the results of recovery experiments.

Also, it will be noted that an increase in weight of only 20% was obtained in the adrenal weight maintenance test by Bornstein over a period of 14 days, during which time the test animal received an amount equivalent to 18 cc of plasma per day (27). This would not seem to be in harmony with the results obtained in the ascorbic acid depletion test. The latter assay would indicate that in the adrenal weight test each animal received an injection equivalent to 2 Y of ACTH per cc of serum, or a total of approximately 35 Y ACTH per day. This, grossly, is equivalent to 0.5 mg of the standard preparation LAIA over the period of 14 days. With this quantity of ACTH, an increase in weight of more than 20% would be expected. However, this argument cannot be carried too far, since there is no direct parallel between adrenal weight increase and ascorbic acid depletion.

So far there is no answer to the problem just discussed. Nevertheless, the following recommendations may be made:-

a) the extraction of blood or serum must be carried out in the shortest time possible after collection (22).

b) adequate amounts of blood or serum extract must be injected into each test animal (3).

c) the extract should not be allowed to stand in the dry state more than 12-24 hours after preparation (27).

The problem of ACTH assay is not a simple one and the methods so far developed take considerable time to carry out and often require a great amount of blood to show a positive reaction. Therefore they appear, in the mind of the present writer, to be lacking the qualities necessary for a practical application.

PART TWO : RELATIONSHIP OF ACTH AND THE MELANOPHORE EXPANDING HORMONE

In 1952 Sulman brought forth evidence to show that ACTH and the pituitary melanophore expanding hormone - also called intermedin - were intimately associated, if not identical, and that the assay of intermedin could be used as an index of ACTH activity. If this theory is correct then the difficulties associated with the investigation of ACTH activity in mammaliam blood would be nearer solution, as the assay of the melanophore expanding hormone is a relatively simple procedure.

In the following pages the facts which have been presented in support of the identity of ACTH and intermedin will first be summarized, then the more cogent arguments against this theory will be presented.

1. IDENTITY OF ACTH AND MELANOPHORE EXPANDING HORMONE

Experimental data of G. Sulman

Injecting into the dorsal lymph-sac of the normal frog, Hyla arborea, different preparations of anterior and posterior pituitary hormones, Sulman (36,37) was the first to report that adrenocorticotrophic hormone was able to induce the change of colour of the total dorsal skin of the animal previously adapted to light. The change was observed with doses as small as 1.0 γ to 0.01 γ. "The quantitative differences between various commercial preparations corresponded roughly to their declared potency in international units" (37). The ACTH preparations were the only ones among the pituitary hormones studied to produce this effect with such low dosages. The minimal effective dose recorded for

the other hypophyseal principles tested ranged from 10 γ for certain preparations of pitressin to 100 γ for TSH. Particularly noteworthy was the fact that 30 γ of intermedin, prepared from hog anterior pituitary powder according to the method of Zondek and Krohn (39), were necessary to give the same reaction, although a particular fraction, representing the pooled precipitates discarded by these investigators in their original extraction, was active at the dose level of 1.5 γ (38).

It was subsequently shown by Sulman (40) that the melanophore expanding effect was obtainable in adrenalectomized, intact and hypophysectomized frogs without significant difference between the three groups as to the time of appearance of the reaction, its duration and intensity of colour at similar dose levels.

The melanophore expanding property, which can be obtained by this technic with intermedin (41), was also tested in vitro with a number of pituitary hormones, including four different preparations of ACTH (42). The results obtained on isolated frog skin maintained in a suitable medium in the presence of the corticotrophin grossly confirmed the results obtained in vivo. The dose, however, of any hormone necessary to give the reaction was much higher - 1 mg for the ACTH preparations, and up to 4 mg for the intermedin as prepared by the Zondek technique already mentioned.

From this series of experiments Sulman concluded that the melanophorotrophic effect obtained in vivo with such minute doses of adrenocorticotrophic hormone is strong support for the assumption of its

identity with intermedin. The obtaining of a positive response with other anterior pituitary principles, in much larger doses, was considered to be explicable on the basis of a high degree of contamination with ACTH. Sulman discussed the possibility of contamination of ACTH with intermedin but thought it to be improbable, since the effective dose for ACTH (0.01 Y) was of the same magnitude as the dose given for intermedin in the literature.

Clinical data of G. Sulman

In the meantime Sulman proposed a simple test, the details of which will be given in the experimental part of this thesis, in which the melanophore expanding property of an extract of blood could be used as an indication of its ACTH content (43). Although the detailed results of his investigation of the chromatophorotrophic factor in the blood of some hundred patients have not as yet been published by the author, it is possible to give here some of the conclusions which he reached (44).

The chromatophorotrophic factor was abundant in the blood of some patients suffering from Addison's disease, Cushing's disease and pregnancy. It was also high for short periods of time in patients with acute conditions known to produce an increase of ACTH secretion, such as mental stress, heart disease and surgical operations.

Sulman puts forward the hypothesis that the chromatophorotrophic factor is one component of ACTH, which would thus explain its fluctuation

in the physiological and pathological conditions investigated, where an increase of ACTH in the blood is to be expected.

Johnsson and Hogberg's evidence

Findings similar to Sulman's were reported at the same time by another group of workers.

S. Johnsson and B. Hogberg in Sweden (45,46), using the frog Rana temporaria adapted to light, found that as small a quantity as 1/20,000 international unit of ACTH (0.01 ϒ of Armour LAIA Standard) gave a clearly visible expansion of the melanophores. Control experiments on hypophysectomized frogs gave the same results. The melanophore expanding factor, related or identical with ACTH, was claimed to be present in the blood of patients with Addison's disease and in patients the day after a surgical operation. It was not present in the serum of individuals under normal conditions.

Moreover, the preliminary report of these authors provides some interesting data for comparison of ACTH and the pigment hormone so far as certain of their chemical and physiological properties are concerned.

i) ACTH and intermedin, isolated from the pituitary glands of cattle and pigs, were both inactivated by nitrite, formaldehyde, trypsin and homogenates of liver, kidney and adrenal gland (46).

ii) Different ACTH peptides, prepared by the method of Li et al, were shown to have the same activity by the frog and the

Sayers assays.

iii) The ACTH peptide and intermedin could be eluted in the same way with glacial acetic acid, after adsorption on a carbon column (47).

iv) An hormonal preparation was isolated from the pituitary by the technique used for the isolation of intermedin, and was administered to a patient in daily doses corresponding to 20 mg of ACTH for three days. The eosinophils fell 66%, the urinary potassium rose 100% and the urinary corticoids increased from a previous level of 0.4 mg/24 hours to 1.1 mg/24 hours. The test was subsequently repeated with corresponding doses of ACTH, the corticoids rising from 0.6 to 0.9 mg (46).

Both Thing (48) and Sulman (49) have independently proposed the degree of melanophore expansion in the frog as a test for the standardisation of ACTH. The former of these authors has extensively studied the conditions in which the test may be used and the different factors which can influence it.

2. CHARACTERISTIC DIFFERENTIATION OF ACTH AND INTERMEDIN

The claim that intermedin is identical with ACTH must be considered in the light of what is known of the chemical properties and the physiological actions of the two hormones. Within a short time, the conception of Sulman and of Johnsson and Hogberg was criticized. In

a sense, the case of the ACTH-intermedin relationship became a scientific "cause celebre", during which the difficulties relating to the extraction of so-called "pure" pituitary hormones became once more evident. Let us examine some of these criticisms.

i) Common chemical and bio-physical properties of ACTH and intermedin

A superficial similarity in the chemical behaviour of ACTH and intermedin can easily be drawn. Some examples have already been given above in the presentation of Johnsson and Hogberg's work.

Waring and Landgrebe (50) have shown that the melanophore hormone is highly soluble in water, fairly heat stable, and destroyed by tryptic but not by peptic digestion, and think that the molecular weight is probably not much greater than 2000. All of these properties can also be attributed to ACTH. But, as Li has pointed out (51), none of them represent sufficiently specific criteria to characterize any polypeptide.

ii) Intermedin as a factor of the ACTH molecule

Reinhardt and Li (51) designed an experiment in which the rat adrenal ascorbic acid depleting activity of three ACTH preparations was correlated directly with their melanophore expanding activity tested on hypophysectomized frogs. Their results showed no direct correlation between the ACTH and intermedin activities of the three preparations. As a matter of fact, there was an inverse relationship; the most potent ACTH containing 200 international units/mg, had to be injected into the frog

at a dose of 5 γ to produce the minimal melanophore expansion, whereas the least potent ACTH, containing 30 IU/mg gave the same reaction in the frog at the dose level of 0.1 γ.

This experiment by itself cannot rule out the possibility that intermedin might be a factor of low molecular weight of the ACTH molecule (44). It should not be forgotten, moreover, that the ascorbic acid depleting test of Sayers is concerned with a property of the hormone which is poorly understood and is not related, except by hypothesis, with what is known of the major function of the hormone, namely the stimulation of the secretion of adrenocortical hormones.

Li himself has pointed out (52) that there was no direct correlation between the potency of certain ACTH preparations when they were compared by the ascorbic acid depletion test and the adrenal weight maintenance test. In this experiment also an inverse relationship existed when the results of the two assays were examined. Furthermore, the preparation which exhibited the highest ascorbic acid depletion potency was the least active in causing reduction in weight of the thymus gland and cervical lymph nodes. That is to say, there was an apparent lack of correlation between ascorbic acid depletion activity and stimulation of the secretion of the adrenocortical hormones, since the latter are known to cause a reduction in weight of the thymus and lymph nodes.

At the same time, Moore and Young (53) found that by using an ion exchange column, ACTH could be separated into a major fraction, almost devoid of ascorbic acid depletion activity, and a minor fraction,

which was a basic substance (probably a peptide) highly potent in ascorbic acid depletion activity. As a tentative explanation of this fact, Young (54) proposed the existence of two factors in ACTH preparations, the one being responsible for adrenal weight (Adrenal Weight Factor, or A.W.F.) and the other for ascorbic acid depletion (Ascorbic Acid Factor, or A.A.F.).

It was against such a background that Sulman felt free to postulate the existence of a third factor of ACTH, this third factor being, as has been already mentioned, intermedin itself. The criticism which can be raised against this conception is, as will be seen later on, that it is difficult to think of a factor, whatever it may be, which would be intimately associated to the ACTH molecule, but which would not need the presence of the adrenal cortex in order to act.

iii) Relative purity of ACTH and intermedin preparations

The most serious objection to the conception of an identity between intermedin and ACTH comes certainly from the biochemical studies of the hormones. Among these objections some are of minor interest, whose validity is still open to question. But others are of primary importance, and positively indicate that the two hormones can be differentiated.

a) One curious and unexplained property of the melanophore hormone is that by heating it with 0.1N NaOH for a short time (5 minutes in a boiling water bath) its actual biological potency is increased (50). On the other hand, if such treatment is applied to an ACTH preparation it

loses in a considerable proportion its ascorbic acid depletion potency (51). There was also noted, in an experiment by Hugerford and Li (55) an important discrepancy between the ascorbic acid depletion potency and eosinopenic activity of the same preparation. After heating an ACTH preparation with 0.1N NaOH for 10 minutes in a boiling water bath, these authors observed an almost complete inactivation of the hormone as judged by the adrenal repair test and a 90-95% inactivation of the ascorbic acid depletion potency. However, the eosinopenic activity of the hormone measured on hypophysectomized rats was completely preserved. They concluded that there was present in certain ACTH preparations an eosinopenic component which is stable after treatment with alkali and heat, and which was not identical with the ascorbic acid depleting factor.

b) Landgrebe (56) showed that intermedin was adsorbed on carbon and could be eluted from it by glacial acetic acid. However, different authors disagree as to the efficacy of obtaining intermedin in this way free of ACTH. Morris (57) states that intermedin can be recovered free of ACTH by such an elution.

Although we do not know of any ACTH test applied to the intermedin fraction obtained by Landgrebe, it is probable that the hormone recovered by his method has a low corticotrophin contamination, since it is treated by sodium hydroxide and brought to the boiling point for ten minutes before adsorption on charcoal. This treatment is supposed, as was seen above, to destroy the ACTH, or at least its ascorbic acid depletion potency, to a large extent. Johnsson (47) reports that in his

experience the fact that ACTH cannot be recovered by glacial acetic acid elution from charcoal holds true only for ACTH protein and not for ACTH peptide, which can be eluted in the same way as intermedin. This author, however, does not mention if the hormone in his experiment was previously submitted to NaOH treatment, which is, of course, of prime importance. Otherwise his observation merely confirms Reinhardt's report (51) that "by use of charcoal columns it is possible to obtain a highly purified ACTH in which the intermedin activity is also enhanced". Indeed, the last author concludes that as an isolated example the activity of such preparations would lend support to the claims of a connection between the two hormones.

It is perhaps of some interest to recall the potency of the Landgrebe-Waring intermedin, which is of the order of 85,000 Landgrebe-Waring units per milligram of dry powder (56). The contamination of the hormone was checked only for its pressor and oxytocin content, which are rather low. The potency of this preparation will sustain comparison with a recent intermedin of the Armour Laboratories (about the method of extraction of which we have no information), which has a potency of about 100,000 frog units per milligram of dry weight, and contains 1 ACTH LAIA unit equivalent per milligram of active substance (58).

c) Partial separation of the two activities (adrenal ascorbic acid depletion factor, and melanophore-stimulating factor) was obtained by Li (51), who employed zone electrophoresis on paper. By use of this technique, the bulk of the ascorbic acid depleting activity was found at

the origin and in the neighbourhood of the cathode area. When solutions were obtained by extracting various sections of such papers, the region of greatest melanophore expansion activity was found to be near the anode, where not more than a small percentage of the total ACTH activity could be demonstrated.

Definite separation was also reported by the same author with fractions isolated from cellulose columns by a discontinuous pH gradient (51). Almost all the ACTH activity resided in the second of three peaks obtained in this method. Intermedin activity was almost completely concentrated in the first peak.

Using the method of adsorption on oxycellulose for the preparation of concentrated corticotrophin, Astwood (59,60) has demonstrated that from the weak acetic acid solution of the "crude corticotrophin" (fraction obtained after precipitation by ether from the glacial acetic acid extraction of acetone dry pituitary powder) three pituitary hormones were adsorbed on oxycellulose and could subsequently be eluted from it: ACTH, intermedin and Adipokinin (or fat-mobilizing factor). However, by counter-current distribution or solvent partition between an organic solvent and water, the intermedin, which is more hydrophilic than ACTH, could be obtained largely free of the latter.

Although neither of these two investigators has as yet succeeded in obtaining an ACTH preparation which is entirely free of melanophore expanding activity, their data indicate that such a separation might eventually be achieved.

It will be pointed out, however, that the results obtained by these investigators, offer a new proof of the great difficulties encountered in the attempt to isolate one pituitary hormone free of another. By the procedures used until now it is quite surprising to realize how great a contamination with melanophore expanding hormone there exists in ACTH preparations. This is all the more disturbing when one considers the great care that was taken to investigate the physiological action of pituitary hormones such as TSH, GH and ACTH, carefully isolated the one from the others. During more than three years now, all the studies reported on ACTH activity have been performed with preparations highly contaminated with an hormonal principle of practically unknown physiological significance.

iv) <u>ACTH activity not mediated by the adrenal cortex</u>

The conception of the identity of intermedin with pituitary corticotrophin actually postulates a mechanism of endocrine gland relationship which is in direct opposition with what is thought to occur in mammals. Certainly pituitary hormones which act directly on one or more peripheral organs without the intermediary of a peripheral endocrine gland are known. This is probably the case of the posterior pituitary hormones. On the other hand, it is difficult to think of a pituitary hormone which in the normal organism produces an effect through stimulation of a peripheral gland and at the same time a direct metabolic effect on peripheral tissues (although such a mechanism seems to be possible, when one considers the recent literature dealing with

growth hormone)(63).

The theory proposed by Sulman, and Johnsson and Hogberg in their attempt to explain the pigmentation encountered in conditions where an increased ACTH output is expected to take place, encounters certain inconsistencies when the two conditions of Addison's disease and pregnancy are considered. If the pigmentation of adrenal insufficiency is to find its explanation in an increased activity of ACTH acting, let us say, at the level of the skin, in the absence of a normal adrenocortical function, then one is immediately obliged to admit that the "pigmentogenic" effect of the hormone is an inherent one which is normally inhibited by the adrenal steroids. It seems, in fact, fairly well established, as will be seen later on, that if the adrenal glands play a role in the physiology of certain types of pigmentation, it is indeed in the sense of an inhibition. Since the extensive use of cortisone acetate in Addison's disease and in the cases of adrenalectomy performed in the hope of arresting the evolution of breast cancer or malignant hypertension, there have been reports of the total prevention or disappearance of pigmentation, which had remained unaffected when desoxycorticosterone alone was used. Pregnancy, another condition in which pigmentation occurs, may be opposed to adrenal insufficiency in the sense that there is a characteristic increase in output of the cortical type of steroids from adrenal or placental origin (61). It is difficult in the light of our present conception of hypophyseal-peripheral hormone balance, to consider that there is a sufficient increase of ACTH free from the "pigment-inhibiting" action

of the circulating cortical hormones, to induce the pigmentation that accompanies pregnancy.

Conclusions

A number of conclusions can be drawn from a consideration of the preceding data.

a) The majority of ACTH preparations obtained to date from the anterior pituitary gland of animals contain a fraction which can be measured, even in small amounts, by the classical test used for determination of the melanophore hormone (37,42).

b) The activity of the fraction measured by the change of colour (or degree of melanophore cell expansion) in cold-blooded animals does not depend on the presence of the adrenal cortex (40).

c) The active principle can be partially separated from the ACTH activity but has not yet been obtained in pure form. It is apparent that the potency of the ACTH fraction measured by the ascorbic acid depletion test in hypophysectomized rats does not run parallel to its content of "pigmentogenic" factor (51). The ACTH potency can apparently be concentrated to a great extent (80, and recently 300 LAIA units per milligram of dry powder have been recorded) (62) without a corresponding concentration of melanophore hormone, in spite of the fact that such preparations still contain a very significant amount of the latter. Conversely, the melanophore hormone can be concentrated (up to 100,000 frog units per milligram) without becoming richer in adrenocorticotrophin (56,58). Here also even the most potent intermedin preparation contains

up to one LAIA unit of ACTH activity per milligram. In other words, on the basis of dry powder weight, it is possible to increase the two activities independently of each other but impossible so far to separate them completely.

d) These findings strongly suggest that ACTH and the melanophore expanding hormone, intermedin, are two separate factors and permit of the speculation that some of the metabolic and hormonal actions attributed to ACTH in the course of investigations with the highly contaminated preparations available might, in fact, be due to the melanophore hormone.

e) If the hypothesis is no longer tenable that the two hormones are identical, the observations of Sulman, and Johnsson and Hogberg remain interesting in that intermedin appears in the blood in conditions where the adrenal cortex is being stimulated and also in other conditions where there is an adrenal insufficiency (44,46).

If their observations are confirmed, the following problems should be examined. Does a balance exist between the activity of the adrenal cortex and the secretion of intermedin by the pituitary? If so, how can the increase of intermedin secretion by the pituitary occur in states of both hypo- and hyperactivity of the adrenal cortex? What is the actual relationship between the adrenal cortex and the pigment hormone?

Such questions are difficult to answer because of our meagre knowledge concerning the mechanism of action of intermedin. Because it has generally been believed (50) that the hormone plays a part in the synthesis of the melanin pigment, its possible relationship with the

adrenal cortex was thought worthwhile to question in connection with the physiology of pigmentation.

PART THREE : ACTH, ADRENAL-CORTICAL HORMONE AND INTERMEDIN IN PIGMENTATION

The subject of pigmentation represents in itself a very broad chapter of physiology which, if properly reviewed, would necessitate an excursion through the fields of physics, biochemistry, nutrition and metabolism. Extensive reviews of the subject are to be found in the publications of Bloch (64), Raper (65), De Just (69), Burgess (70) and in the recent article of Lerner (67). After a brief exposition of our actual knowledge of melanin formation, an attempt will be made to correlate a few studies in which the melanophore hormone and the cortical hormones have been shown to influence this process. The pigmentation in human subjects will be discussed only in those cases where there is a possible association with the adrenal cortex or intermedin. Because the metabolism of the tripeptide, glutathione, has been demonstrated by several investigators to be influenced by ACTH, and at the same time is considered to play a role in the formation of melanin, an attempt will be made to examine their inter-relationship in the question of pigmentation.

1. BASIC FACTS RELATED TO THE BIOCHEMISTRY OF MELANIN FORMATION

Considering our present conception of melanin synthesis, the following information obtained over the past 50 years by different investigators appears to be of particular significance.

In 1927 Bloch (64) made the first important step toward an understanding of this question when he found melanin granules appearing in the

cytoplasm of cells located in the basal layer of the epidermis following incubation of human skin sections in a 1/1000 solution of the naturally occurring amino acid, 3,4-dihydroxyphenyl-l-alanine, or DOPA. These cells, called melanoblasts by Bloch, were subsequently proven to originate from the neural crest region (107,108).

Further investigation by the same author provided evidence that the oxidation of DOPA to melanin was due to the presence in the tissues of an enzyme which was called DOPA-oxidase. Although this work was amply confirmed by other workers, the conception of DOPA as a starting point in the physiological synthesis of melanin has not been accepted without reserve, chiefly because it has not been possible to demonstrate its presence in mammalian tissues.

Later, tyrosine, which is known to occur in all tissues including the skin, was shown by Raper (65) to form melanin in the presence of the enzyme tyrosinase, obtained from mealworms.

In spite of these findings, the problem of melanin synthesis in the mammalian skin remained unsolved for many years because pigment formation was not obtained as it had been under the same conditions with DOPA when tyrosine was used as a substrate in a preparation of skin section. In addition, tyrosinase activity could not be demonstrated in normal pigmented mammalian tissue. Within the last ten years, following the investigations of Hogeboom et al and Lerner et al (66,67) from mouse, horse and human nelanomas were all shown to contain both tyrosinase and DOPA-oxidase activity. More recently, the necessity for the participation

of more than one enzyme was questioned when Lerner and Fitzpatrick (67) showed that a tyrosinase prepared from mouse melanoma was able to catalyse the oxidation of both tyrosine and DOPA to melanin in the Warburg apparatus.

Although many points regarding the biochemical and enzymatic mechanism of melanin formation remain to be proven, the following facts appear to be established (68) :-

The synthesis of melanin pigment taking place in the epidermis depends on the available concentration of three substances - i) the enzyme tyrosinase (or an enzymatic system composed of tyrosine and DOPA-oxidase), the activity of which seems to be intimately related to the presence of copper; ii) a suitable substrate, usually tyrosine or DOPA; and iii) molecular oxygen. In the presence of tyrosinase and molecular oxygen, tyrosine is oxidized to DOPA. The reaction when studied in vitro is usually slow at the onset and then becomes very fast. This induction period seems to correspond to the time necessary for a certain concentration of DOPA to be produced. When this concentration is reached, DOPA itself acts as a catalyst and hastens the oxidation of tyrosine. DOPA is oxidized enzymatically by a reversible reaction to DOPA-quinone. The subsequent reactions involving the formation of dihydroxydihydroindole carboxylic acid, its corresponding quinone (hollochrome), 5,6-dihydroxyindole, and finally the indole, 5,6-quinone, which then polymerizes to melanin, proceed rapidly in the absence of the enzymes. It is of interest to note here that the indispensable role of the enzyme

system in this series of reactions is only apparent at the very beginning of the chain, although it has also been shown to have an influence on increasing the rate of the reactions following the formation of DOPA.

Many in vivo or in vitro inhibitors of melanin formation have been described in the literature. Only glutathione and ascorbic acid will be mentioned here because of the particular interest which they have recently aroused in connection with the hypophyseal-adrenal axis, and as possible physiological inhibitors of pigmentation in the living organism.

In the presence of ascorbic acid, melanin cannot be formed by the action of tyrosinase on tyrosine or DOPA until all the ascorbic acid is oxidized (71). On the other hand, the oxidation of ascorbic acid seems to be inhibited by sodium chloride. Lea (72) found that the in vitro inhibition of melanin formation in the tyrosine-tyrosinase preparation was less important when the concentration of sodium chloride was 0.5% than when it was 0.9%. Similarly, an increased rate of oxidation was recorded with the same system in the Warburg apparatus when the poorer concentration of NaCl was studied. However, it must be noted that the difference in oxidation is not very great (14%). The author concludes that there may be a possible relationship between sodium chloride lost, ascorbic acid and the pigmentation of Addison's disease. This is difficult to correlate with the observation that desoxycorticosterone alone, which is thought to have its chief action on sodium metabolism, has

very often no effect on the Addisonian pigmentation.

Large doses of ascorbic acid have been claimed to decrease the pigmentation in patients suffering from Addison's disease (73,74), or to reduce the melanin in the skin to a relatively light-coloured substance (72).

Glutathione belongs to a rather large group of organic sulphur-containing compounds (cysteine, thiouracil, thiourea, phenylthiourea etc.) thought to inhibit the reaction of melanin formation by their own reaction with copper ions which, as was previously mentioned, are necessary for tyrosinase activity. However, in spite of the very important role attributed to glutathione as an inhibitor of pigmentation, it is necessary to point out that the action of the tripeptide which has been demonstrated by many in vitro studies has never been shown in vivo.

2. ROLE OF INTERMEDIN IN PIGMENTATION

i) In vitro studies

The relationship between melanin formation and activity of a specific pituitary hormone remains obscure. Some primary studies have been attempted in order to throw some light on this problem.

Fostvedt (75), measuring the oxygen uptake of a tyrosine-tyrosinase preparation in the Warburg apparatus in the presence of various intermedin-containing pituitary extracts, reached the following conclusions:-

a) Beef posterior pituitary extract increased the oxygen uptake 60% as compared with the tyrosine-tyrosinase system alone. This

extract was previously treated with strong NaOH with the view of destroying its oxytocic and pressor activity.

b) No increase of oxygen uptake was obtained as compared with the tyrosine-tyrosinase preparation alone when an extract obtained from whale anterior pituitary lobe was treated with trypsin. Its intermedin content, as judged by the frog test, was destroyed.

c) Extracts of beef muscle, liver, kidney and lung did not appreciably increase the oxygen uptake.

d) The melanophore rich extract free of oxytocic and pressor activity was found to decrease the spontaneous oxidation of DOPA which normally takes place in vitro at pH 7 (67).

The work of Fostvedt was criticized because of the wide range of increase of oxygen uptake (23 to 80%) obtained with different pituitary extracts. The author explains these variations by the difference of activity of the enzymatic preparations. Different tyrosinases extracted from plants and lower animals are known to be less specific in their action on tyrosine and DOPA than mammalian tyrosinase. Although the pituitary preparations used were free of oxytocic and pressor activity, their possible contamination with other pituitary hormones capable of inhibiting the reaction was considered by the author.

Fostvedt's experiment remains an interesting attempt in this field. It has, unfortunately, not been repeated and is at present the only indication of a possible action of melanophore hormone on the chemical reaction which leads to melanin synthesis.

ii) Presence and fate of intermedin in the animal body

The preceding observations must be carefully examined in the light of what is known of the behaviour of intermedin in the animal body.

Levinson (82) detected a pronounced diurnal fluctuation in the melanophore hormone content of rat blood. Jores (81) was able to show the presence of a melanophore expanding principle in the blood of rabbits kept in darkness. Landgrebe (83), also studying rabbits kept under different conditions of illumination, was able to get a melanophore expansion in Xenopus by injecting the equivalent of 3 to 6 cc of blood extract, but felt that he could not ascribe this reaction unequivocally to intermedin. For instance, if the equivalent of 3 cc of blood raised the melanophore index to 2.5, twice the dose had no greater effect, whereas graded doses of melanophore hormone give graded responses. When intermedin ("B" containing pituitary extract of Landgrebe) was injected intravenously at the dose level of 50,000 units approximately, 5,000 units could be recovered from the urine of the animal after $2\frac{1}{2}$ hours. The urine removed from untreated male rabbits and injected directly into Xenopus had no melanophore expanding activity (83).

When 10,000 units of B were injected subcutaneously or intramuscularly into rabbits and samples of blood taken at various intervals of time, only a slight rise of melanophore index was recorded. The author expresses the doubt whether any measurable quantities were detected in the circulation. After intravenous injections the hormone could

easily be detected in the blood within a few minutes. But after five minutes only 30% of the dose could be recovered; after fifteen minutes 15%; and after half-an-hour only 3%. These interesting observations give an idea of the very rapid rate of removal of the hormone from the circulation.

In Landgrebe's opinion the failure to find a significant amount of intermedin in mammals would be explained by the necessity of pre-treating the blood, which is associated with an inevitable loss of the hormone (84). On the other hand, the blood of fish and amphibia can be injected without preparation into the frog and the melanophore hormone more readily appreciated.

The preceding findings are of interest when confronted with the reports of other workers in the human being.

iii) Clinical data

It is quite surprising that intermedin, in spite of its very well known action on the melanophores of fish, amphibia and reptiles, should have been so little studied in connection with the pigmentation occurring in other animal species, including man itself. Only by inference from the fact of its wide distribution in the pituitary gland of many species (rats: 400,000 units per gram of wet weight; sheep: 100,000 units; hog: 600,000 units) (106) including man (114) can we suppose that it plays a part in the phenomenon of pigmentation in a much wider range of living organisms. Some of the available data which tend to indicate the action of the melanophore hormone in the human will now be presented.

One of the first observations that might implicate the pituitary in human melanogenesis concerns an arthritic patient of Sprague (76) who developed a marked pigmentation at the site of the palmar creases, axilae, dorsum of the hands and feet, and in a recent operative scar, while receiving ACTH. "The preparation, however, was found to contain appreciable melanophore hormone on bioassay".

In five patients without adrenal disease receiving ACTH, McCracken and Hall (76) reported an increase in melanin content of the skin as measured by the Hardy reflectance spectrophotometer. No melanin increase was seen following cortisone therapy. Bioassay in hypophysectomized frogs demonstrated a high intermedin content of the ACTH preparations used.

Hall (96), using the same electrophoretic method, showed that cortisone administration did not prevent the pigmentary changes of the skin following total adrenalectomy in man. On the other hand, cortisone reversed towards normal the pigmentary changes observed in adrenal insufficiency. It is stressed that "excess pituitary secretion of intermedin might be responsible for pigmentation in Addison's disease".

When intermedin is considered independently of ACTH, the observations of Sulman (44) and of Johnsson and Hogberg (46), showing the presence of a melanogenic factor in the blood of Addisonians (see the second part of this thesis), must be taken as an indication of the presence of melanophore hormone in the blood of patients suffering from

adrenal insufficiency.

Some other data, which are somehow difficult to correlate with pigmentation, at least give some clue to the presence of intermedin in the human body. These are the findings of Raza and Spurrell (78) who demonstrated the existence of a melanophore-like principle in the urine of pregnant women. The injection of 1 to 2.5 cc of human pregnancy urine into the dorsal lymph sacs of pale frogs produced an expansion of the melanophore cells in 98% of the cases. The urinary melanophore-expanding factor possessed at least one of the chemical characteristics usually considered specific of intermedin - an enhancement of its potency after treatment with alkali. However, it could not be recovered after adsorption on kaolin, and it was not destroyed by trypsin. The interest of this observation seems to be limited by the criteria chosen for a positive response: the melanophore expansion was arbitrarily classified as "strong" if the melanophore index exceeded 2, and as "weak" if the change lay below that figure. In 45 patients studied, 30 strong and 15 weak reactions were obtained. Recently a similar experiment (79) demonstrated the presence of melanophore hormone in the blood and urine of some normal and pathological pregnant patients. Unfortunately it has not been possible to obtain the details of this work.

Other studies by Dietl (109), Ehrhardt (110), Drouet (111) and Konsuloff (112) showing the presence of a melanophore expanding factor in urine have been published. Such data have been criticized separately

by Landgrebe (83) and Stehle. The last author particularly questioned their value on the basis that "Zondek and Krohn (39) could not detect intermedin in normal urine and could not even detect it when added to urine unless the added quantity was very large" (113).

3. ROLE OF ADRENOCORTICAL HORMONES IN PIGMENTATION

i) Animal experiments

What is actually known of the possible role of adreno-cortical hormones in pigmentation comes essentially from two sources: clinical observation and a few animal experiments (usually not designed to study pigmentation as such). Although the idea that the adrenal cortex might play a part in the phenomenon of pigmentation came first from the clinical observation of the particular skin colour developed in adrenal insufficiency, the experimental data will be presented first.

Hamilton (85) explanted into a clot composed of embryonic extract mixed with plasma, pieces of skin removed from the dorsal mid-line of a six-day old chick embryo. When desoxycorticosterone acetate in the concentration of 50-333 γ per cc was added to the medium, no melanophore cells were seen to differentiate. Control cultures showed the differentiation of these cells, the development of which was apparently inhibited by the presence of the DOCA. Moreover, when skin from seven-day old embryos, or older, already containing many black melanophores, was incubated in the presence of the same concentrations of desoxycorticosterone acetate, the melanophore cells present were

observed to contract, round up and die. New cells were not differentiated.

A similar effect was reported by Ralli et al (86). Rats of the Long-Evans strain normally present pigmented bands of the dorsal skin which can be characterized on microscopic examination as an accumulation of melanin in the massed hair bulbs and follicles. When such rats are maintained on a diet deficient in filtrate factors of vitamin B1 varying degrees of graying fur is noted and, after shaving, the skin is uniformly pink, presenting no trace of pigmentation. However, if the animals are adrenalectomized, a marked transitory hyperplasia of the hair bulbs and follicles occurs, accompanied by the increased deposition of melanin to such a degree that the entire surface of the back skin assumes a bluish colour. The DOPA-oxidase reaction which was absent from the skin of unoperated animals on deficient diet was positive in the adrenalectomized animals.

In further studies (87) the author noted that if the adrenalectomized rats were maintained with desoxycorticosterone, the stimulating effect of adrenalectomy on melanin formation was suppressed. The adrenal cortical extracts were active in the same way but less so in the doses used.

These observations furnish, so far as we are aware, the first experimental proof of the in vivo action of the adrenal cortex on melanin metabolism. It is to be regretted that the experiments were not repeated after hypophysectomy and injection of melanophore hormone.

Butcher (88) confirmed these experiments and showed by trans-

plantation of the adrenal glands into the kidney (where cortical tissue persisted, but the medulla died) that the inhibition of melanin deposition was specifically due to the maintenance of the adrenal cortex. He also showed that adrenalectomy resulted in an increase of oxygen consumption in the skin of the rats (89).

The inhibition of melanin deposition in the skin has been demonstrated by Whitaker and Baker (90) in normal rats by local application of cortical hormone on the previously shaved skin. These authors remark that a dark pigment is present in the skin during the time that dark hair is being formed. By rubbing the skin every day with adreno-cortical extract, or cortisone (1 ml. of the former; 100 Y of the latter) they could totally abolish both the hair growth and the appearance of pigmentation in the ear during six weeks of treatment.

ii) Pigmentation of Addison's disease

In spite of these experimental findings the increase in melanin deposition characteristic of adrenal insufficiency remains unexplained. It is found in those parts of the body which are normally subject to greater pigmentation, such as the axillae, areolae, anogenital region and areas usually exposed to mechanical irritation such as the palms of the hands, or to light, such as the face. Increase in temperature (such as might occur in the protected parts of the body) and increase in exposure to ultraviolet light (such as obtains for the uncovered parts) are known to increase melanin formation, but cannot be considered as the more important factors. It is in fact probable that the lack of cortical hormones

affect the appearance of pigmentation in Addison's disease. Since the use of cortisone in the treatment of Addison's disease, very marked pigmentations have been observed to decrease and in some cases completely disappear (109). This was not noticed when desoxycorticosterone alone was used. At that time, pigmentation of the patient was claimed to be the most persistent symptom of the disease. This fact is worth emphasizing because it is in complete contradiction to the in vitro and in vivo experiments, in which desoxycorticosterone is apparently able to interfere with the development of melanoblasts and melanin deposition.

The manner in which cortisone might affect the pigmentation of Addison's disease remains completely obscure. It would not be unreasonable to think that it might have an effect by interfering at some point in the chain of reactions leading to the formation of melanin as another steroid hormone, estrone, has been shown in vitro to release the inhibiting action of glutathione on this reaction (80).

There are no clinical studies which correlate the degree of pigmentation with the state of adrenal insufficiency. It is, however, certain that such pigmentation only develops in cases of chronic insufficiency and has never been noted in the acute crises.

If the presence of intermedin can be confirmed without any possible doubt in the blood of Addisonians, the influence of this hormone will have to be studied. It will then be necessary to postulate some relationship between the adreno-cortical hormones and intermedin, whereby adrenal hormones inhibit the release of intermedin by the pituitary, or counteract

its effect at the level of the tissues. Whatever it may be, it is almost certain, when one considers the multiple physiological factors capable of influencing in one way or another the development of pigmentation, that the process of melanogenesis depends on more than one factor. However, since by ignorance of the total picture one is obliged to attack the problem from some particular and hypothetical angle, it is hoped that the study of individual compounds known to have an action on the biochemical process of pigment formation may shed some light on the subject.

4. THE INHIBITING ACTION OF GLUTATHIONE ON MELANIN SYNTHESIS

i. In vitro studies

One such physiological and widely-distributed organic compound thought to play a possible role in pigmentation is the tripeptide, glutathione. It will be recalled that glutathione inhibits in vitro the formation of melanin pigment by neutralization of the tyrosinase and most probably by binding with the copper ion, which has been shown to be essential for enzymatic activity. This fact would remain without very great importance had not a number of studies indicated that the adrenal glands may play a part in the metabolism of sulphydryl compounds.

Frank, in 1941 (91) demonstrated that small amounts of glutathione (from 100 ϒ to 1 mg. per 20 cc of substrate) definitely inhibited the in vitro enzymatic oxidation of tyrosine in the presence of tyrosinase and atmospheric oxygen. It was suggested that both tyrosinase and tyrosine could be present in a cell without leading to melanin synthesis if a sufficiently strong inhibitor such as glutathione was present.

Inhibition of pigment formation was obtained by Ginsberg (92) using a crude aqueous extract of guinea pig skin, and by Rothman and Krysa (93) with a similar extract of human skin. Twenty mg. of extract, when added to a measured amount of a tyrosine-tyrosinase preparation at pH 7.4, caused a decrease of melanin formation from 178 γ to 40 γ per 10 cc of solution after 48 hours of incubation. When iodoacetamide, a specific poison for -SH groups was added to the epidermal extract, the melanin formation was not inhibited but, indeed, was enhanced to a significant extent. Iodoacetamide alone in a similar concentration did not modify melanin formation in the tyrosine-tyrosinase system. In a subsequent article (94) Flesch and Rothman showed that the inhibitory principle of human skin extract was dialysable, heat-stable and counteracted by chloromercuribenzoic acid, another poison for -SH groups. The degree of inhibition of melanin formation varied directly as the logarithm of the molar concentration of the -SH group when the degree of inhibition caused by the skin extracts was compared to their sulphydryl content. The same relationship was demonstrated to occur in vivo after ultraviolet irradiation of rabbit skin. The increase of pigmentation caused by this treatment was found to be preceded by a decrease in the -SH content of the skin.

ii. Relationship of ACTH, intermedin and glutathione

According to the preceding studies it would not be unreasonable to think that the concentration of glutathione (or more generally, of -SH group compounds) in the skin, controls to a certain extent the degree of

pigmentation. Of particular interest in this connection is the observation of the French physiologist, Leon Binet (95) who showed a decrease in sulphydryl compounds in the blood of patients suffering from Addison's disease. Such an observation raises the question of the action of ACTH and adreno-cortical hormones on the sulphydryl compounds.

In a study of the diabetic syndrome appearing in normal subjects receiving ACTH, Conn (97) noticed a fall in blood glutathione and claims that the sugar metabolism was brought back to normal by injection of this compound. Kass (98) pointed out that glycosuria may occur during ACTH administration, associated with a diminished blood glutathione level. The hyperglycemia was not constant. The ACTH effect on carbohydrate metabolism could be reversed by injection of glutathione. The sulphydryl compound did not affect the rate of corticoid excretion when ACTH was continued. Hess (99) reported a decrease in blood glutathione level following ACTH injection, but the fall obtained after administration of cortisone was not judged significant. The total blood glutathione value was not affected by the hormones but the decrease in GSH was brought about mainly by its oxidation. Sprague (100) found little alteration of the blood glutathione level in three patients who received ACTH, and a slight increase during cortisone therapy. Changes in blood glutathione were not obtained by Joiner (101) during ACTH therapy. On the other hand, Kinsell (102) noted an increase in total urinary sulfate and organic sulphur after the injection of whole ACTH and ACTH peptide to an arthritic patient. The method used for the determination of urinary

cysteine also measured glutathione. In the discussion of this paper, Conn (102) remarks that different ACTH preparations give different patterns of sulphur elimination. He wonders if this fact is due to a difference on the basis of total ACTH present in the preparation or on the basis of actual qualitative differences in the compounds. Goldzieher (103), by means of intravenous administration of ACTH to 15 patients during one to three days observed a significant depression of blood -SH at four hours, with a return to normal at eight hours despite continued administration of the hormone. Correlated changes of blood ascorbic acid could not be demonstrated.

The preceding data are not always consistent. Care must be taken to avoid too generalised a conclusion when one considers that the values of glutathione can fluctuate with change in the hematocrit, and according to the more or less specific method used for determination of -SH group in body fluids.

This review of the recent literature concerning the role of ACTH in glutathione (or sulphur) metabolism leads to the following observations. If it is possible to show with relative ease that ACTH causes a decrease in blood glutathione and an increase in urinary organic sulphur, then the same phenomenon would be expected to occur following the administration of an adrenal cortical hormone. Curiously enough, this fact has never been reported conclusively in man, with the adrenal hormones used now in clinics, although it has been noted by Lazarow in the rat by use of cortisone (104,105).

In spite of the importance accorded to glutathione in explaining many different kinds of pigmentation occurring in man (68), we do not know of any study which has investigated the possible role of the melanophore hormone on the metabolism of this compound. Considering the high degree of contamination with intermedin of most ACTH preparations so far used in clinical investigations, it would be extremely interesting if the relationship of intermedin and glutathione were to be explored in some detail.

EXPERIMENTAL DATA

PART ONE : ACTH-LIKE ACTIVITY IN HUMAN BLOOD

ACTH-like activity in the human blood was investigated in a small number of patients in an attempt to repeat the work of Bornstein. The following techniques were employed:

i. Preparation of the blood extract

The preparation of the blood extract was essentially the same as that employed by J. Bornstein and Trewhella (16).

Twenty ml. of blood (instead of 10, as used by the above-mentioned authors) were withdrawn from the cubital vein into a heparinized syringe and centrifuged as soon as possible.

The plasma was drawn off with the help of a pipette and added to four volumes of acid acetone (25 ml. of analytically pure HCl in 975 ml. of redistilled acetone). The resulting precipitate was allowed to stand at room temperature for eight hours, being shaken at intervals, and then centrifuged. The supernatant fluid was taken off. To it was added 1 ml. of saturated NaCl, and the concentration of acetone brought to 94%, to allow precipitation, which takes place at 4°C. over a period of twelve hours.

The resulting mixture was centrifuged and the supernatant fluid discarded. The precipitate was washed with acetone and dried in a desiccator. This precipitate was dissolved in distilled water at the

time of injection, in such a quantity that 0.5 ml. of the solution corresponded to 1-2 ml., and in some cases to 7.5 ml. of serum.

Occasionally the extracts prepared in this way are toxic and care was taken to inject the solution very slowly, intravenously.

A modification of this technique was sometimes employed, consisting of a double extraction of the serum, pooling the supernatants and adding four times their volume of pure acetone. This was followed by centrifugation after twelve hours to obtain the precipitate (3).

ii. Operative techniques

Hypophysectomy. Hypophysectomy was performed using a classical method. After producing surgical anesthesia with ether, the rat was placed on its back and a medial incision made in the neck, commencing at the submental papilla and continuing distally for about 2 cm. The salivary glands were then separated and retracted by means of forceps. After exposing the angle made by the sternohyoid, thyrohyoid and digastricus muscles, the forceps, by blunt dissection in an anterior and cranial direction, were placed on either side of the spleno-occipital synchondrosis. Here they served as retractors for an assistant. The splenoid bone was then cleaned of all soft tissue by means of a cotton pledget, and drilled to the level of the dura mater with a dental burr. The dura was then torn with a dental pick and the exposed hypophysis removed by gentle suction through a glass cannula. A small pledget of fibrin foam was placed in the drill cavity to hasten coagulation and seal the opening. After withdrawing the forceps, the skin incision was closed with interrupted sutures

of cotton thread. The entire operation was performed within 45 seconds, during which time the animals were unable to breathe, and often artificial respiration was necessary to aid resucitation.

The completeness of all hypophysectomies was verified by autopsy examination of the sella turcica.

On completion of the operation, the animals were put back in their cages and given the ordinary purina fox chow, to which bread was added. A solution of 5% glucose was given as drinking water.

Hypophysectomy was performed 21-24 hours before the injection of the blood extract.

All the rats weighed between 100 and 120 grams. They belonged to a colony of hooded rats inbred in the department since 1925.

Adrenalectomy and extract injection

The following morning the rats were anesthetized by an intraperitoneal injection of nembutal (4 mgm/100 gm. body weight). An incision was made in the left side bisecting the angle between the vertebral column and the last rib. The ligament of the adrenal was then grasped by means of thumb forceps and the adrenal dissected free with iris scissors. Upon removal, if the adrenal was not dissected immediately it was placed in a Petri dish for a few minutes, the filter of which contained wet filter paper, to avoid drying of the tissue. In no case did the adrenal stay in the Petri dish more than two minutes.

The jugular vein was exposed and the blood extract was injected directly into it during a period of 1-3 minutes. The volume of the

extract injected was approximately 0.5 ml.

After the injection the animal was placed in a box on a layer of cotton wool. Care was taken to avoid chilling of the animals by placing a previously warmed metal plaque beneath the cotton wool, or by directing the light of a 100-watt bulb on the animals.

The adrenal gland was carefully dissected free of peripheral fatty tissue with a pair of fine eye-scissors, and weighed on a precision balance as quickly as possible. It was immediately dropped into a 15 ml. conical graduated centrifuge tube containing a few grains of sterile sand and 12 ml. of 4% trichloracetic acid.

If the adrenal capsule was opened during the dissection, the gland was discarded and the animal was not used for the test.

iii. Ascorbic acid determination

Ascorbic acid determination was made by the method of Roe and Kuether (115).

The adrenal gland was first finely ground in the centrifuge tube with the aid of a glass stirring rod.

Approximately 0.3 gm. of acid-washed norrit was added to the tube, which was shaken for a short period of time. The mixture was then filtered into a small Erlenmeyer flask.

Four ml. of the filtrate were pipetted into each of two Evelyn colorimeter tubes, to which was added one drop of an alcoholic solution of thiourea. This solution was prepared by dissolving 10 gm. of thiourea in

a 50% ethyl alcohol water mixture.

One ml. of 2-4 dinitrophenyl hydrazine solution (prepared by dissolving 2 grams of 2-4 dinitrophenyl hydrazine (Brickman and Co.) in 100 cc of 9 Nsulphuric acid) was added to both tubes. The tubes were gently shaken, corked and placed in an incubator at 37°C. for three hours.

Two blank tubes, containing the same amounts of thiourea and and trichloracetic acid but no dinitrophenyl-hydrazine, were allowed to stand at room temperature until the second part of the determination.

At the end of three hours all the tubes were immersed in ice water. Using a graduated burette, 5 ml. of 85% H_2SO_4 were run slowly into each tube, kept at a cold temperature in a beaker containing ice water. During the addition of the acid, each tube was gently agitated to avoid an abrupt increase in the temperature, this procedure taking more than one minute. The tube was then replaced in the basin containing ice water until the acid had been added to all of them.

Finally 1 ml. of dinitrophenyl-hydrazine was added to each of the two blank tubes.

The tubes were removed from the ice water and allowed to stand at room temperature for thirty minutes.

The measurement of the color developed was carried out in an Evelyn Colorimeter, using a filter of 540. The results were interpreted by reference to a standard curve established for values of ascorbic acid from 10 to 80 γ.

The standard curve used in these experiments has been verified in repeated compilations (see Fig. 1).

iv. Preliminary studies

As a preliminary study the content of ascorbic acid in the adrenal gland of four groups of animals was studied:

a) 10 normal animals, killed by cervical fracture, the adrenals of which were studied separately for their ascorbic acid content.

b) 10 hypophysectomized animals, whose adrenals were removed at the same time after the animals were killed with nembutal.

c) 10 hypophysectomized animals receiving 3 ml. of isotonic saline intravenously immediately after the first adrenalectomy. The second adrenalectomy was performed one hour later.

d) 10 hypophysectomized animals receiving 3 ml. of 0.1N HCl intravenously immediately after the first adrenalectomy. The second gland was removed one hour later.

The following table includes the results of the experiments, together with those found in a fifth group of ten rats, who had been incompletely hypophysectomized and who received 3 ml. of isotonic saline intravenously.

No. of rats	Description	Injection	Ascorbic acid depln.: Total in γ.	left gland-right gland per 100 grams of adrenal tissue
10	normal	none	2.5	20 mg.
10	hypophysectomized	none	1.5	20 mg.
10	"	3cc isotonic saline	5.5	34 mg.
10	"	3cc .1/N HCl	1.75	45 mg.
10	incompletely hypophysectomized	3 cc isotonic saline	19.5	170 mg.

Examination of the results permits the following conclusions:

a) that a value of ascorbic acid depletion lower than 20 mg./100 gm. of adrenal tissue falls within the range of the normal ascorbic acid difference between the left and right glands, as seen in normal, non-stressed, hypophysectomized rats. The ascorbic acid differences of the left and right adrenal of hypophysectomized non-injected rats are presented in Table I. These values are expressed as per cent of the ascorbic acid content of the left adrenal. Compared to the values published by Sayers (15) they are very significantly higher.

b) that even when the animals are well hypophysectomized a large amount of saline or weakly acid solution (3 ml. in these experiments) injected intravenously can significantly increase the ascorbic acid difference between the left and right glands. Three ml. of fluid represents for these rats, weighing between 120-215 grams, more than half the blood volume.

c) in incompletely hypophysectomized rats the injection of 3 ml. of saline causes an ascorbic acid depletion equivalent to as large a dose as 10 γ of ACTH injected intravenously, as may be seen by comparison with the standard curve for ACTH later on.

From these observations, further conclusions may be drawn:

a) small volumes of extracts must be injected whenever possible.

b) where the ascorbic acid difference is less than 40 mg./100 gm. adrenal tissue, it is unsafe to conclude that ACTH activity in the extract is responsible for the depletion.

v. Recovery experiment

Blood of a normal subject (C.G.), which had been demonstrated in a previous test to contain no demonstrable ACTH activity when the equivalent of 2.2 ml. of serum had been injected per animal, was used for this recovery experiment.

Seven ml. of plasma were taken and, immediately before acid acetone precipitation, 3 γ equivalent of LAIA standard of the corticotrophin Canada Packers Lot No. 1 were added per ml. The blood was extracted by the method already described.

A group of six rats was injected intravenously, each rat receiving an amount of extract corresponding to 1 ml. of serum, containing 3 γ of ACTH.

The mean ascorbic acid depletion of the group was found to be 105 mg./100 gm. adrenal tissue, in comparison with 123 mg./100 gm. when 3 γ of the same ACTH was dissolved in saline and injected into the animals.

This value corresponded to a recovery of 85%. The details of the assay will be found in Table II.

vi. Standard curve

A standard curve was established, using a preparation of ACTH Canada Packers Lot. No. 1, at the following dose levels: 0.25 γ, 0.5 γ, 1.5 γ, 3.0 γ, 5.0 γ and 8 γ LAIA equivalent. The details of the animal assays for each dose level will be found in Table III. A straight line

was obtained on plotting the mean ascorbic acid depletion in six animals against the logarithm of the dose (see Fig. II).

The following observations may be made as a result of establishing this standard curve:

a) there is no difference between the mean ascorbic acid depletion of 0.25 Υ injected rats and hypophysectomized rats who received no injection, in each case the mean depletion being 20 mg./100 gm. adrenal tissue.

b) there is no differentiation between the ascorbic acid depletions seen at the 0.50 and 0.75 Υ injected levels. In one case the value is 34 mg/100 gm. adrenal tissue, and in the other 36 mg.

c) the results obtained with the Canada Packers preparation indicate that it is not possible to measure ACTH activity below the range of 0.75 Υ of LAIA equivalent. Comparing the ascorbic acid depletion produced by 1 and 2 Υ of LAIA equivalent of an Armour ACTH Lot. No. 17409 with the depletion caused by 1.5 and 5 Υ of LAIA equivalent of the Canada Packers corticotrophin, it was noted that in terms of LAIA standard the Armour preparation was definitely more potent. Technical difficulties did not permit, however, the establishing of a second standard curve using the Armour ACTH.

There is the further possibility that more reliable values could have been ascertained if a larger number of animals had been used for each dose level. However, an examination of the table which gives the details of the experimental findings at each dose level reveals that the

results are more consistent from animal to animal when the doses are greater than the equivalent of 0.75 Y LAIA.

According to the preceding considerations, the standard curve here presented cannot be used for ascorbic acid depletion lower than the one produced by 0.75 Y LAIA.

vii. Subjects studied

The blood of two normal subjects and eight patients was studied for ACTH activity.

Whenever possible, blood was taken in a fasting condition. However, in the case of patients coming to the Outdoor Clinic of this hospital, the blood was obtained about three to four hours after breakfast.

In every case care was taken to centrifuge the blood, separate and precipitate the serum by acid acetone in the shortest time possible. Following the preparation of the extract, less than twenty-four, and often less than fifteen hours elapsed before its injection into the test animals.

The following table gives for each patient the clinical diagnosis, the therapy received at the time of blood collection, the equivalent of serum extract injected per animal and the result of the assay.

Name	Sex	Diagnosis	Therapy	Serum equivalent injected per animal	Sayer's test
M.C.	M	Normal	-	2 cc	0
C.G.	M	Normal	-	2.2 cc	0
C.T.	M	Addison's disease	12.5mg cortisone/day	1.5 cc	0
L.E.	M	Addison's disease	12.5mg cortisone BID	2 cc	0
C.H.D.	M	Addison's disease	off cortisone 8 days	6.5 cc	0
P.F.	M	Addison's disease	off cortisone 3 days	1.5 cc	76 γ ACTH per 100 cc plasma
C.A.R.	F	Cushing's syndrome	-	2 ml.	245 γ ACTH per 100 cc plasma
M.T.	M	Amputation left arm	-	2 ml.	0
Y.	F	Insulin resistant diabetes	off insulin - no ketosis	5.5 ml.	0
A.R.	M	Diabetes	Insulin 40 units/day	2.5 ml.	0

In view of these results it was not judged necessary to give an outline of the clinical observations and the laboratory data for each patient. The main clinical and laboratory data obtained for the two patients whose ACTH assay gave positive results are as follows:

a) P.F. was treated for Addison's disease and maintained on 12.5 mg. of cortisone daily. Before treatment, this patient presented

a marked pigmentation. The value of the biological corticoids, as measured by the Venning assay, was 15 glycogenic units/24 hours. At the time of the collection of blood, the patient had been withdrawn from cortisone for three days and complained of fatigue and anorexia. Neither the blood pressure nor the serum electrolytes showed a change as compared with the values obtained immediately before suppression of cortisone.

b) C.A.R. presented the typical clinical features of a Cushing's syndrome - obesity, amenorrhoea, purple striae, easy bruisability, increased pilosity. Her blood pressure was 142/100. The biological corticoids were 100 glycogenic units/24 hours; the 17 ketosteroids 21 mg/24 hours. The blood was obtained for the Sayer's test before any treatment was decided. Subsequently, an unilateral subtotal adrenalectomy was performed. The microscopic examination of the gland revealed hyperplasia of the adrenal cortex. The surgical exploration of the second adrenal was postponed.

The details of results obtained in individual animals in the Sayer's test will be found in Table IV.

viii. Discussion of the assay and its application to clinical material

I. The number of animals used per assay in the clinical investigations presented is, in most cases, very unsatisfactory. In such a bioassay not less than five, or even six animals should be injected for each blood tested. This rule has been difficult to apply throughout the present work for the following reasons:

a) the extract of blood prepared by the acid acetone method

sometimes presents such a degree of toxicity that the animal may die soon after the injection; or, during the injection, the animal may suffer from a period of apnea, necessitating the interruption of the intravenous injection and revival by artificial respiration, which is not always successful. Often, one or two animals have been lost per assay in this manner.

b) none of the literature dealing with this subject is able to state with certainty the amount of serum extract which should be injected by animal. We therefore decided on as large an amount as possible of extract for each animal. If the equivalent of 3 ml. of serum is injected per animal in a group of six, the amount of blood collected has to be greater than 40 ml. It is often difficult to obtain this quantity of blood from hospitalized patients.

c) in some cases one or two animals injected were proven to possess hypophyseal remnants when the sella turcica was examined after the assay. Such animals could not be used in the calculation of the results.

Although these conditions represent the usual difficulties attendant on the Sayer's test, they assume a greater importance when one is dealing with a limited amount of extract than when preparations of ACTH are to be assayed.

II. In many cases a wide range of ascorbic acid depletion was observed between the individual animals. For instance, in the case of one patient, values of -31, -119, 74 and 203 were recorded, giving an average depletion of 30 mgm/100 gm. of adrenal tissue. This value is obviously meaningless.

The reason for these individual variations is not known. However, a tentative explanation can perhaps be advanced.

a) it may be that the completeness of hypophysectomy, as checked by macroscopic examination, was inadequate and that some hypophyseal remnant was missed. This might offer some explanation where aberrant positive values of ascorbic acid depletion are considered, but cannot account for negative values.

b) a similarly wide range of ascorbic acid depletion, expressed by 100 gm. of adrenal tissue, was observed when the glands of hypophysectomized non-treated animals were studied. At the time this was thought to be accounted for by the probable imperfection of adrenal gland dissection. An incomplete removal of peripheral fat from the gland would greatly multiply the error when the ascorbic acid is calculated per 100 gm. of adrenal tissue. However, this explanation is not entirely satisfactory. After the most careful dissection, a difference in weight of more than 1 mg. between the two glands has been noted and sometimes could be correlated with the inconsistency of the ascorbic acid results. It is possible that in the conditions of this experiment and with the strain of rats used, the ascorbic acid content of the two adrenal glands of any given animal is not the same twenty-four hours after hypophysectomy. This supposition finds some support in Table I where the ascorbic acid of the right adrenal gland of hypophysectomized untreated rats is expressed as a percentage of the ascorbic acid of the left gland. As has already been pointed out, this table shows that our

rats present a much wider range of adrenal ascorbic acid content between the two glands than do the animals of Sayers.

c) the impression was gained that when one is concerned with values of ascorbic acid depletion lower than 50 mg/100 gm. of adrenal tissue, there is a higher degree of individual variation among the rats injected with the same amount of extract than when dealing with higher values of ascorbic acid depletion. Consequently, when blood extracts are studied, the sensitivity of the assay, reached by such investigators as Sayers himself, may not be completely satisfactory, unless more animals than the usual six could be injected, or a larger quantity of extract injected per animal.

III. It is also in the realm of possibility that the inconsistency of our results can be partly explained by the management of the animals before and throughout the test. For instance, the temperature to which the animals were exposed after hypophysectomy could not be controlled, a constant temperature box being unavailable.

IV. In our opinion, it is not possible to draw conclusions from the results obtained in these experiments and no attempt has been made to compare these data with the rather conflicting results found in the recent literature dealing with this subject. In the two patients who gave a positive value the animals showed a variation in their individual responses. The fact that these two results were obtained, one in a patient suffering from Cushing's syndrome and the other in an Addisonian deprived of cortisone for three days, is probably of some significance.

PART TWO : INTERMEDIN-LIKE ACTIVITY IN HUMAN BLOOD

A. MELANOPHORE EXPANDING PROPERTY OF PITUITARY PREPARATIONS

An attempt was made to repeat the results of Sulman (37,38) by testing the melanophore-expanding property of different preparations of pituitary hormones in frogs.

Experiment Conditions

The female frog Rana pipiens was chosen as the test animal. The frog was prepared for the experiment by being allowed to stand in the laboratory under the following conditions, which were designed to obtain as complete a melanophore cell contraction as possible:

i) each animal was placed in an individual beaker of one litre size, containing about 40 ml. of water. The water was changed two or three times a week, always being replaced by water at room temperature.

ii) the beakers were kept on a table which was covered with a white sheet, and surrounded by white cardboard.

iii) each beaker was incompletely covered with a square of glass, so that a space was left for air.

iv) two 100-watt bulbs, placed at a distance of approximately 25 cm. from the top of the beakers, provided a bright light, which was kept on night and day.

v) the temperature of the water was maintained at 25°C. as

constantly as possible. A lowering of the temperature was rapidly followed by a darkening of the animals.

vi) no food was given to the animals when they were used for a period of a few weeks to two months. Animals which were kept longer were force-fed fresh liver twice a week.

These conditions were easy to realize during the summer months, but during the winter it became very difficult to keep the temperature constant, and at this time the mortality was very high. Also, during the winter adaptation to light required a longer time to occur.

The frogs weighed between 35 and 50 grams. No special care, however, was taken to use animals in a narrow range of body weight because it had been demonstrated by Thing (48) that the same response to the same dose of ACTH was obtained in frogs of widely differing body weights.

Method of Assay

The frogs were injected with a 22-gauge needle introduced from the hind leg through the gluteus region into the dorsal lymph sac.

The preparations injected were dissolved in isotonic saline, each dose given being contained in approximately 0.5 ml. The control animal received the same volume of saline. Amounts of saline up to 1 and 1.5 ml. could be injected with no change in the existing state of the melanophore cells.

After injection, the frog was immediately replaced in its beaker, subject to the same conditions of light as before.

The melanophore expanding potency of the preparation was measured by the minimal amount necessary to affect a change of colour of the total dorsal skin in the hour following injection. Although such a method has been criticised, its degree of accuracy seemed sufficient when a certain number of animals was used in establishing the minimal dose.

The dose injected was calculated on the basis of ACTH LAIA potency for each preparation. In each case the actual weight of the powder injected was also recorded. No toxic reactions were observed.

The results were recorded thirty and sixty minutes after the injection. Observations taken beyond this time were not necessary when measurement of the potency was calculated on the basis of the minimal quantity of hormone able to change the colour of the skin. The minimal quantity was tested on at least four animals; but in view of the limited number of frogs available, doses which were expected to give a very positive reaction were tested on a smaller number.

The results are presented in Table V.

Presentation and Discussion of the Results

Examination of these results will show that the minimal quantity necessary to darken the frog's skin is remarkably constant for the different preparations of ACTH used when expressed in terms of LAIA Standard. Indeed, the melanophore-expanding activity of the different preparations seems to fluctuate less when expressed in these terms than

when expressed as weight of powder actually injected. For instance, the minimum activity for the Nordic Biochemicals Acton X is between 0.13 and 0.06 γ of LAIA equivalent, and for Connaught Lot 62, 0.13 γ LAIA; when expressed as weights of powder injected, the value for Acton X is 0.01 γ and for the Connaught preparation 0.47 γ. Seven out of ten preparations tested showed a minimal activity at the dose level of 0.13 γ, or between 0.06 and 0.13 γ.

Thus it would seem that the melanophore-expanding property followed quite closely the potency of the preparation as expressed in terms of LAIA Standard.

Another example of this correlation is seen in the test using the Thyrotropic preparation of Armour. This preparation, containing the equivalent of 0.02 USP units of corticotrophin per milligram, had its minimal melanophore-expanding effect at the dose level of 5-13 γ by weight, which corresponds to 0.10 and 0.25 γ of LAIA Standard.

The same observation holds true for the Growth Hormone prepared by Armour. The results obtained by the ascorbic acid depletion of Sayers with this preparation varied from 1 to 4 (0.01 to 0.04 mg. of LAIA Standard/mg. solid powder). If we choose a mean ACTH contamination of 0.02 LAIA/mg. as the basis of our calculation, the minimal dose for melanophore-expanding activity calculated in terms of LAIA would be 0.18 γ.

The preparation P7 from the Nordic Biochemicals laboratories was also investigated. This fraction is obtained in the course of

corticotrophin purification according to the Astwood method, after adsorption on oxycellulose. P7 remains in the supernatant, from which it is isolated by precipitation. When assayed by the Sayers' test, this fraction is found to contain a certain proportion of ACTH. We tested the preparation on the frog before this contamination was known, and found it to be quite rich in intermedin (melanophore-expanding activity). When the results of the Sayers' test were obtained, through the courtesy of Nordic Biochemicals laboratories, the same relationship was observed that has already been described between the LAIA Standard and the melanophore-expanding activity.

When a preparation of intermedin was studied, it was noted that the minimal active dose corresponded to 0.92 Y by weight of the powder itself, a quantity which is significantly greater than the minimal active doses of the ACTH preparations when these are considered in terms of weight of powder injected. However, this observation may not be very suggestive, since it was not possible to know how the unit of intermedin activity was established. Neither was anything known about the possible contamination of this preparation with ACTH.

The Astwood preparation, containing "about 80 LAIA units per milligram" (116) was tested, using two different samples.

The first sample gave the minimal reaction at a dose of 0.01 Y of weight of the preparation, and the second at 0.03 Y. If one calculates the ACTH potency on the basis of 80 LAIA units/mg., the two doses represent 0.8 Y and 2.4 Y of LAIA Standard, which are significantly higher

than the values of the other ACTH preparations used. Unfortunately, according to Dr. Astwood, the potency of these preparations has not been systematically studied.

The same conclusion might also hold for the Armour Beef ACTH preparation, but no information concerning its LAIA content could be obtained.

The results of this investigation on the different pituitary preparations, with the exception of the last two, agree with the findings of Sulman. If intermedin and ACTH are two different hormones, the proof is not to be found at least in a comparison of their melanophore-expanding activities. As far as the present test is concerned, despite the criticisms which may be raised to its accuracy, the conclusion must be reached that the intermedin potency of ACTH preparations is closely related to their corticotrophic activity when expressed in terms of LAIA equivalent.

It is even possible that the contamination of any pituitary hormone by intermedin is closely related to the presence of ACTH and that where intermedin is present in a preparation, ACTH is also present. The fact that when ACTH is adsorbed on oxycellulose, intermedin is also adsorbed, and that when part of the intermedin remains in the supernatant of oxycellulose a certain proportion of ACTH also remains, would seem to corroborate this opinion.

As far as the Astwood preparation of ACTH and the Armour intermedin are concerned, these are two instances where the possibility

of getting a concentration of ACTH or intermedin independently of each other is realized. The melanophore-expanding potency for the Astwood preparation expressed in terms of LAIA equivalent contained in the minimal dose is up to 2.4 γ; for the Armour intermedin it is very low, being less than 0.0125 γ.

B. CORTISONE AND COMPOUND F : THEIR RELATIONSHIP TO THE MELANOPHORE-EXPANDING PROPERTY OF ACTH IN THE FROG

Because cortisone is able to decrease the pigmentation in subjects suffering from Addison's disease, an experiment was designed to test whether the adrenal cortical hormones, Compounds E and F, were able to interfere with the melanophore-expanding property of ACTH. To this end, the melanophore index of Hogben (117), which measured the degree of melanophore cell expansion by microscopic examination of the frog-web was chosen.

Method

Following the example of Hogben, the melanophore range was designated numerically as 1, 2, 3, 4, or 5, depending on the degree of expansion observed. Usually the degree of expansion was uniform for all cells examined, but occasionally the intermingling of two adjacent groups was seen, a degree of expansion of 2, for instance, co-existing with a degree of expansion of 3 for adjacent cells. In such a case, the degree of expansion was designated as 2.5, and so on.

Under ideal experimental conditions, where the frogs employed

are fully adapted to light (being quite pale) the melanophore index is 1. The cells appear completely contracted, and are visualized as a series of round black dots on a very clear background. Illustrations of the appearance of the melanophore cells designated by the melanophore indices (1-5) may be seen in the original work of Hogben (117) or in the more recent publication of Waring, Landgrebe (50) and Thing (48).

At the time of the test, the skin of the web is examined and if the degree of contraction of the melanophore cells is judged satisfactory, its index is recorded. This is repeated every thirty minutes for several hours in order to be sure of the stability of the index. The melanophore index was always recorded on the web joining the first and second digits of the left posterior limb. Examination was first made with the low power, x5 ocular, to obtain a general impression of the index, and then repeated with x10 ocular to determine more accurately the index.

Once the control index was established, an injection of ACTH was given; 90 minutes later, when the melanophore expansion was at its maximum, an injection of cortisone or Compound F was given; 60 minutes later, a second injection of ACTH, the same dose as the first, was given; a third injection of ACTH was usually given one hour before the end of the period of observation, which lasted approximately six hours.

A group of five frogs was used for each experiment, and each animal acted as its own control.

Results

As may be seen from an examination of the curves (Fig. 3), 1 Υ of ACTH (Armour Lot 17409) produced in 60-90 minutes the full expansion of melanophore cells of frogs adapted to light. Injection of 3 mg. of Compound E or F Acetate at this time produced a sharp decrease of the melanophore index, sometimes in as short a period as 15 minutes. A subsequent injection of ACTH (1 Υ) failed to produce the usual melanophore expansion (totally, where Compound E Acetate had been injected previously; partially in the case of Compound F Acetate). The period of inhibition was limited, being longer by cortisone than by Compound F, and a third ACTH injection (2 Υ) performed three hours after the cortisone or Compound F injection, produced the usual increase of melanophore index.

In these experiments smaller doses of adreno-cortical hormone failed to exert the above inhibiting action. On the other hand, even with the doses employed, the inhibition would not seem to be as complete as the change in melanophore index would indicate. The colour of the total skin of the back became lighter after injection of Compound E or F, but changed only from a dark olive colour to a sort of intermediate greyish colour, which is darker than the light-adapted frog at the beginning of the experiment. This observation permits one to wonder if the melanophore index is in this case a good criterion of the behaviour of the melanophore cells on the whole surface of the dorsal skin.

These experiments are unable to shed much light on the physiological

relationship between the adrenal steroids and the melanophore-expanding property of ACTH, since the disproportion between the doses of the pituitary (1.0 Y) and adrenal cortical hormones (3 mg.) is obvious.

C. MELANOPHORE-EXPANDING PROPERTY OF HUMAN BLOOD EXTRACTS

The melanophore-expanding property of blood extracts was investigated according to the method proposed by Sulman (43).

Method of Blood Extraction

i) 20 ml. of blood are withdrawn from the cubital vein of a patient and immediately transferred to a bottle containing 20 ml. of distilled acetone and 5% glacial acetic acid.

ii) the suspension is filtered with suction through a 1-2 inch Buchner funnel lined with Whatman filter paper no. 1, and the precipitate is washed with 5 ml. of 50% acetone.

iii) the pooled filtrates (approximately 25 ml.) are carried over to a 100 ml. bottle and 60 ml. of distilled acetone are added. A precipitate slowly forms within an hour. In order to facilitate precipitation and sedimentation, 10 ml. of ether are added at the same time, and the bottle is allowed to stand overnight in the refrigerator.

iv) the next morning the supernatant fluid is decanted; the precipitate is washed with about 5 ml. of ether, the ether decanted and the residue freed from traces of ether by allowing it to stand for about 30 minutes.

v) the precipitate is dissolved quantitatively in 0.8 ml. of N/20 NaOH with the addition of 1-2 drops of a standard bromthymol blue indicator solution. If the precipitate does not completely dissolve, the bottle should be shaken in a shaking apparatus. Finally, a few drops of N/4 acetic acid are added until the solution turns green, indicating that the pH is about 8.

In the test reported here a frog control was injected with the same volume of solvent used for the precipitate and at the same pH. No expansion of melanophore cells was seen in the control animal (when examined microscopically) nor was there any visible change of colour.

In one or two cases, serum alone was extracted according to the method proposed by Bornstein (see above) and the final extract was dissolved in distilled water before injecting into the animal.

Double extraction of the serum by acid acetone (see above) was also tried.

Method of Assay

Two methods were used to determine the melanophore-expanding property of the blood extracts.

i) Inspection by the naked eye : the same procedure was adopted as described above for the visual evaluation of the change of colour of the total dorsal skin of the frog when the ACTH preparations were investigated. The only modification used here for the blood extracts was that the period of observation was prolonged $1\frac{1}{2}$ to 2 hours, with the thought

that the hormone contained in the blood extract might possibly act more slowly than the purified hormone preparation.

ii) Microscopic examination of the frog web : this is the same method as used for the ACTH and intermedin preparation, described above.

The test was used in a qualitative manner to determine the presence or absence of intermedin in these extracts. The total blood extract was injected in a volume of 0.5-1 ml. per frog. When the measurement of the melanophore cell expansion was done, the animal was used as its own control.

Recovery Experiments

Recovery experiments were performed with blood from normal subjects since previous studies had shown that such blood does not contain intermedin-like activity when less than 30 ml. equivalent are injected in one animal.

Ten ml. of blood were taken from each normal subject. Different quantities of the corticotrophin Armour Lot 17409 (from 0.13 Y to 1 Y of LAIA equivalent) were added, and the extraction performed immediately. The extract was injected into the frog when ready, and the change of colour of the total dorsal skin of the frog as measured by the naked eye was the criterion used in evaluating the test.

0.5 Y of ACTH added to blood and injected into 8 frogs gave a positive reaction in 4 animals (50% of the cases); 0.25 Y injected into 8 frogs gave a positive reaction in 13 animals (70%); 0.13 Y gave a

uniformly negative reaction.

In an attempt to clarify the lack of correlation of the preceding results, ACTH was added at different steps during the extraction procedure.

When the ACTH was added to the blood before the acid acetone precipitation, complete loss of activity sometimes occurred.

When ACTH was added to the filtrate of 10 ml. of blood, as small a quantity as 0.25 γ and, in a few cases, 0.13 γ could be recovered. These results tend to show that the acid acetone precipitation of the serum does not extract such small amounts of the added hormone, or perhaps destroys them. However, this does not explain why it is possible to recover the activity in some cases and not in others, when the hormone is directly added to serum, since the same procedure was followed in every case and the loss of activity could not be attributed to differences in the method or the reagent used. It is also difficult to believe that the hormone added to serum, being a relatively pure preparation, would for that reason be more easily destroyed by a method of extraction as crude as the acid acetone precipitation. If such were the case, the destruction would have been the same throughout. The question remains as to whether the hormone is more readily destroyed by certain bloods than by others.

Clinical Cases

1. Normal Subjects (see Table VI)

Twelve normal individuals were studied. In five

subjects, 20 ml. of whole blood were collected, and the total extract, prepared as above, injected in one animal per subject. The tests were negative.

In three subjects, 30 ml. of whole blood were collected and the total extract injected in one animal per subject. In one case the test was positive. The other two gave a negative response.

In four subjects, 40 ml. of whole blood were collected, and the total extract injected as in the other tests. In only one case was the test positive.

2. Pregnancy (see Table VII)

a) Normal pregnancy.

Nineteen normal pregnant women were studied. The patients were seen in the Outdoor Clinic, where they had reported for a routine check-up. 15-20 ml. of blood were collected per person, and injected into separate frogs. Table VII gives the results obtained. Sixteen gave negative results; in the other three patients there was slight activity in the extracts :

i) R.Y. - 7th month - Melanophore index 3.5

ii) P.I. - 4th month - Melanophore index 2.75

iii) R.F. - 7th month - Melanophore index 3

b) Abnormal pregnancy

Seven cases were those of women hospitalized for mild signs of late toxemia in the 8th and 9th months of pregnancy. An extract

equivalent to 20 ml. of blood was injected per frog per person. In three cases the tests were positive.

3. Addison's Disease (see Table VIII)

Seven patients suffering from Addison's disease were investigated. In only two cases was it feasible to suppress the supportive hormonal therapy for several days prior to the collection of the blood sample.

Table VIII gives the results of these tests. Four of the seven patients gave a sub-positive degree of melanophore expansion, the extract of 20 ml. of blood equivalent being injected per frog. The melanophore index is in each case of the same value as the one obtained with the other subject found positive, i.e. from 2.5 to 3.5

The result obtained in the patient P.F. is of some interest. Blood which was taken during cortisone therapy was negative, but blood taken five days after suppression of cortisone therapy produced a marked darkening of the frog and gave a full melanophore expansion lasting several hours. At the time of blood collection the patient was complaining of fatigue and loss of appetite. His blood pressure and serum electrolytes did not change significantly from their values during cortisone therapy. This patient is the only one among the Addison's disease cases studied who also gave a positive result with the Sayers' test (76 γ of ACTH-like activity per 100 ml. plasma).

Unfortunately, difficulties in suppressing the cortisone therapy of patients suffering from adrenal insufficiency did not permit parallel

studies of intermedin and ACTH-like activity in the blood of untreated Addisonians.

DISCUSSION

In the light of the results obtained, the investigation of the melanophore-expanding property of human blood extracts has proved disappointing.

The few "positive" results obtained in normal pregnancy and in mild toxemia of pregnancy cannot be correlated either with the period of pregnancy or the state of severity of the toxemia.

In the cases where the increase of melanophore index has been chosen as the criterion, the values recorded as positive represent a moderate increase of this index in relation to the large amount of blood extract injected (15-20 ml.). It is that which makes one question whether the melanophore hormone was really present in the blood at a very low level, or whether the values observed had no significance.

The recovery experiment leads to the conclusion that the method of extraction is far from ideal. Landgrebe, in his article (83), was very sceptical as to the significance of the results he obtained in his investigation of the melanophore-expanding property of rabbit blood. The values recorded by this author for the melanophore index are of the same order as the ones reported here. Landgrebe also utilizes the results obtained with human blood and urine, and stressed the inadequacies of the method of extraction in use at present. It is also possible that the rapid rate of disappearance of intermedin from the peripheral circulation

or its inactivation, in part explains these findings.

Although the values of the melanophore index obtained with blood extracts from patients with Addison's disease are only slightly greater than those recorded with blood extracts from normal and mildly toxemic pregnant patients, evidence of melanophore-expanding activity seems more easily demonstrable in the former group. If such values of the melanophore index could be accepted as adequate criteria of the melanophore-expanding activity of the extract, one would tend to believe that intermedin is demonstrable in the peripheral circulation of Addisonians. The very positive result obtained in one patient deprived of cortisone for 3 days will sustain this opinion. It is almost unnecessary to point out that such patients, although they may present the most favourable conditions for the study of intermedin in peripheral human blood, are extremely rare.

SUMMARY

1. Ten subjects were investigated for ACTH-like activity in blood by the Sayers' test : two normal individuals and eight patients.

2. Positive results were obtained in two cases:

a) One case of Addison's disease, removed from cortisone therapy three days before the test, in which an estimation of 76 Y ACTH/100 ml. plasma was made.

b) One case of Cushing's Syndrome, untreated, shown by later biopsy to have hyperplasia of the adrenal cortex, in which an estimation of 245 Y ACTH/100 ml. plasma was made.

3. The following difficulties were observed in the clinical use of the Sayers' test:

a) Toxicity of the blood extracts.

b) Practical difficulty of obtaining sufficiently large quantities of blood for assay.

c) Wide range of ascorbic acid depletion in individual animals with the same quantity of material injected.

d) Decreasing sensitivity of the test with the assay of small quantities of ACTH.

e) Limited range over which activity can be measured.

4. Forty-five subjects were investigated for intermedin-like activity in blood by the Sulman frog test : twelve normal individuals; nineteen

cases of normal pregnancy; seven cases of mild toxemia of pregnancy; seven cases of Addison's disease.

5. Positive results were obtained in eleven cases:

a) One normal subject where an extract equivalent of 30 ml. of blood was injected per frog.

b) One normal subject where an extract equivalent of 40 ml. of blood was injected per frog.

c) Three cases of normal pregnancy, two in the 7th month, one in the 4th month.

d) Three cases of mild toxemia of pregnancy, one in the 7th, one in the 8th, and one in the 9th months.

e) Four cases of Addison's disease, the strongest response being present in the Addisonian removed from therapy for three days, who also gave a positive response with the Sayers' test.

6. Most of the positive responses recorded above represented a very slight degree of melanophore expansion.

7. A recovery experiment indicated that the method of extraction of melanophore-expanding activity from the blood is not wholly satisfactory.

8. There was a generally good, but not invariable, correlation between the melanophore-expanding activity and the adrenal ascorbic acid depletion activity (as measured in LAIA units) of the various preparations studied, which included ACTH from different laboratories, TSH, growth hormone.

TABLE I

ASCORBIC ACID CONCENTRATION OF ADRENALS OF UNTREATED HYPOPHYSECTOMIZED RATS

Adrenal ascorbic acid total in γ			Adrenal ascorbic acid mg. per 100 grm. fresh tissue		
Left	Right	Difference % of the left adrenal	Left	Right	Difference % of left adrenal
52	48.5	-6.6	472	475	0.5
44.5	39.1	-12.1	483	476	-1.4
56.5	49.2	-13.0	487	502	3.0
54.5	52.3	-4.2	592	622	5.0
66	72	9.1	647	679	5.0
24	28.9	20.3	557	627	12.5
38.5	34.6	-10.1	601	540	-10.0
54.5	53.5	-1.9	545	581	6.5

TABLE II

RECOVERY EXPERIMENT - EACH RAT RECEIVED 1cc. SERUM EXTRACT CONTAINING 3 γ OF ACTH CANADA PACKERS LOT. I

Rat no.	Adrenal weight	Diff. weight	Ascorbic acid per gland in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
I	*L 10.4 mg. *R 9.4	1	54.75 35.25	19.5	526 375	151
II	L 9.6 R 8.8	.8	56.5 44.0	12.5	588 500	88
III	L 10.2 R 9.8	.4	52.5 40.5	12.0	514 413	101
IV	L 8.1 R 7.7	.4	45.25 36.0	9.25	558 467	91
V	L 12.2 R 11.3	.9	63.75 49.0	14.75	522 433	89
VI	L 17.4 R 13.6	3.8	80.62 48.0	32.62	463 352	111

Mean of 6 rats : L-R = 105 mg./100 gram of adrenal tissue Recovery : 85%

Standard deviation : 22

*L = left adrenal gland
*R = right adrenal gland

TABLE III

FALL OF ASCORBIC ACID IN THE RIGHT ADRENAL

GLAND OF HYPOPHYSECTOMIZED RATS RECEIVING ACTH CANADA PACKERS LOT NO. I

Rat no.	Adrenal weight mg.	Diff. weight mg.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 0.25 γ ACTH/100 gm. body weight			
I	*L 9.9	.9	64	0	646	-66
	*R 9.0		64		712	
II	L 8.2	-.8	71	9.5	866	183
	R 9.0		61.5		683	
III	L 8.4	.8	60.5	4	720	-23
	R 7.6		56.5		743	
IV	L 8.6	0	64.75	4.25	753	50
	R 8.6		60.50		703	
V	L 6.4	-1	47.5	0	742	99
	R 7.4		47.5		643	
VI	L 10.8	1.4	71.0	2.5	657	-125
	R 9.4		73.5		782	

Mean of 6 rats : L-R = 20 mg. per 100 gram body weight

Standard deviation : 103.2

*L : left adrenal
*R : right adrenal

TABLE III (continued)

Rat no.	Adrenal weight mg.	Diff. weight mg.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 0.5 γ ACTH/100 gm. body weight			
I	L 9.8 R 10.1	-.3	43.5 38.25	5.25	444 379	65
II	L 10.9 R 9.2	1.7	48.65 40.25	8.4	446 438	8
III	L 10.7 R 10.1	.6	49.5 46.25	3.25	463 458	5
IV	L 9.0 R 8.4	.6	48.25 40.25	8	536 479	57
V	L 11.4 R 10.0	1.4	53.75 50.25	3.5	471 502	-31
VI	L 6.8 R 7.2	-.4	41.75 37.12	4.63	614 516	98

Mean of 6 rats : L-R = 34 mg. per 100 gram body weight

Standard deviation : 43.6

TABLE III (continued)

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 0.75 γ ACTH/100 gm. body weight			
I	L 7.8	.4	40.5	7	519	66
	R 7.4		33.5		453	
II	L 9.7	.5	49.0	4	505	16
	R 9.2		45.0		489	
III	L 8.0	.1	41.5	10	519	120
	R 7.9		31.5		399	
IV	L 8.9	1.6	47.0	4.75	528	2
	R 7.3		42.25		526	
V	L 6.8	-.2	35.75	3.5	525	64
	R 7.0		32.25		461	
VI	L 7.0	1.4	33.25	3.75	475	-52
	R 5.6		29.5		527	

Mean of ascorbic acid depletion left gland-right gland : 36 mg/100 gram adrenal tissue

Standard deviation : 54.8

TABLE III (continued)

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 1.5 γ ACTH/100 gm. body weight			
I	L 8.8 R 8.0	.8	44.75 33.0	11.75	508 412	96
II	L 8.2 R 8.0	.2	44.32 34.0	10.32	540 425	115
III	L 11.8 R 11.6	.2	54.0 38.0	16.0	458 328	130
IV	L 8.1 R 7.4	.7	33.15 25.37	7.78	409 343	66
V	L 7.0 R 6.4	.6	41.25 32.5	8.75	589 508	81
VI	L 8.7 R 7.9	.8	45.75 40.0	5.75	525 506	19

Mean of 6 rats L-R = 84.5 mg. per 100 grams adrenal tissue

Standard deviation : 36

TABLE III (continued)

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 3 γ ACTH/100 gm. body weight			
I	L 9.3	1	45.5	15.15	490	124
	R 8.3		30.35		366	
II	L 7.9	.5	37.25	10.75	472	114
	R 7.4		26.50		358	
III	L 7.8	.5	35.85	11.1	460	121
	R 7.3		24.75		339	
IV	L 9.4	.9	39.5	14.35	420	124
	R 8.5		25.15		296	
V	L 8.4	.4	44.87	12.37	535	129
	R 8.0		32.5		406	
VI	L 8.2	1	51.25	15.38	625	127
	R 7.2		35.87		498	

Mean of six animals L-R = 123 mg. per 100 grams of adrenal tissue

Standard deviation : 4.8

TABLE III (continued)

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 5 γ ACTH/100 gm. body weight			
I	L 7.6 R 8.0	-.4	33.85 24.35	9.5	445 304	141
II	L 8.7 R 7.3	1.4	47.88 30.75	17.13	550 421	129
III	L 8.5 R 7.2	1.3	40.25 27.60	12.65	474 379	95
IV	L 9.2 R 8.6	.6	44.5 31.0	13.5	484 360	124
V	L 7.6 R 7.9	-.3	49.0 31.5	17.5	645 399	246
VI	L 7.3 R 7.3	0	38.85 23.5	15.35	322	147

Mean of 6 rats L-R = 147 mg. per 100 gm. adrenal tissue

Standard deviation : 47.2

TABLE III (continued)

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			DOSE - 8 γ ACTH/100 gm. body weight			
I	L 9.6 R 6.0	3.6	45.25 24.50	20.75	472 408	64
II	L 9.0 R 7.8	1.2	41.5 25.5	16.0	462 327	135
III	L 10.2 R 9.2	1	62.0 33.5	28.5	609 364	245
IV	L 10.0 R 6.8	3.2	58.50 31.0	27.5	585 456	129
V	L 12.6 R 9.3	3.3	74.25 38.0	36.25	590 409	181
VI	L 9.0 R 7.6	1.4	54.75 36.25	18.5	608 477	131

Mean of 6 rats L-R = 148 mg. per 100 gm. adrenal tissue

Standard deviation : 17.5

TABLE IV

CLINICAL STUDIES WITH SAYERS' TEST

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			Subject M.C. (Normal)			
I	L 15.4 R 12.0	3.4	63.5 45.4	18	412 380	32.0
II*	L 10.0 R 8.2	1.8	49.5 42.5	7		
III	L 10.5 R 8.4	2.1	56.5 46.5	10	538 554	-16.0
IV	L 9.2 R 8.0	1.2	56.5 52.5	4	614 656	-42.0
			Mean of 3 rats L-R = -26 mg/100 gr. adrenal tissue			
*incomplete hypophysectomy						
			Subject C.G. (Normal)			
I	L 8.8 R 9.0	-.2	60.75 57.25	3.5	690 637	53.0
II**	-		-	-	-	-
III	L 12.6 R 9.4	3.2	68.50 53.0	15.5	544 564	-20.0
IV**	-		-	-	-	-
			Mean of 2 rats L-R = 16.5 mg/100 gr. adrenal tissue			
**dead during assay						

TABLE IV (continued)

Rat no.	Adrenal weight mgm	Diff. weight mgm	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			Patient Y - Insulin resistant diabetes			
I	L 12.0	.6	92.75	-2	773	-59
	R 11.4		94.75		832	
II	L 7.0	1	58.0	11	829	-121
	R 6.0		47.0		950	
III*	L 8.0	-	-	-	-	-
*dead during assay			Mean of 2 rats L-R = -90 mgm/100 gm. adrenal tissue			
			Patient C.H.D. - Addison's disease			
I	L 6.2	.1	51.25	7.25	827	107
	R 6.1		44.0		720	
II	L 8.2	.4	70.0	4.5	856	17
	R 7.8		65.5		839	
III	L 7.6	0	65.25	8.0	858	-28
	R 7.6		57.25		886	
Mean of 3 rats L-R = 32 mgm/100 gm. adrenal tissue						

TABLE IV (continued)

Rat no.	Adrenal weight mgm.	Diff. weight mgm.	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			Patient C.T. - Addison's Disease			
I*	-	-	-	-	-	-
II	L 8.0 R 6.3	1.7	44.0 34.5	9.5	550 541	3
III**	L 9.9 R 9.1	.8	50.0 33.0	17.0	550 363	187
IV*	-	-	-	-	-	-
V	L 10.1 R 10.0	.1	58.5 52.5	7.0	579 525	54

Mean of 2 rats L-R = 28 mg./100 gr. adrenal tissue

*dead during assay; **incomplete hypophysectomy.

			Patient L.E. - Addison's Disease			
I	L 9.2 R 8.2	1	53.0 49.5	3.5	576 603	-27
II*	L 9.6	-	-	-	-	-
III	L 8.4 R 9.6	-1.2	59.0 56.75	2.25	702 592	110
IV**	L 11.4 R 10.8	-.6	68.5 49.75	18.75	-	-
V*	-	-	-	-	-	-

Mean of 2 rats L-R = 41.5 mg./100 gr. adrenal tissue

*dead during assay; **incomplete hypophysectomy

TABLE IV (continued)

Rat no.	Adrenal weight mgm	Diff. weight mgm	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			Patient P.F. - Addison's disease (off treatment)			
I*	L 10.0 R 8.7	1.3	51.5 32.0	19.5	515 369	146
II	L 10.3 R 9.4	.9	58.75 48.25	10.5	570 514	66
III	L 7.9 R 7.8	.1	48.0 39.0	9	608 501	107
IV	L 8.8 R 8.3	.5	54.0 45.5	8.5	614 547	67
V**	-	-	-	-	-	-

Mean of 3 rats L-R = 80.0 mg/100 gram adrenal tissue = 76 γ ACTH activity per 100 ml. plasma

Standard deviation : 19.1

*incomplete hypophysectomy; **dead during assay

			Patient C.A.R. - Cushing's syndrome			
I	L 7.2 R 7.6	-.4	35.5 31.0	4.5	492 408	84
II*	L 8.5	-	-	-	-	-
III	L 8.0 R 7.5	.5	39.5 29.25	10.25	494 395	199
IV	L 8.8 R 7.2	1.6	45.25 34.75	10.50	515 482	133

Mean of 3 rats L-R = 138 mg/100 gram adrenal tissue = 245 γ per 100 ml. plasma. Standard deviation 47.1

*dead during assay.

TABLE IV (continued)

Rat no.	Adrenal weight mgm	Diff. weight mgm	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
			Patient M.T. - Amputation, left arm			
I	L 9.8	1.2	58.5	4	597	-37
	R 8.6		54.5		634	
II	L 10.0	1.6	64.75	.5	647	-119
	R 8.4		64.25		766	
III	L 9.6	.2	56.0	17.5	484	74
	R 9.4		38.5		410	
IV	L 7.8	-.6	66.0	12.0	846	203
	R 8.4		54.0		643	
V*	L 9.8	.4	-	-	-	-
	R 9.4					

Mean of 4 rats L-R = 30.25 mg./100 gram adrenal tissue

*dead during assay

TABLE IV (continued)

Rat no.	Body weight	Adrenal weight mgm	Diff. weight mgm	Ascorbic acid total in γ	Diff. per gland in γ	Ascorbic acid per 100 gm. tissue	Diff. per 100 gm. tissue
				Patient A.R. - Diabetes			
I	104	L 7.8 R 7.4	.4	38.0 34.5	3.5	488 466	22 mg.
II	92	L 9.6 R 8.2	1.4	42.0 35.5	6.5	437 433	4 mg.
III	89	L 8.8 R 6.7	2.1	40.5 35.5	5.0	461 530	-69 mg.
IV	96	L 8.3 R 6.8	1.5	43.5 35.5	8.0	525 522	3 mg.
V	104	L 8.4 R 8.5	-0.1	41.0 41.50	-0.5	489 489	0

Mean of 5 rats L-R = -8 mg. per 100 gram adrenal tissue

TABLE V

MELANOPHORE-EXPANDING ACTIVITY - SULMAN FROG TEST

ACTH preparation	Equivalent LAIA injected/frog	Actual weight of powder injected per frog	No. of animals	Reaction	Minimal reaction
Armour Lot 17409	1 Y	0.52 Y	5	++	
	.25	0.13	4	+	
	.13	0.06	5	+	+
	.06	0.03	4	-	
Armour Lot 19907	.75 Y	0.16 Y	3	++	
	.25	0.05	3	+	
	.13	0.025	5	+	+
	.06	0.013	2	-	
Armour Lot J16609	.75 Y	0.28 Y	2	++	
	.25	0.09	2	+	
	.13	0.05	10	±	+
	.06	0.025	2	-	
Connaught Lot 6:2	1 Y	3.76 Y	2	++	
	.25	0.94	2	+	
	.13	0.47	7	+	+
Connaught Lot 5:1	1 Y	1.6 Y	2	++	
	.25	0.40	2	+	
	.13	0.20	5	+	
	.06	0.10	3	±	+
Connaught Lot K.C.	.5 Y	0.7 Y	2	++	
	.25	0.35	2	+	
	.13	0.18	5	+	+
Nordic Biochemicals Acton X	.5 Y	0.08 Y	2	++	
	.25	0.04	2	+	
	.13	0.02	5	+	
	.06	0.01	4	±	+

TABLE V (continued)

ACTH preparation	Equivalent LAIA injected/frog	Actual weight of powder injected per frog	No. of animals	Reaction	Minimal reaction
Canada Packers Lot. no. 1	1 γ		2	+	
	.75		4	+	+
	.5		5	-	
Intermedin	.0125 units	2.32 γ	3	+++	
	.001	1.85	3	++	
	.0008	1.48	3	++	
	.0005	.92	4	+	
	.0003	.55	3	±	+
	.00013	.24	2	-	
Armour Beef ACTH	1 γ	2.8 γ	2	+++	
	.13	.35	3	++	
	.06	.18	2	++	
	.03	.09	3	+	
	.01	.03	4	±	+
Astwood's ACTH	approx. 20 γ	.25 γ	3	+++	
	" 4	.05	2	++	
	" 2	.025	2	++	
	" .8	.01	3	+	+
	" .4	.005	2	-	
Nordic Biochemicals preparation P.7 (Lot. 104)	.85 γ	5 γ	1	+++	
	.34	2	1	++	
	.17	1	1	++±	
	.09	.5	5	+	
	.05	.25	3	±	+
T.S.H. Armour Lot. K23101	approx. 1 γ	50 γ	1	+++	
	" .50	25	2	++	
	" .25	13	2	+	
	" .1	5	4	±	+

TABLE V (continued)

ACTH preparation	Equivalent LAIA injected/frog	Actual weight of powder injected per frog	No. of animals	Reaction	Minimal reaction
Growth Hormone (Armour)	1.75 to 7 Y	172 Y	1	+++	
	.35 to 1.4	34.4	2	+++	
	.09 to .36	8.6	4	+	+
	.02 to .08	1.7	2	-	

TABLE VI

NORMAL SUBJECTS

Subject	Blood extracted	Colour change of the frog	Response
H.E.	20 c.c.	0	0
B.Y.	20	0	0
J.G.	20	0	0
W.C.	20	0	0
J.P.	30	0	0
C.M.	30	++	pos.
K.L.	30	0	0
C.J.	40	++	pos.
D.D.	40	0	0
J.A.	40	0	0
C.C.	40	0	0
P.J.	40	0	0

TABLE VII

A. NORMAL PREGNANCY

Patient	Month of pregnancy	Melanophore index from 1 to	Response
P.I.	4	no change	-
S.Z.	4	"	-
T.R.	5	3	+
B.E.	5	no change	-
K.Y.	5	"	-
C.G.	6	"	-
M.L.	6	"	-
E.D.	6	"	-
M.I.	6	"	-
T.D.	6	"	-
B.T.	6	"	-
P.J.	7	"	-
R.Y.	7	3.5	+
P.E.	7	no change	-
R.F.	7	3	+
H.R.	7	no change	-
H.J.	7	"	-
K.J.	7	"	-

TABLE VII (continued)

B. MILD TOXEMIA OF PREGNANCY

Patient	Month of pregnancy	Colour change of the frog	Response
D.R.	9	0	0
J.D.	8	++	pos.
L.J.	8	0	0
Mc.E.	9	+	pos.
P.T.	7	0	0
C.L.	7	0	0
L.L.	8	0	0
W.E.	7	++	pos.

TABLE VIII

ADDISON'S DISEASE

Patient	Sex	Treatment	Melanophore index from 1 to:	Response
C.I.	M	cortisone 12.5 mg. B.I.D.	2.5	+
P.E.	M	cortisone 12.5 mg. daily	no change	0
P.E.	M	off cortisone 3 days	5	+++
L.E.	M	cortisone 12.5 mg. B.I.D.	no change	0
M.R.	M	cortisone 12.5 mg. B.I.D.	no change	0
D.B.	M	cortisone 12.5 mg. T.I.D.	3.5	+
C.P.	M	off cortisone 2 days	3.5	+

TABLE IX

Reference	Blood From	Blood Preparation	Equivalent inject.per animal	Assay method	ACTH/100 ml. Plasma	ACTH/100 ml. Blood
Jores 1935 (7)	Cushing's syndrome	Deproteinized heated & concentrated serum.	12 cc.	Increase adrenal weight & histologic change in intact mice	Pos.	value not stated
Sievert 1938 (34)	Hypertension a)benign b)malignant c)nephritic	Plasma ultrafiltrate	3-6 cc.	Disappearance of sudanophobe zone in intact mice	Pos. " Neg.	"
Paschkis 1940 (25)	Cushing's syndrome. Adrenalcortical tumor. Hypertension	Not stated	Not stated	Method of Jores (above)	Pos. Neg. "	"
Rakoff 1941 (26)	Cushing's syndrome	Not stated	Not stated	Method of Jores (above)	Pos.	"
Cabeza 1942 (14)	Diabetes	Deproteinized heated & concentrated serum	20 cc.	Increase adrenal weight & histologic change in intact mice.	"	"
Bartelheimer 1942 (8,24)	Diabetes a)Cushing type b)Acromegalic type c)Insulin hypoglycemia.	Deproteinized heated & concentrated serum	20 cc.	Method of Cabeza (above)	Neg. Pos. Pos. Pos.	"

TABLE IX (continued)

Reference	Blood from	Blood Preparation	Equivalent inject.per animal	Assay method	ACTH/100 ml. Plasma	Blood
Golla & Reiss 1942 (12)	Pregnant mare	i) acetone dried serum ii) boiled serum	not stated	i)Increase adrenal weight in hypophysectomized rats. ii)Increase adrenal weight in 2day-old chick.	Pos. "	value not stated
Faber 1945 (32)	Adrenalectomized rats	Untreated serum	10 cc.	Increase adrenal weight & histologic change in hypophysectomized rats	Neg.	"
Crooke 1948 (20)	Normal	Acetone precipitate of plasma	5-10 cc.	Sayers' assay	Pos.	"
Taylor 1949 (17)	Normal Adrenal hyperfunction. Addison's disease	Untreated serum	5-10 cc. 2.5 2.5	Sayers' assay	Neg. " Pos.	" " "
Sayers 1949 (6)	Normal Normal + intravenous ACTH	Lyophilized serum	2 cc. "	Sayers' assay	> 10Y	

TABLE IX (continued)

Reference	Blood from	Blood Preparation	Equivalent inject.per animal	Assay method	ACTH/100 ml. Plasma	Blood
Bornstein 1950 (16)	Normal	Acid acetone extract	1.2 cc	Sayers' assay	197±39 Y	
	Diabetes				202±27 Y	
	Cushings' syndrome				451±104 Y	
	Simmonds' disease				28 Y	
	Congestive heart failure					
	i) in failure				412±41 Y	
	ii) controlled				192±25 Y	
	After operation				337±35 Y	
D. Parrott 1951 (19)	Normal	Acid acetone extract	Not stated	Sayers' assay	81 Y	
	Diabetes				55 Y	
	Cushings' syndrome				212 Y	
	Diabetic pregnancy				194 Y	
	Adipose gynism				214 Y	
	Simmonds' disease				0 Y	
Gemzell (3)	Normal rats	Acid acetone extract	6 cc.	Sayers' assay	0 Y	
	Adrenalectomized rats (1 week after opern.)				28 Y	
Bornstein Parrott D. (27)	Normal	Acid acetone extract	9cc. x 2 daily	Adrenal weight maintenance	Pos.	value not stated

TABLE IX (continued)

Reference	Blood from	Blood Preparation	Equivalent inject.per animal	Assay method	ACTH/100 ml. Plasma	ACTH/100 ml. Blood
Sydnor & Sayers (18)	Normal rats	Oxycellulose (Astwood's method)	12 cc	Sayers' assay		2-3 Y
	Adrenalectomized rats.		6 cc			10-12Y
	Normal subjects					>1 Y
	Addison's disease					2-4 Y
Ceresa, F. 1953 (33)	Normal	Bornstein's method	not stated	Sayers' assay	97.2 to 17.3 Y	
	Rheumatoid arthritis	Acid acetone			0 to 56 Y	

FIGURE I

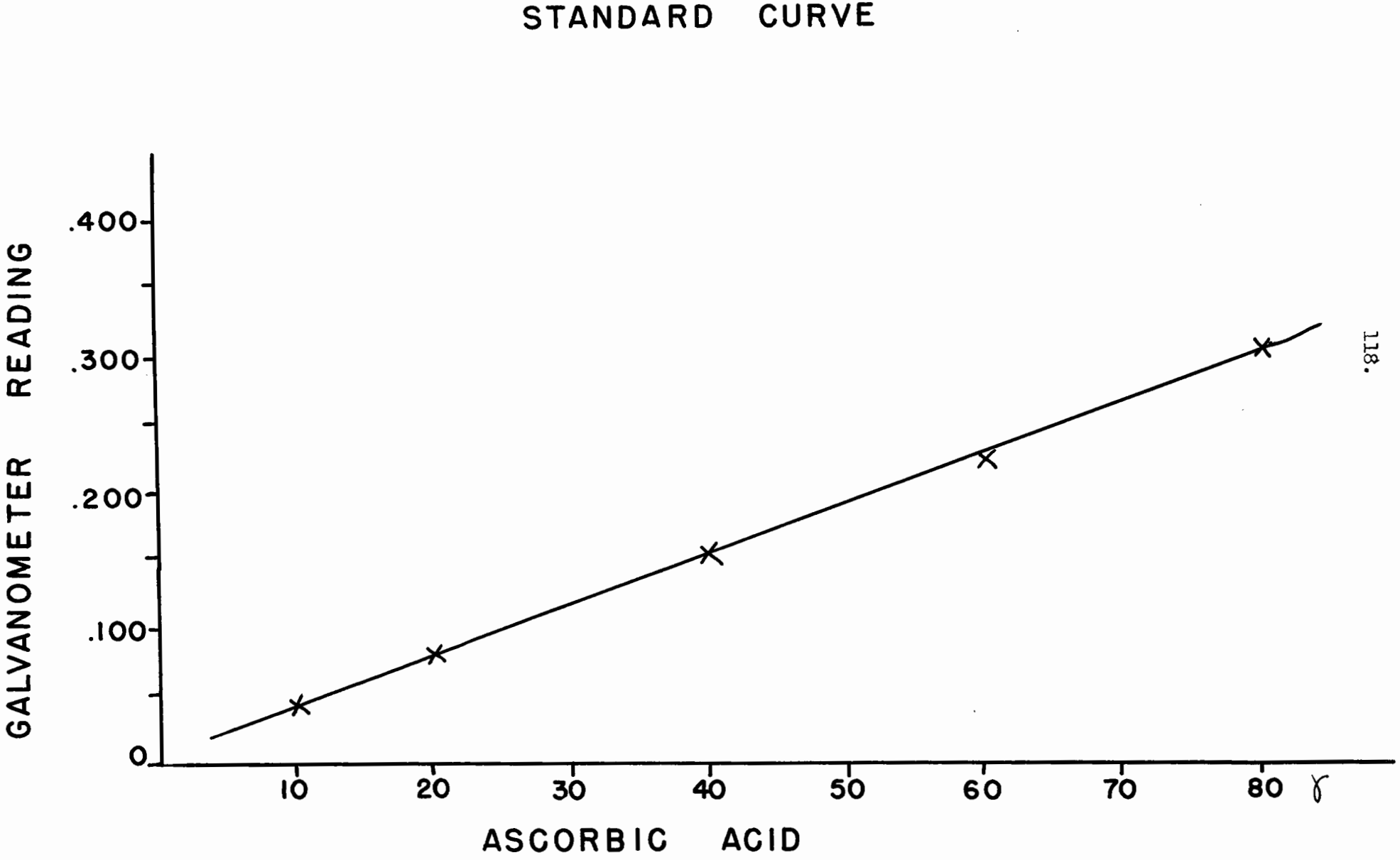

FIGURE I

SAYERS ASSAY

STANDARD CURVE

ACTH CANADA PACKERS
LOT NO. I

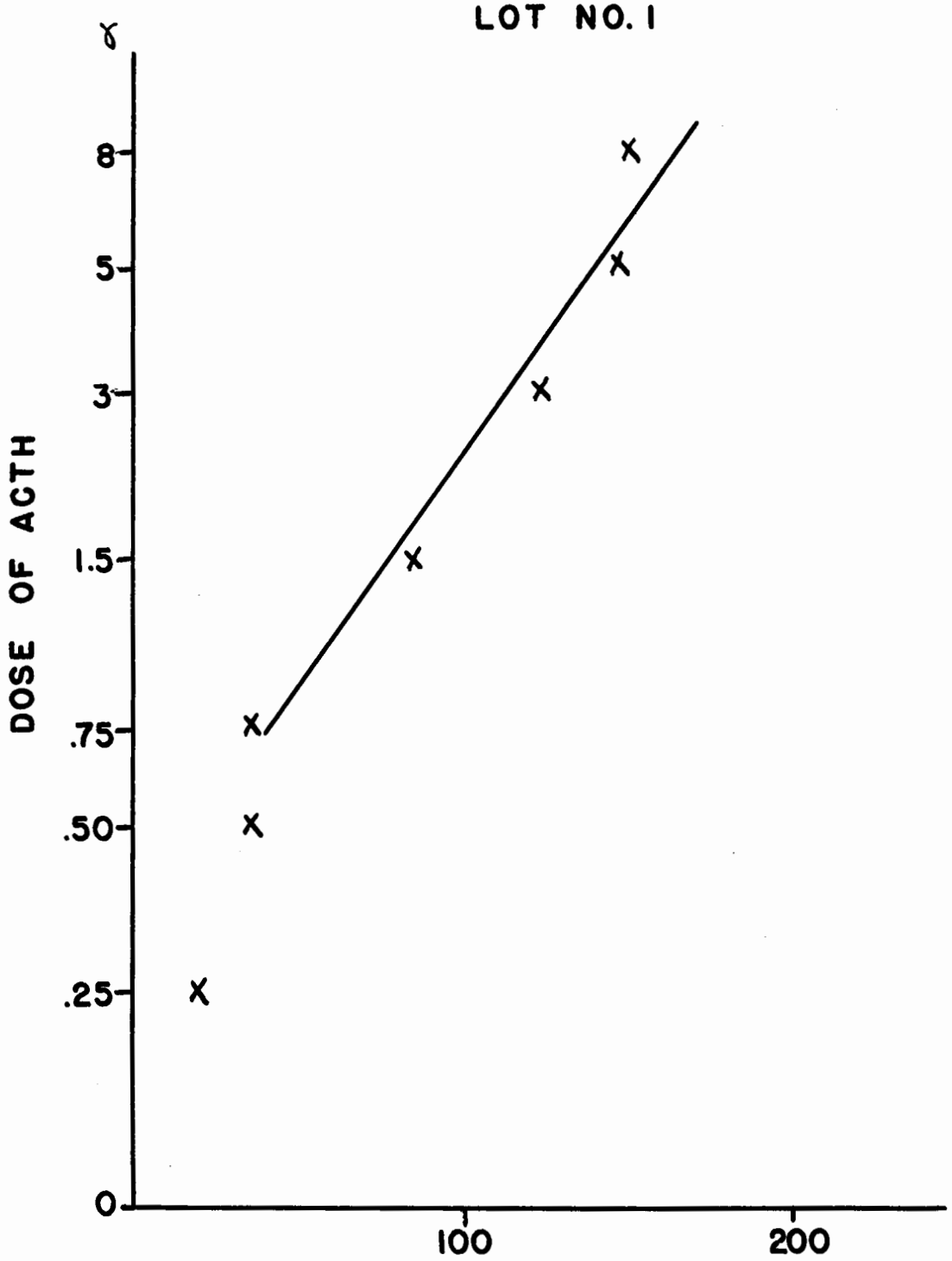

FIGURE II

COMPOUND "F" — CORTISONE ACETATE INHIBITING EFFECT ON PIGMEN — TATION CAUSED BY ACTH (MEAN VALUES OF GROUPS OF 5 FROGS)

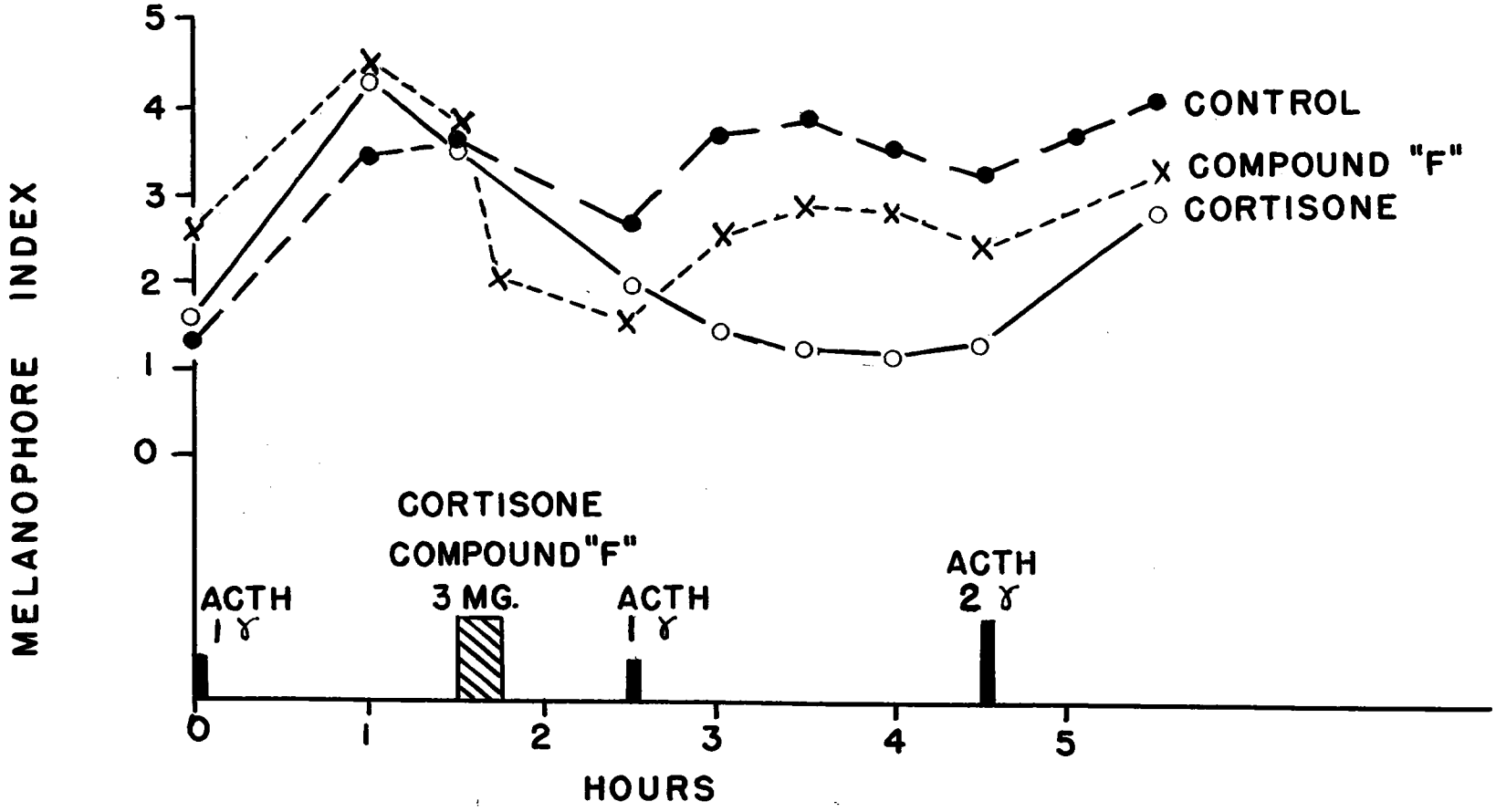

FIGURE III

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