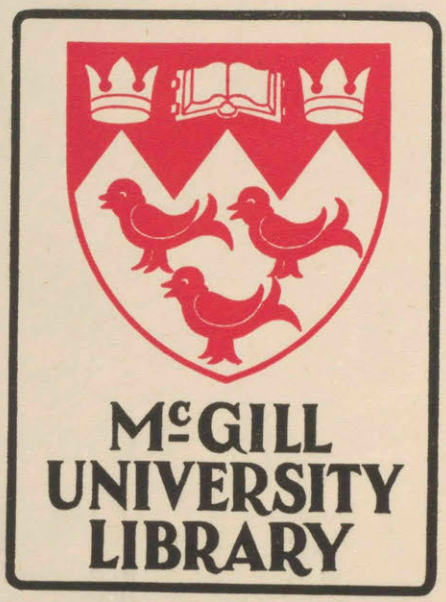

An Attempt to Locate Steroids in Tissues with Special Emphasis on The Distribution of Radio-Iodo-Estradiol in Cancer-Susceptible Mice

by

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PART I

HISTORICAL BACKGROUND

Genesis of the Present Study

One of the achievements of modern endocrinology has been the discovery of the multiple actions of steroid hormones. Of these, the male and especially the female hormones (Bullough, 1946) stimulate growth not only of the sex accessories, but also of almost all body tissues, and may even induce cancer. The adrenal steroids, on the other hand, are concerned with a wide range of metabolic processes, since they play a role in the behavior of water, salts, carbohydrates and proteins.

The isolation of steroid hormones from the gonads, adrenals and placenta has provided undeniable proof of their production in these organs. Furthermore, their excretion products in the urine have also been thoroughly investigated. The pathways of metabolism between production and excretion are, however, as yet relatively unknown, since little work has been done to determine how these compounds circulate, where they are deposited and what chemical changes they undergo.

This aspect of steroid metabolism was approached by two widely different techniques. The first was histological, and consisted in an attempt to locate steroids in tissue sections; it was thus hoped to identify not only their sites of formation, but also their preferential localizations. The second method took advantage of the ease with which radio-elements may be followed throughout the body and excreta, and consisted in tracing α-estradiol iodinated with radio-iodine.

The first systematic attempts to trace ketosteroids with the aid of histochemical methods had been made by the Boston group of histologists (Wislocki, Bennett, Dempsey). Using phenylhydrazine and later fuchsin-sulphurous acid, the so-called "ketosteroid reagents", they found intense reactions in the organs producing hormonal steroids. This was confirmed in our preliminary experiments, but some results cast doubt on the theory that ketosteroids were the responsible agents. It was necessary, therefore, to establish whether such steroids themselves or some other substances were being stained. Furthermore, it soon appeared that Bennett's reaction, although intense at the sites of steroid production, supplied no information concerning the behavior of these compounds in other tissues of the body.

A different approach to the problem of the fate of steroids was used in the laboratories of Miescher by Tschopp and in that of Lacassagne by Berger. These investigators, however, worked with artificial estrogens. They determined the fate of such estrogens either by feeding them to rats and assaying the organs (Tschopp), or by injecting artificial estrogens labeled with radioactive isotopes (Berger). Due to the differences known to exist in the treatment by the body of natural and artificial estrogens, the results obtained did not provide information applicable to those produced normally in the body. In this laboratory the problem is approached by synthesizing naturally occurring steroids labeled with radioactive isotopes. These are administered to animals and traced throughout the organs and tissues of the body. The best method would have been to use radioactive carbon (C_{14}) for labeling. Unfortunately, until very recently, this element was not available in Canada, while radioactive iodine (I^{131}) was easily procurable. It was

found that α-estradiol could be easily iodinated with elemental iodine and it soon became evident that this iodo-estradiol behaved like a phenol and seemed to have retained its steroid structure. The first experiments on the distribution of this compound revealed the dominant role of the bile and gastro-intestinal tract in its excretion. It was then found that certain organs, such as mammary glands, skin, etc., could selectively localize the iodinated estradiol.

This present work is a contribution toward the solution of the problem of the "intermediary metabolism" of steroids. Of the two methods used, histochemical and radioactive, the latter was by far the most productive. Even when some of the results were not up to expectations, the field has been cleared for further progress.

Because many of our investations were carried out on cancer-susceptible and tumor bearing animals, a brief resume of the relation of steroids and cancer will be given as well as a review of the literature on the distribution of steroids in organs and tissues of the body.

Role of Steroids in Tumorigenesis

The incidence of certain types of cancer is quite different in men and women. Thus, mammary cancer is common in females while gastric neoplasms occur more often in males. The frequent involvement of the accessory sex organs (mammary gland, uterus and uterine cervix in females, and prostate in males) led to the belief that the gonads were instrumental in the production of such tumors, long before it was realized that they elaborated hormones (Schinzinger, Beatson).

The relation of the gonads to cancer was clarified by experiments carried out in mice of various strains. It was noted that in some strains, as in humans, females showed a high incidence of breast cancer, while males, with their atrophic mammae, were immune (Loeb, 1907). If the mammary glands were prevented from developing by keeping the female animals from breeding or by spaying them, the occurrence of mammary cancers could be decreased or even suppressed. Conversely, the implantation of ovaries into males from susceptible strains produced tumors similar to those observed in breeding females (Cori, 1927; Murray, 1928). It remained for Lacassagne (1932, 1933) to show that mammary cancer could be induced in male mice of cancer-susceptible strains following the injection of large doses of estrogens. The observations of these pioneer investigators have been amply confirmed and widely extended during the past 15 years. Estrogens[1] have produced tumors of the mammary gland in mice and rats; of the pituitary in mice, rats and rabbits; of the testis and lymphoid tissue in mice; of the cervix in mice and monkeys; of the connective tissue in mice and rabbits, as well as uterine and extra-uterine fibroadenomata and ovarian neoplasms in guinea-pigs.

1 - A complete bibliography on this subject may be found in monographs by J. L. Hartwell (1941) and W. U. Gardner (1944).

While there seems to be no doubt that in certain animals tumors can be induced by estrogens, only sporadic and isolated cases of cancer have been reported in women under estrogen treatment (Zondek, 1947).

Small amounts of estrogens have a definite influence on the rate of mitosis in practically all organs and tissues of the body (Bullough, 1946). In comparison, the growth stimulatory effects of the less active luteal and testicular hormones are less striking and more or less confined to the accessory sex organs. Accordingly, the tumorigenic action of these hormones is less than that of estrogens. Androgens have only produced mammary and uterine tumors in mice and sarcomata in mice and rats (Lacassagne, 1937, 1939a, 1939b; Lacassagne and Raynaud, 1939; McEuen, 1939). Only one report could be found of the production of tumors by the luteal hormone, namely, that of Symeonidis (1947) who described mammary tumors in pregnant mice given large doses of progesterone. The adrenal cortical hormones, which possess no growth stimulatory powers, have not been found to induce tumors (Hartwell, 1941).

The Distribution of Steroid Hormones in Organs, Body Fluids and Excreta

In interpreting the results of experiments designed to determine the presence of a steroidal hormone in tissues, one must distinguish between the actual demonstration of that compound in, and its effect on, a particular tissue or organ. It is well known that estrogens cause enlargement of the mammary glands, uterus, adrenals and pituitary, yet to say that they do so by direct action on these organs is not valid unless the estrogenic substance can be traced into the target organ. Evidence of a direct action may be demonstrated by the persistence of morphological effects (change in weight and cytology) in the absence of all glands known to be concerned with the development and maintenance of the organ under investigation. Chemical extraction and biological assay of receptor organs may also reveal the presence of the active agent, but the relative insensitivity of these tests and the minute amounts of stimulatory agent present made it necessary to develop methods which are both specific and sensitive.

In the following review of the literature an attempt has been made, wherever possible, to follow a definite plan. The source of endogenous steroid hormones, transportation by means of the blood stream, distribution throughout body organs and fluids and mode of excretion will be discussed. The same plan is adhered to in discussing the fate of administered hormones. In the case of the estrogens these topics have been discussed in greater detail, because the latter half of the thesis is mainly concerned with these problems.

Estrogens*

Under Physiological Conditions

Under normal conditions, the ovary is the main source of estrogenic hormones in the female. It was shown long ago that ovarian extracts and transplants could reawaken the suppressed sexual cycle of spayed animals. The isolation of α-estradiol from sow's ovaries and its behavior in a manner similar to ovarian extracts provided the final piece of evidence, thus leading to the postulate that this was the principle estrogen. In an attempt to localize the site of production the idea was advanced that the interstitial cells were responsible. On the basis of results obtained from histological investigations and from extraction and implantation experiments this concept was largely modified. Since only traces of these hormones are present in prepubertal and senile animals, the maturing follicles, not present under these conditions, seemed to be implicated. Further evidence was provided by the observation that the injection of hypophyseal gonadotrophin greatly augments follicular maturation and thereby induced the elaboration of these hormones.

It has been much more difficult to determine whether granulosa or thecal cells are chiefly responsible. In large ovaries the various parts of the follicle can be separated and direct bioassays indicated that follicular fluid, granulosa and thecal cells contain high concentrations. Destruction of the highly sensitive granulosa cells by X-rays did not suppress estrus in mice providing thecal tissue was present. Treatment of hypophysectomized rats with luteinizing hormone failed to stimulate the granulosa, but caused marked proliferation of the theca accompanied

*For the sake of clarity all values have been converted to and expressed as International Units (I.U.), while the original values have been included in parenthesis. The following conversion figures have been employed (averages taken from Glandular Physiology and Therapy, 1942):
1 - 1 M.U. equals 2.28 I.U.
2 - 1 R.U. equals 20.84 I.U.
3 - Estrone: α-estradiol : : 1 : 4
4 - Estriol: Estrone : : 1 : 10
5 - 1 mg of estrone equals 10,000 I.U.

by estrus. The bulk of the evidence favors the view that the theca is the main source, but it is possible that the granulosa and interstitial cells also play a role, if not in elaboration then in storage of the hormones or their precursors. In addition to the follicles of the ovary, the corpora lutea constitute another source of estrogenic hormones. This subject has been very ably discussed by Claesson and Hillarp (1947b) and Selye (1947). Both α-estradiol and estrone have been isolated from the adrenal cortex and the testis (Selye, 1947). The exact site of elaboration in the latter organ has not been established. In the dog, the characteristic features of estrogenic stimulation have been found accompanying Sertoli cell tumors (tubular adenoma), thus indirectly pointing to these cells as a possible source (Innes, 1942). During pregnancy, when ovarian function is markedly diminished, the placenta assumes an active role in the production of estrogens (Philipp, 1929; Abderhalden, 1942), two of which have been characterized as α-estradiol and estrone (Selye, 1947). An attempt has recently been made to implicate the synciteal layer of the trophoblast (Dempsey and Wislocki, 1944).

Following elaboration in these organs, estrogens are distributed throughout the body by means of the blood stream; their presence in this fluid has been demonstrated by many workers. Under normal physiological conditions the estrogenic titer of the blood is rather low, ranging from 20 to 60 I.U. per 100 cc. (Szego and Roberts, 1946; Roberts and Szego, 1946), and varies with the different phases of the menstrual cycle (Fluhmann, 1934). Somewhat higher values have been reported by Krichesky and Glass (1947a), but since their bioassay method is not specific for estrogens (Krichesky and Glass, 1947b), the results must be regarded with suspicion. In the blood it seems that most of the estrogenic material is closely associated with the lipoprotein fraction (III 0 of Cohn), the protein portion consisting mainly of β-globulins.

During pregnancy there is a tendency for the estrogenic level of the blood to increase, reaching values as high as 570 I.U. per 100 cc. (Kemp and Pedersen-Bjergaard, 1933-34; Cantarow, Rakoff, Paschkis, Hansen and Walkling, 1943). Of this activity about 60 per cent is found in the plasma and 40 per cent in the cells (Kemp and Pedersen-Bjergaard, 1932). Following termination of pregnancy blood estrogens fall precipitously; values of 36, 18 and 9 I.U. per 100 cc. have been reported at three, four and seven days post-partum (Cantarow, Rakoff, Paschkis, Hansen and Walkling, 1943).

Due to the normally low blood levels it has not been possible to demonstrate estrogenic activity in the organs and tissues of the body. But because of the increased level during gestation, activity has been found in the liver[1] (Parker and Tenney, 1938), colostrum and milk of pregnant females (Bruhl, 1929; Winter, 1932). The latter finding prompted investigators to assay the products of conception.

Umbilical blood (Fels, 1926; Bruhl, 1929), amniotic fluid (Bruhl, 1929) and the mammary secretion of the newborn (Joseph, 1929) showed estrogenic material. In addition, various organs and excreta of the fetus were found to give positive assays. Meconium (Gsell-Busse, 1929) and liver (Parker and Tenney, 1938) were the richest, containing 12,504 I.U. (600 R.U.) and 38 to 912 I.U. (16.6 to 400 M.U.) per 100 gm. respectively. The blood, ovaries, testes and kidneys contained much less. The exact source, undetermined as yet, was presumed to be maternal. This whole subject has recently been reviewed in a monograph by Price (1947).

Since estrogens and their metabolic products are demonstrable in the feces and urine, they must be eliminated from the body via the gastrointestinal tract and kidneys. Most of the fecal estrogens appear to be derived from the bile, since considerable activity could be found in this fluid in the non-pregnant and pregnant mammal. Values ranging from 167 to

1 - 114 to 342 I.U. (50 to 150 M.U.) per 100 gm.

1250 I. U. per 100 cc, depending on the species, have been reported (Gsell-Busse, 1929; Cantarow, Rakoff, Paschkis, Hansen and Walkling, 1943; Pearlman, Rakoff, Cantarow and Paschkis, 1947). Part of the estrogenic activity found in the bile must be resorbed in the gastro-intestinal tract, as demonstrated by the fact that only a small per centage was recoverable in the feces. Normal women excrete about 1600 I.U. of estrogenic material during the entire menstrual cycle (Siebke and Schuschania, 1930), while normal and castrate men excrete from 68 to 160 I.U. per day (Kemp and Pedersen-Bjergaard, 1933-34).

Estrogens have also been found in the feces of mice[1] (Dorfman and Gardner, 1944) as well as in those (cloacal in origin and probably contaminated with urine) of the fowl (Gustavson, 1931; Berdnikoff, 1936). During pregnancy the amount of estrogens in the feces was found to increase markedly in the human[2] (Dohrn and Faure, 1928), the mare[3] (Kemp and Pedersen-Bjergaard, 1933-34) and the cow[4] (Levin, 1945). In the latter this material was shown to exist in an unconjugated form.

In contrast to the small amounts of biologically active estrogens found in the feces, relatively large amounts are excreted in the urine. Normal and castrate men excreted less than 2.28 I.U. (1 M.U.) per 100 cc.of urine and women from 104 to 5206 I.U. (5 to 250 R.U.) per 24 hours (Kemp and Pedersen-Bjergaard, 1933-34; Smith, Smith and Pincus, 1938; Werner, 1941). The fact that castration had very little effect on the urinary excretion in males, suggests that some other gland, possibly the adrenal, is responsible for the elaboration of this substance in the male. A great deal of work has been done on the correlation between urinary estrogen excretion and the phase of the menstrual cycle. Smith, Smith and Pincus (1938) observed two peaks of activity, on the 11th and 19th day. A similar

1 - 0.04 I.U. per day
2 - 6840 I.U. (3000 M.U.)
3 - 951 I.U. (417 M.U.)
4 - 20840 I.U. (1000 R.U.)

cyclicity has been observed by Werner (1941) and Siebke and Schuschania (1930). In an attempt to simplify the detection of urinary estrogens, various colorimetric assay methods have been devised. These have yielded very interesting but as yet unexplained results. Pincus, Wheeler, Young and Zahl (1936) found that the amount detected colorimetrically was from 309 to 2070 percent higher than that obtained biologically. The overestimation was present in both the estrone and estriol fractions. Jayle, Crepy, Vandel and Judas (1946) obtained results similar to those of Pincus and his associates. Both these groups of investigators concluded that inactive chromogenic material, possibly derived from estrogens, was present. The discovery by Häussler (1934) that stallions excreted on the average 38,760 I.U. (17,000 M.U.) of estrogenic material per 100 cc. of urine led to the observation that this mass excretion of follicular hormone is a property exclusively inherent in male equines (horse, zebra, donkey), and not encountered in other male animals (Zondek, 1941). Among laboratory animals it has been found that certain mice[1] excreted estrogenic material in the urine (Dorfman and Gardner, 1944). During pregnancy, in keeping with the general increase in the circulation of estrogens, the amounts excreted in the urine rise considerably. Values approximating 2736 I.U. (1200 M.U.) in women and 22800 I.U. (10000 M.U.) in mares per 100 cc. are not uncommon (Ascheim and Zondek, 1927; Zondek, 1939).

1 - 0.03 I.U. per day

Following Administration of Estrogens

The distribution and excretion observed under normal conditions is emphasized following the administration of natural estrogens and bears a striking resemblance to what occurs during pregnancy. After introduction of synthetic estrogens, on the other hand, a different behavior is found, consisting of a greater and longer retention and, in general, a slower rate of metabolism.

Following subcutaneous injection, the rate of absorption of natural and synthetic estrogens differ. Thus, 48 hours after the administration of from 2280 to 22,800 I.U. (1000 to 10,000 M.U.) of estrone, or 100 I.U. of stilbesterol, less than 10 per cent of the former and 25 per cent of the latter was recovered at the site of injection (Dingemanse and Laqueur, 1937); Zondek, 1941). In this respect, the synthetic estrogen, stilbesterol, resembles natural estrogen esters.

After entering the blood stream natural estrogens are rapidly removed from the circulation. This phenomenon has been observed in the rabbit[1] (Frank, Goldberger and Spielman, 1932), the dog[2] (Stamler, 1937) and the human female[3] (Zondek, 1941; Krichesky and Glass, 1947a). This is exemplified by the experiments of Cantarow, Paschkis, Rakoff and Hansen (1943). These workers showed that, 24 hours after the administration of 250,000 I.U. of α-estradiol to external bile-fistula dogs, only 450 I.U. were in the blood. Of this activity, 300 I.U. were found in portal vein

1 - 57 I.U. (25 M.U.) per 100 cc. recovered following the intravenous administration of from 4560 to 6840 I.U. (2000 to 3000 M.U.) of an unnamed estrogen.
- Krichesky and Glass (1947a) recovered 283 I.U. per 100 cc. following the subcutaneous administration of 1500 I.U. of estrone.

2 - 90 per cent of the estrone administered intravenously disappeared within a few minutes.

3 - Zondek (1941) found less than 100 I.U. per 100 cc. after administration of 3,600,000 I.U. α-estradiol benzoate to a young girl over a period of 60 days.
- Krichesky and Glass (1947a) recovered 357 I.U. per 100 cc. after the subcutaneous administration of 1500 I.U. of estrone to women.

blood, none in hepatic vein blood, 100 I.U. in femoral artery blood and 50 I.U. in inferior vena cava blood. At the end of 48 hours no estrogenic activity could be detected in the circulation.

In contrast to these results, Dingemanse and Tyslowitz (1941) found that diethylstilbesterol, a synthetic estrogen, persisted in the blood for some time, since 2000 I.U. per 100 cc were still present 48 hours after the administration of about 100,000 I.U. (5 mg.). More recently, Berger (1946) found, following the injection of the synthetic estrogen, triphenylethylene labeled with radio-bromine, into mice, considerable amounts present after 15 hours. Indeed, the blood contained more than that present in all the other investigated organs combined. It must be noted, however, that, while a dose of less than 5 micrograms of this substance is estrogenic in the mouse, the extremely large amount of 1 mg. per mouse was employed. It is apparent from these experiments that the blood is freed rapidly from the natural estrogens but more slowly from the artificial ones.

Inability to detect more than traces of natural estrogens, following administration of relatively large doses, reflects the speed with which they are eliminated. In the entire organism (blood, carcass, and fur) of rats treated with large doses of estrone, 10 per cent only was recovered by Dingemanse and Laqueur (1937) at the end of 6 hours. Even lower values, 1 to 2 per cent after 2 hours and 0.2 per cent after 48 hours, have been reported by Zondek (1941). The yield from the entire organism can be increased to 20 per cent after 2 and to 6 per cent after 48 hours by acid hydrolysis, thus suggesting that some of the estrogenic material had been conjugated.

No outstanding concentration has been observed in most of the internal organs, except the liver, from which a small amount has been

recovered in the rabbit[1] (Frank, Goldberger and Spielman, 1932) and in the dog. In the latter animal, Dingemanse and Tyslowitz (1941) using daily doses of 40,000 I.U. (5 mg.) of α-estradiol up to a total of 80,000 to 2,500,000 I.U., observed that the liver contained 80 to 800 I.U. before and 800 to 3000 I.U. after hydrolysis, again indicating that most of the estrogenic material was conjugated. This latter finding has been confirmed by Cantarow, Paschkis, Rakoff and Hansen (1943) who recovered 26 I.U. of both free and conjugated material 24 hours after the administration of 250,000 I.U. of α-estradiol to external bile-fistula dogs, and none thereafter. Traces - less than one tenth of that found in the liver - were also found in the kidneys, heart plus lungs, brain and spleen (Dingemanse and Tyslowitz, 1941).

Following the administration of artificial estrogens, a more widespread distribution has been observed. This is not surprising, since, a relatively high circulating blood level for a longer period has been demonstrated. Dingemanse and Tyslowitz (1941) observed a considerable amount of diethylstilbesterol in the livers of dogs, comparable to that found after the administration of natural estrogens. Similarly, Tschopp[2] (1946) found that, following the administration of 7-methyl-bisdehydro-doisynolic acid to rats, the livers of both sexes fixed a large amount, exceeded only by that found in muscle. Following the injection of radio-brom-triphenylethylene into mice, Berger (1946) observed that the liver contained a total quantity surpassed only by that of the blood, although the concentration was smaller than in several other organs.

Moderate amounts of estrogenic material have been recovered from

1 - 24 hours after the injection of 6840 I.U. (3000 M.U.) of an unnamed estrogen, 4.56 I.U. (2 M.U.) were recovered.

2 - Administered a single dose (1 mg. per kilogram) of 7-methylbisdehydro-doisynolic acid by stomach tube to rats, and bioassayed the organs from the treated animals by feeding them to spayed, female test animals.

the remaining organs after administration of synthetic compounds (Dingemanse and Tyslowitz, 1941; Tschopp, 1946; Berger, 1946). Tschopp (1946) found the greatest concentration of 7-methyl-bisdehydro-doisynolic acid in muscles, fat, heart, salivary glands, etc. Berger (1946) observed similar concentrations of brom-triphenylethylene in kidneys, salivary glands, lungs and brain, but all were lower than that in the blood. The skin was examined by Tschopp (1946) and was found to contain a rather low concentration; however, the total quantity fixed was fairly large, exceeded only by that in the muscle, liver, and gastro-intestinal tract. Somewhat similar observations were made by Bird, Pugsley and Klotz (1947) who found the concentration of synthetic estrogens in the adipose tissue and the skin of the fowl very high.

The female gonads and accessory sex organs were found by Berger (1946) to contain the second highest concentration of estrogenic activity in the body. Of these the ovaries were the richest, a fact interpreted as indicating that the compound used (brom-triphenylethylene) resembled a pro-estrogen and was transformed by the ovary into a true estrogen. The concentration in the mammary glands and especially the uterus increased with time and reached a level comparable to that of the blood. A high uterine concentration was also observed by Tschopp (1946).

Of the male sex accessories, Berger (1946) found that the preputial glands showed the highest concentration of all bodily organs with the exception of the blood. Tschopp (1946), on the other hand, observed that among these accessories the seminal vesicles contained the highest; the preputials moderate; and the epididymis and prostate much lower concentrations, but these were exceeded by the concentrations in other organs.

The administered estrogens (natural and synthetic) were found to be excreted in a manner similar to that found under normal physiological

conditions. Certain subtle differences, however, can be shown to exist between the amounts of natural and synthetic estrogens in urine and feces.

Administered estrogens are cleared from the blood, for the most part, by the liver and kidneys, thus appearing in the bile, gastro-intestinal tract, feces and urine. All these organs and excreta have been intensively studied. Longwell and McKee (1942) recovered 1.3 to 8 per cent of the injected dose (10,000 I.U. or 1 mg) in the bile of dogs. Much higher values were observed by Cantarow, Rakoff, Paschkis, Hansen and Walkling (1943) in a series of well executed experiments. Within three days following the administration of 250,000 I.U. of estrone to external bile-fistula dogs, these investigators recovered 82 to 96 per cent of the injected dose. The same group of workers (Pearlman, Paschkis, Rakoff, Cantarow and Walkling, 1947) found that the bile contained more estrogenic material than either urine or feces following the administration of 1.3 gm. of estrone to the same type of test animals, thus suggesting that part of the material excreted by the bile was continually resorbed in the intestine. On the other hand, the recovery of estrogenic material in the bile of dogs was considerably lower following the administration of diethylstilbesterol (Cantarow, Rakoff, Paschkis, Hansen and Walkling, 1943). In contrast, Berger (1946) found a fairly high concentration of radio-brom-triphenyl-ethylene in the gall bladder of the mouse, while Twombly (1947) also found, following the administration of radio-brom-equilin to rabbits and monkeys, a prompt excretion via the bile.

The Philadelphia group (Cantarow, Rakoff, Paschkis, Hansen and Walkling, 1942, 1943, 1947) found that the estrogenic material excreted into the bile was in a non-conjugated form since it was extractable without hydrolysis. Estrogenic material could also be recovered from the bile even when α-estradiol was introduced into the duodenum of external bile-

fistula dogs. Less than 25 per cent of the injected dose could be recovered over a period of 4 days, but, 20 of the 25 per cent recovered was in a conjugated form. These observations suggested that the non-conjugated material released in the bile is combined in the duodenum.

There is some evidence that the estrogenic activity of the bile consists mainly of non-ketonic, weakly acidic phenols (estradiol?) (Longwell and McKee, 1942; Paarlman, Paschkis, Rakoff, Cantarow and Walkling, 1947), a small amount of strongly acidic phenols (estriol?) and a minor portion in the weakly acidic ketonic fraction (estrone?) (Pearlman, Paschkis, Rakoff, Cantarow and Walkling, 1947).

No estrogenic activity has been recovered so far from the gastro-intestinal tract following the administration of natural estrogens. A high content was found following the injection of the synthetic estrogen, 7-methyl-bisdehydro-doisynolic acid (Tschopp, 1946). A similar observation was made by Twombly (1947), who, 6 hours after the administration of radio-brom-equilin to rabbits, recovered 35 per cent in the intestinal contents. In contrast, Berger (1946) observed a small amount of radioactivity in the duodenum of mice 15 hours after administering radio-brom-triphenylethylene

Rather small amounts of estrogenic activity have been recovered from both feces and urine following administration of natural estrogens. When estrone was administered to rats 0 to 3 per cent was recovered, while following various esters of estrone and estradiol the recovery was 1 to 5 per cent. On the other hand, 25 per cent of injected stilbesterol could be recovered in experiments of comparable duration to those done with the natural estrogens and their esters (Zondek, 1941).

In the feces, Luchsinger and Voss[1] (1929) recovered 0.4 per cent of an oral dose in a woman, amenorrheic for 4 years, while Siebke and Schuschania[2] (1930) were unable to detect any increase above the normal (58 I.U. or 26 M.U.) following oral or parenteral administration of rather low doses. However, this latter investigator observed that, when larger doses[3] were administered fecal excretion rose to 225 I.U. (98 M.U.) per day. In the human male, Kemp and Pedersen-Bjergaard (1933-34) found that, after low doses,[4] the excretion in the feces did not appreciably vary from the controls, but, large oral doses[5] permitted recovery of 4.1 to 14.3 per cent. Following administration of the same dose parenterally, excretion dropped to less than 1.4 per cent. In external bile-fistula dogs, Pearlman, Paschkis, Rakoff, Cantarow and Walkling (1947), following the administration of 1.3 gm. of estrone, recovered a small but significant portion in the feces. This suggested that the gastro-intestinal tract was capable of estrogen secretion. The amount of conjugated material found in the feces by these workers was small but larger than that in the bile. A small fraction of the estrogenic activity present was in the strongly acidic phenol fraction, indicating the possible presence of estriol. Following the subcutaneous administration of the synthetic estrogen, radio-brom-triphenylethylene, only a small amount was recovered in the feces (Berger, 1946).

1 - 1140 I.U. (500 M.U.) of Progynon administered orally and 4.56 I.U. (2 M.U.) recovered.
2 - 2280 to 5700 I.U. (1000 to 2500 M.U.) of Progynon administered orally.
3 - 45,000 I.U. (20,000 M.U.) of Progynon administered over a period of 42 days.
4 - 2280 I.U. (1000 M.U.) of estrogenic material derived from mare's urine administered subcutaneously.
5 - Up to 22,800 I.U. (10,000 M.U.) of estrogenic material derived from mare's urine either per os or parenterally.

In the urine of mammals, appreciably larger amounts of administered estrogens may be found than in the feces. In the human female, Luchsinger and Voss (1929) were able to recover 4 per cent of an injected estrogen (Progynon) within 24 hours. Siebke and Schuschania (1930) found that by increasing the oral dose, urinary secretion was effectively raised. On the other hand, Frank, Goldberger and Spielman[1] (1932), administering small doses of estrogen during a normal cycle, detected no variation from the usual excretion pattern of women. Zondek (1941) observed that 3 per cent of administered estrone was excreted by the kidneys, and found the urinary level to be independent of the dose. With small doses[2] in the human male, Kemp and Pedersen-Bjergaard (1933-34) could only recover traces of activity in the first 12 hours and none thereafter. When larger doses[3] were administered orally, 5.7 to 6 per cent were found; when given subcutaneously the recovery dropped to 2.8 per cent. Heard and Hoffman (1941) were able to retrieve 10.4 per cent following the injection of a massive dose (250 mg) to a young man.

1 - 11,400 I.U. (5000 M.U.) of an unnamed estrogen administered to a woman on 9th., 15th., and 26th. day of the cycle.
2 - 2280 I.U. (1000 M.U.) of estrogenic material derived from mare's urine.
3 - 20,520 to 22,800 I.U. (9,000 to 10,000 M.U.) of estrogenic material derived from mare's urine administered orally and 1202 to 1336 I.U. (527 to 586 M.U.) or 5.7 to 6 per cent recovered over a period to 60 hours. When the same dose administered subcutaneously 581 to 677 I.U. (255 to 297 M.U.) or 2.6 to 2.8 per cent was recovered.

Great specie differences in urinary excretion have been demonstrated. Following the injection of 3420 I.U. (1500 M.U.) of estrogenic material into monkeys, 20 per cent could be regained (Frank, Goldberger and Spielman, 1932). Slightly lower amounts, 6.5 to 16 per cent, has been recovered in dogs in the three days succeeding injection (Dingemanse and Tyslowitz,[4] 1941; Longwell and McKee,[5] 1942; Cantarow, Rakoff, Paschkis, Hansen and Walkling,[6] 1942). Of this material about 60 per cent was in a conjugated form (Dingemanse and Tyslowitz, 1941). Some of the activity in the urine following estrone administration has been demonstrated in the ketonic fraction (Pearlman, Paschkis, Rakoff, Cantarow and Walkling, 1947). Of the 10.4 per cent recovered by Heard and Hoffman (1941) following the injection of α-estradiol, 3.9 per cent was unchanged and 6.4 per cent was estrone.

4 - 50,000 I.U. (5 mg.) of estrone.
5 - 10,000 I.U. of estrone.
6.- 10,000 to 250,000 I.U. of estrone or 250,000 I.U. of α-estradiol.

Androgens

That the testes of mammals are the chief source of the male sex hormone has been amply demonstrated in the past by the effect of castration on the prostate and other sex accessories and their return to normal by testicular extracts and testosterone. The Bouin school showed that in the testes the interstitial cells were the main site of hormone elaboration, by demonstrating that vasoligation, x-ray treatment, exposure to heat and many other interventions destroyed the seminiferous epithelium but leave the interstitial cells intact and did not interfere with the endocrine activity of the testis (Bouin and Ancel, 1923). This was finally proved by the selective action of pituitary fractions (luteinizing hormone) on the interstitial cells and the male sex accessories; while these extracts had no effect on the accessories in the castrate.

The nature of the male sex hormone was further clarified by the isolation of the ketosteroid, testosterone, from the testis of the bull (Laqueur, David, Dingemanse and Freud, 1935; David, Dingemanse, Freud and Laqueur, 1935; Goldberg, 1938), and the horse (Tagmann, Prelog and Ruzicka, 1946). A non-crystalline substance has recently been isolated from hog testis showing the same infra-red absorption as testosterone (Prelog, Tagmann, Lieberman and Ruzicka, 1947). The pregnene derivative, Δ^5-pregnenolone, has been isolated from porcine testes (Ruzicka and Prelog, 1943). Whether this hormonal steroid, represents another type of male sex hormone or a precursor of testosterone has not been determined.

The state in which the testicular hormone or hormones exist in this gland is not clear. Goldberg (1938) was able to extract testosterone from bull testes without using the drastic measures employed by previous workers (for instance, concentrated acid), indicating that probably some of the testosterone,

in this species at least exists in a free state. McCullagh (1939) claimed that the androgenic activity of bull testis exists in a state soluble in dibutyl ether, in which all known active androgens are soluble, and that this ether soluble material, unlike testosterone, lost most of its activity on boiling with 5 per cent sulphuric acid for 15 minutes. On the other hand, extraction of the testes with alcohol, in which inactive androgens are soluble, and then re-extraction with dibutyl ether yielded androgenic material. The portions extracted with alcohol (but not re-extracted with dibutyl ether) were boiled with acid and showed more activity than the ether soluble fraction. The conclusion is that either more than one androgen exists, or, if only one is present, part exists in a free and part in a bound state. In view of the similar results obtained by Goldberg (1938) the latter hypothesis is probably correct.

In addition to the testis, a certain amount of androgenic material is also produced by the adrenal cortex and ovaries. This has been demonstrated in the adrenal cortex by chemical extraction of androgens, (-androstenedione, adrenosterone and androstane- 3(β), 11( )-diol-17-one) and biological experimentation (Selye, 1947). The ovaries of some animals (pig, guinea-pig, rat and mouse) have been shown to furnish sufficient testcid material to maintain normal size and function of the sex accessories of castrate males (Lipschutz, 1932; Parkes, 1937; Hill, 1937; Deansley, 1938).

The androgenic material released into the circulation has been demonstrated in the blood stream by the capon-comb test. Sacchi and Fraschini (1935) found more activity in spermatic vein blood than in the general circulation. McCullagh and Osborn (1938) recovered 4 I.U. (equivalent to 6 micrograms) of testosterone per 100 cc. from the blood of normal young men. This androgenic

material existed in an inactive form, but could be activated by boiling with acid or the addition of tissue extracts. Further evidence of androgen transportation by the blood has been provided by Shipley, Meyer and Biddulph (1943). These workers observed that when 300 micrograms of testosterone propionate were administered to one partner of castrate rats in parabiosis, the equivalent of 8 micrograms of testosterone propionate crossed to the uninjected parabiont. Normal seminal vesicles and prostate glands were obtained in the non-injected member when 1,000 micrograms were administered. In contrast, Dingemanse and Tyslowitz (1941) found no androgenic activity in 70 cc.of blood from dogs as early as one half hour following a dose of 10 mg of testosterone propionate.

Testoid activity also has been demonstrated in the anterior hypophysis of cattle[1] (Prelog and Beyerman, 1945). With the exception of the epididymis (Goldberg, 1938), no androgenic activity has been found in any of the remaining organs, even after the administration of large doses of testosterone propionate[2] (Dingemanse and Tyslowitz, 1941).

The majority of the excreted androgenic material has been found in the urine (Pincus and Pearlman, 1943) and only traces in the feces[3] (Samuels and Bittner, 1947). Both testicular and adrenal androgens are excreted mainly as 17-ketosteroids, (androstenone, etiocholanolone, androsterone, iso-androsterone, and dehydro-iso-androsterone). Normal adult men excrete 20 to 120 I.U. of testoid material per day, normal adult women 20 to 50 I.U., castrate men 3 to 13 I.U. and ovariectomized women 6 to 16 I.U. (Pincus and Pearlman, 1943; Selye, 1947). These findings indicated that, following gonadectomy, androgen

1 - 0.5 capon comb units (equivalent to 50 micrograms of androsterone or 7.5 of testosterone) per kilogram.
2 - 810 mg.of testosterone propionate administered over a period of 81 days.
3 - 17-ketosteroids were reported in the feces of mice of the cancer susceptible "C3H" and "A" strains.

excretion of men and women approached the same level, and indicated that the adrenal cortex produced similar amounts in both sexes. Androgenic and 17-keto-steroid excretion of animals was found to vary from 1 to 5 I.U. per liter of urine and therefore was considerably below that of men (Selye, 1947).

Following the administration of testosterone or its esters, androgenic material may be recovered from the urine. The percentage of recoverable urinary androgens paralleled the amount injected, which is in contrast to estrogen excretion where dosage did not seem to influence percentage recovery. For instance, Dorfman and Hamilton (1939) observed that, following the administration of a single dose of 5 mg. of testosterone to male and female monkeys, less than 1.7 per cent was recovered.[1] Following the introduction of 20 mg. in a single dose, 1.2 to 6.4 per cent was retrieved. When 70 to 80 mg. was injected over a period of 30 days, 7.8 to 11 per cent was present in the urine. After the administration of 340 mg. in divided doses on alternate days for a month, between 5.4 and 15 per cent was retrieved. More recently Hoffman, Sabin and Desbarats (1947) were able to recover 40 per cent of a massive dose of testosterone (5 gm.) administered to a man and a rabbit. In addition to the usual keto-steroids, these workers identified two alcoholic steroid metabolites, etiocholanediol-3(α), 20(α) (man and rabbit) and androstanediol-3(β), 17(α) (rabbit). In man, the alcoholic metabolite comprised 3 per cent of the recovered material.

1 - calculated as androsterone.

Progesterone

The luteal hormones, principally progesterone, are produced for the main part by the lipid laden cells of corpora lutea. Progestational changes in the uterine endometrium occur only in the presence of functional corpus luteum tissue, while extirpation of these structures causes a breakdown of the progestational endometrium even in the presence of the remaining ovarian tissue. Certain extra-ovarian tissues, especially the adrenal (Selye, 1943) and the placenta (Adler, de Fremery and Tausk, 1934;Ehrhardt, 1934; Selye, Collip and Thomson, 1935; Abderhalden, 1942) can also produce luteal hormones. The placenta assumes an important role in the production of progesterone, or similar hormones, during pregnancy, since in some animals it completely takes over hormone production from the ovary. As yet it has not been possible to demonstrate luteoid activity in any of the other organs and tissues of the body with the exception of the blood.

Allende (1940), using a bioassay method based on intrauterine injection into infantile and estrogen primed rabbits, demonstrated the presence of luteal hormone in the blood of monkeys throughout the menstrual cycle, and found that the curve gradually fell during the second half. She calculated that a maximum of 0.25 to 2.5 micrograms and a minimum of 0.06 to 0.12 micrograms of hormone was present in 1 cc of blood. Hoffmann and Lam (1942) using a similar bioassay method, demonstrated luteoid activity in the blood of pregnant women. Abderhalden (1942) found minute amounts of activity in 10 liters of pig blood and none in 500 cc of pregnancy blood. Reynolds and Ginsburg (1942), employing ultraviolet absorption spectrophotometry, were unable to detect progesterone in the blood serum of rabbits. They estimated that if any were present, it must be

in quantities less than 0.008 to 0.010 micrograms of progesterone-equivalents per cc. Forbes and Hooker (1948) found that random samples of blood plasma from pseudopregnant rabbits, pregnant mice, a pregnant woman, and a monkey in the luteal phase of the cycle contained 5.5 to 8.0 micrograms of progesterone per cc. Approximately 90 per cent of the hormone was biologically active without fractionation and was readily extracted with ether or acetone. The remaining 10 per cent appeared to be bound to protein and showed no activity until freed by partial hydrolysis with acid. These workers employed a highly sensitive and fairly specific bioassay method based on stromal nuclear changes in a uterine segment of a spayed, young, adult mouse following the injection of progesterone into that isolated segment.

The chief metabolic product of ovarian progesterone, pregnanediol, is found almost exclusively in the urine occurring, in the human, as sodium pregnanediol glucuronidate (Venning and Browne, 1937). Other metabolic products such as pregnanolone and its isomers have been claimed as end products of progesterone metabolism (Selye, 1947). The excretion of sodium pregnandiol glucuronidate (5 to 10 mg per day) commences one or two days after ovulation and reaches a maximum about 1 week prior to the onset of bleeding. (Jayle and Libert, 1947).

Following the injection of 5 mg. of progesterone to infantile rats, Zondek (1941) recovered 20 per cent of the dose in their bodies at 6 to 12 hours, less than 20 per cent at 24 hours and none thereafter.

Apparently exogenous progesterone is metabolized in the same manner as the endogenously produced hormone, since, Heard, Bauld and Hoffman (1941) were able to retrieve 7 to 11 per cent of a dose of 300 mg. administered to female rabbits in the urine as pregnanediol. The rate of conversion in the human is similar to that described in the rabbit by the above mentioned investigators.

Adrenal Cortical Hormones (Corticoids)

The adrenal cortex is the main source of steroid hormones possessing corticoid activity. Of the many isolated, all but a few are ketosteroids (Selye, 1943; Miescher, 1946). The compounds, corticosterone, dehydro-corticosterone, 17-hydroxy-corticosterone, 17-hydroxy-11-dehydro-corticosterone, desoxycorticosterone and 11-desoxy-17-hydroxy-corticosterone are representative of the type elaborated. An oxygen at C11 in the form of a hydroxyl or ketone denotes a carbohydrate regulatory function, while the absence of the C11 oxygen indicates a mineral and water regulatory function. Giroud, Martinet and Bellon (1940) employing an assay method based on the ability of cortical hormones to produce a slow contraction of the pigmented cells of the carp, found activity to be predominantly present in the zona fasciculata and reticularis, while the glomerulosa possessed only minute amounts. This suggested that cortical hormones were predominantly formed in the first two areas. Recently, Greep and his associates (Greep, 1946; Deane and Greep, 1946a; Deane and McKibbin, 1946b; Deane and Shaw, 1947a) have demonstrated by means of morphological and cytochemical studies, that the zona fasiciculata may atrophy; may become hyperactive; or may become exhausted without any marked alteration in the glomerulosa. These results were interpreted as indicating that the cortex was composed of two separate secretory zones, the glomerulosa possibly secreting the 11-desoxy type, and the fasciculata the 11-oxy type of cortical hormones. In addition to the adrenal cortex, small amounts of corticoid activity may be found in the corpus luteum, probably because progesterone itself possesses this property to a limited extent.

Following elaboration, the corticoids pass into the blood stream as shown by Giroud, Ratsimamanga and Chalopin (1941) and Vogt (1943). The latter investigator, Vogt, calculated that the average output of one adrenal per

minute per kilogram of body weight was equivalent to the activity extractable from 0.6 gm. of adrenal tissue. Under certain conditions of stress, the corticoid activity of 1 cc of suprarenal plasma may be ten times as high as that extractable from 1 gm.

Cortical hormones, as determined by the assay method of Giroud and his associates (1941) appear to have a widespread distribution throughout the body. The adrenal cortex, the site of elaboration of these hormones, contained 4500 Chromatophore Units (C.U.), the corpus luteum 100, the anterior hypophysis 20, duodenal mucosa and spleen 7, while the lymphatic nodules and blood contained about 5 C.U. The observation of modest amounts in the lymphatic nodules is in keeping with the well recognized inhibitory action of cortical hormones on this tissue. The remaining organs examined, brain, spinal cord, cardiac and skeletal muscle, carotid artery, pancreas, liver, testes and ovary (denuded of corpora lutea) possessed only traces of cortical hormone activity.

It appears that under normal conditions substances possessing corticoid activity may be detected in the urine. The excretion of these corticoids is quite independent of 17-ketosteroid excretion and probably represents metabolic products of the adrenal corticoids. Venning and Kazmin (1946), using liver glycogen deposition in the adrenalectomized mouse as a bioassay method, found that normal males excreted 40 to 85 units[1] and normal females 25 to 65 units of glycogenic corticoids per 24 hours.

1 - 1 glycogenic unit is equivalent to the biological activity contained in 1 microgram of 17-hydroxy-11-dehydro-corticosterone.

There is only scant data on the fate of administered corticoid hormones. Apparently desoxycorticosterone, when administered as the acetate, may be recovered in human urine like progesterone, as sodium pregnanediol glucuronidate (Cuyler, Ashley and Hamblen, 1940) and as unconjugated pregnanediol in the rabbit (Hoffman, Kazmin and Browne, 1943). The rate of recovery in the urine is similar to that for progesterone, that is, about 5.6 to 14.6 per cent.

From these experimental data it seems that the non-androgenic cortical hormones are excreted in the urine partly as glycogenic corticoids and partly as inactive pregnanediol.

Resume

Natural estrogens, under physiological conditions, have been determined in sites of formation, blood and liver, but in no other tissues. They are excreted in minute amounts in the urine and feces, suggesting that for the most part they are either eliminated in an inactive form or completely destroyed in the body. Synthetic estrogens are absorbed more slowly, persist longer in the blood and are generally metabolized at a slower rate. Consequently, they have been found in most organs and body fluids. They are excreted in a manner similar to the natural estrogens but in larger quantities, suggesting that they are more resistant to inactivation or that they are metabolized in a different manner.

The remaining hormonally active steroids have only been demonstrated in the organs where they are elaborated and in the urine where they are excreted.

One may conclude from this review that only the excretion into the urine of biologically active steroids (with the exception of pregnanediol) is understood. Distribution and other modes of excretion are almost an untouched problem.

PART II

HISTOCHEMICAL INVESTIGATION

Historical

In the attempt to demonstrate steroids in tissues by histochemical methods, our interest was stimulated by the experiments of Wislocki and his collaborators, especially Bennett and Dempsey. These investigators have adopted the view that ketosteroids may be demonstrated in certain endocrine organs by means of the carbonyl reagents phenylhydrazine and fuchsin-sulphurous acid, although they recognized the fact that acetal lipids may also play an important role in the genesis of these reactions.

It was apparent from the outset of this work that fuchsin-sulphurous acid and phenylhydrazine, in common with other carbonyl reagents such as hydroxylamine, semicarbazide, etc., were only specific for the carbonyl grouping and that several compounds present in tissues might react. While the distribution of compounds with the carbonyl group may not be devoid of interest, it would be more important to be able to assign the observed reactions to one definite compound or group of compounds such as the ketosteroids. Lacking specific chemical techniques, several approaches may be considered. Thus, while several tissue constituents may affect a reagent in vitro, only one or a few of them may be present in detectable concentrations in tissues. Furthermore, suitable experimentation may sometimes complete the chemical data and make possible the identification of a tissue substance. For instance, the histochemical reaction for vitamin C disappears in C-deficient animals and returns upon administration. Thus, it was hoped that the carbonyl reactions might

disappear when the ketosteroid sources (adrenal and gonads) were removed and return upon substitution therapy. It was felt, therefore, that a study of the effect of various physiological, experimental and pathological conditions - age, extirpation of adrenals and gonads, substitution therapy, malignancy - on the fuchsin-sulphurous acid and phenylhydrazine reactions in organs and tissues might provide a better understanding of their meaning.

Preliminary to a discussion of our methods and experimental findings the chemical basis and previous histochemical applications of these two reactions will be discussed.

The Fuchsin-Sulphurous Acid (Plasmal) Reaction

Chemical Basis

In 1866, Schiff demonstrated that aldehydes would recolorize a solution of basic fuchsin rendered colorless by sulphurous acid. The use of fuchsin-sulphurous acid for the detection of aldehydes has since become widespread. The actual reactions between basic fuchsin and sulphurous acid, and between decolorized basic fuchsin and aldehydes have been described by Wieland and Scheuing (1921) and re-examined by Neuman (1944).

In the course of the reaction between basic fuchsin (chloride salt) (Fig. 1 B) and sulphurous acid, the chromophoric quinoid structure is destroyed and the pseudo-leuco-base (carbinol) (Fig. 1 A) is formed. The sulphurous acid then adds on to the central carbon atom giving rise to the compound C (Fig. 1), which with an excess of sulphurous acid is converted to either D or E or both (Fig. 1). On adding an aldehyde, D and E react to form F and G. In either case the quinoid chromophore is restored (Fig. 1 F and G) and the original color reconstituted while, at the same time, the aldehyde is converted to an alcohol combined with a molecule of sulphurous acid.

Although it is usual to classify fuchsin-sulphurous acid as an aldehyde reagent, it is possible to refer to it as a carbonyl reagent, since certain ketones are also supposed to react with this decolorized dye (Lison, 1936; Karrer, 1938). Even such a designation may not be accurate for oxidizing agents, such as cupric salts, also give positive results (Karrer, 1938).

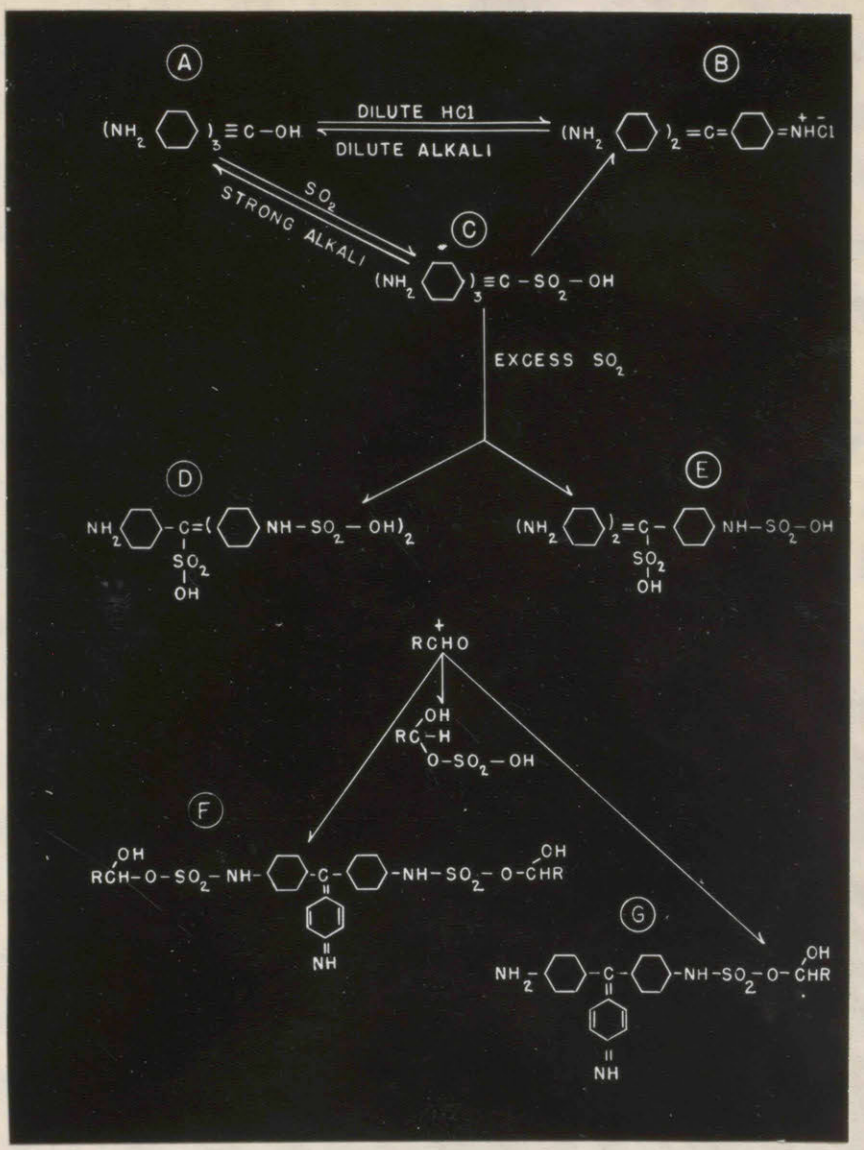


Fig. 1 The Fuchsin Compounds (taken from Neuman, 1944)

Histochemical Application

Fuchsin-sulphurous acid has been employed in histochemistry as a basis for three separate reactions, Feulgen's "nucleal" reaction, Bauer's reaction for glycogen and the Feulgen "plasmal" reaction examined in this work. In all of these reactions, preliminary to staining, the carbonyl group of the reactant material must be unmasked. The method of unmasking determines the compound to be stained. In Feulgen's "nucleal" reaction, for the demonstration of desoxyribonucleic acid, mild acid hydrolysis for a short time (5 to 15 minutes in N-HCl) separates the purine and pyrimidine bases from the rest of the molecule, thus allowing the desoxyribose to react with the fuchsin-sulphurous acid. In Bauer's reaction for glycogen, oxidation in 4 per cent chromic acid for 1 hour converts the glycogen into a substance, undetermined as yet, which gives a violet-red color. Finally, in the "plasmal" reaction of Feulgen, the aldehydes of the higher fatty acids contained in the acetal phosphatide, plasmalogen, are liberated by the action of mercuric chloride for a short time or by acid hydrolysis for a much longer time (3 hours at room temperature). The aldehydes then react with the fuchsin-sulphurous acid yielding a violet color (Lison, 1936). It is the "plasmal" reaction of Feulgen (synonymous with fuchsin-sulphurous reaction in this thesis) that has been employed in this histochemical investigation, since the conditions employed in the staining technique are those recommended by Lison (1936).

The following review is a resumé of the previous histochemical applications of the "plasmal" reaction.

A positive reaction with fuchsin-sulphurous acid may be obtained with unfixed tissues or tissues fixed in copper sulphate, mercuric chloride and neutral formalin, while either mercuric chloride or platinum chloride

may be used as the unmasking agent (Lison, 1936).

Stoeltzner in 1923 observed that a dark red-violet color was produced in human and rabbit muscle. Feulgen and Voit (1924) confirmed this observation and called the lipid-soluble reactant material present in the cytoplasm, plasmal, and its precursor, plasmalogen, and the whole staining procedure the "plasmal" reaction. Since then this substance has been found throughout the whole range of the animal kingdom (Immhauser, 1927), but has not been demonstrated in plants (Neuman, 1944).

The earliest systematic analysis of the tissue distribution of the "plasmal" reaction was carried out by Immhauser (1927), and since that time his observations have been amply confirmed and extended by many other workers.

Among the endocrine organs the most intense reactions were found in the adrenal cortex, adrenal medulla, corpus luteum, interstitial and thecal cells of the ovary, and the interstitial cells of the testis (Immhauser, 1927; Verne, 1929; Motta, 1931; Becher, 1939; Voss, 1940; Aboim, 1943; Dempsey and Bassett, 1943; Deane and Greep, 1946a). Less intense reactions were to be found in the thyroid, pituitary (Immhauser, 1927; Verne, 1929) and in the syncytial layer of the placenta (Dempsey and associates, 1944, 1947; Wislocki and associates, 1946a, 1946b, 1947).

Considerable differences of opinion exist with regard to the location of the "plasmal" reaction in the adrenal. Immhauser (1927) found positive reactions in both the medulla and cortex, while Verne (1929) and Voss (1940) observed that the most intense reactions occurred in the medulla and zona glomerulosa and not at all in the fasciculata. On the other hand, Becher (1939), Aboim (1943) and Deane and Greep (1946a) found only a slight reaction in the medulla with intense coloration in the fasciculata and glomerulosa. Indeed, Becher examined the adrenals of

many species of mammals and found that, although the intensity differed, the general staining pattern was always the same. In an attempt to explain these discrepancies, Hayes (1947) demonstrated that sections of fresh, unfixed adrenal glands exhibited positive reactions in the zona glomerulosa and medulla, while sections of formalin-fixed glands reacted throughout the cortex. Like Feulgen, Hayes believed that plasmalogen could be detected by reactions effected only on fresh tissues. Formalin fixation was considered to destroy the true "plasmal" reaction and give rise to some other artificially formed aldehyde.

The fuchsin-sulphurous acid reaction has also been examined in a few male and female accessory sex organs. As yet, positive reactions have been found only in the alveoli and ducts of the mammary gland (Verne, 1929; Dempsey, Bunting and Wislocki, 1947) and in uterine endometrium during pregnancy (Wislocki and Dempsey, 1945).

Reactions of varying intensity were found among the remaining organs and body tissues. In the liver, Feulgen and Voit (1924) had reported that the parenchyma was practically negative. This finding was supported by Verne (1929) who observed a positive reaction only in the cells of the bile canaliculi. On the other hand a definite reaction in the hepatic cells was reported by Immhauser (1927).

A positive reaction also has been found in the kidney (Immhauser, 1927; Verne, 1929; Oster and associates, 1944, 1946). Verne found that the convoluted tubules and loops of Henle were stainable, while the glomeruli and tubes of Bellini were negative. This latter finding has been questioned by Oster who observed that, in the kidney cortex, all the glomeruli stained lightly, and in addition the innermost zone (intercorticomedullary) stained most intensely. The staining reaction follows the same pattern in the kidneys of all mammals examined, however, there is great specie variations

in staining intensity.

In the nervous system intense reactions have been found in the myelin sheaths, while axis-cylinders, neuroglia and ependyma are negative (Immhauser, 1927; Verne, 1929).

In general, the gastro-intestinal tract does not stain. An occasional reaction, however, has been observed in the salivary glands (Verne, 1929).

In the lung a positive reaction in the alveolar cells has been reported by Verne (1929).

The weakest reactions were found in the connective tissue of parenchymatous organs. A fairly intense reaction has been observed in elastic tissue, especially the internal elastic membrane of arterial walls (Feulgen and Voit, 1924; Voss, 1927).

The skin was found by Voss (1941) to give a positive reaction, located for the most part in the stratum germinativum. Sweat gland ducts, but not sebaceous glands, reacted. The same author (1927) observed a strong reaction in the fat laden cells of the ear cartilage of the mouse and in the amphibian fat-body, though he found no reaction in mammalian adipose tissue. Mockel (1943) confirmed the latter finding in resting fat cells, but observed that developing fat cells and those found during starvation gave positive reactions.

No reaction has been found to occur in blood cells, lymphatic tissue and thymus (Verne, 1928a).

The distribution of the "plasmal" reaction in body fluids has been investigated by Stepp, Feulgen and Voit (1927). These workers found that cells in the saliva, as well as pus, blood serum, milk and colostrum were positive, while urine (concentrated in vacuo), cerebrospinal fluid and the aqueous humor of the eye were negative.

Some interesting observations have been made regarding the behavior of "plasmal" reaction under various physiological and experimental conditions. Age seemed to cause marked differences as indicated by the fact that the blood plasmalogen level of human babies and young calves was found to be much lower than in older animals (Immhauser, 1927, 1928), while sex, pregnancy and lactation did not seem to affect the blood level.

A relation between steroid hormones and the "plasmal" reaction has been observed by Oster (1946). This investigator found that in the kidney male sex hormones seemed to be specific for intensification of the reaction, while estrogens had the opposite effect.

That the "plasmal" reaction may serve as an indication of the lipid shift in the adrenal cortex under various experimental conditions was shown by many investigators (Tonutti, 1941; Greep and Deane, 1947; Deane and her associates, 1946a, 1946b, 1947a, 1947b). Tonutti observed a marked increase in the "plasmal" reaction of the adrenal cortex following the administration of diphtheria toxin and corticotropic hormone. A similar but less extensive increase was observed following pregnancy and castration. He suggested that these intensified reactions reflected increased functional activity in this gland. Greep, Deane and their associates found that pantothenic acid and thiamine deficiency, hypo- and hyperthyroidism caused a diminution in the staining reaction of the zona fasciculata of the adrenal. Hypo- and hyperthyroidism also caused a decrease in the intensity of the reaction in the zona glomerulosa, while following hypophysectomy the reaction diminished in the fasciculata but not in the glomerulosa; on the other hand, desoxycorticosterone acetate suppressed the reaction in the zona glomerulosa in both intact

and hypophysectomized rats.

Some very interesting generalizations can be made from all these observations. With the exception of the myelin nerve sheaths, the steroid hormone producing organs, known to be rich in lipid substances, give the strongest reactions. Furthermore, a substance possessing the ability to recolorize fuchsin-sulphurous acid has been shown to have a widespread distribution throughout the body and to be affected by various experimental conditions. Its importance to body economy can be partially deduced from this widespread distribution. The causal agent, as yet in dispute, may be plasmalogen, possibly ketosteroids (as suggested by Dempsey and his associates), or some as yet unidentified substance.

Significance

In the experimental investigations involving the use of the "plasmal" reaction, prior to 1943, it was generally accepted that the acetal phosphatide, plasmalogen, was the causal agent, since it released the aldehyde, plasmal. This interpretation was questioned by Dempsey and his associates (1943) who suggested that this reaction, in addition to a battery of 5 other histochemical reactions, revealed the presence of ketosteroids in endocrine organs. One of the fundamental arguments brought forward by these workers was the intense reactions found in the steroid producing organs, ovary, adrenal, testis and placenta.

There is no doubt that aldehydic steroids, if present - the only one so far isolated being testalolon (Hirano, 1936) - may react with fuchsin-sulphurous acid. Whether the more common ketosteroids also react will be discussed below.

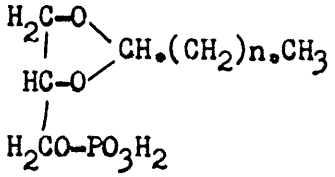
Since the main point of this discussion is to decide whether ketosteroids or aldehydes, such as plasmal, are the cause of the reactions observed in tissue sections, it was decided to outline briefly the available knowledge concerning plasmal and its precursor, plasmalogen.

Plasmalogen, as well as plasmal, (the aldehyde released from it by acid hydrolysis or the action of mercuric chloride), was found in the phosphatide fraction of tissues (Feulgen, Immhauser and Behrens, 1929). Steam distillation of muscle phosphatide carried away some of the plasmal, which after condensation with semicarbazide was found to consist chiefly of palmitic aldehyde semicarbazide, contaminated with a small amount of stearic aldehyde semicarbazide. Using an improved method for preparation of plasmal Behrens(1930) was able to isolate 1 to 1.5 gm.of plasmal semicarbazide from 10 kg.of horsemeat. Ehrenstein and Britton (1937) demonstrated the presence of sodium acid palmitate in testicular and adrenal

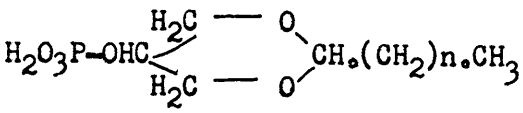
cortical extracts. Recently, Anchel and Waelsch (1942) were able to isolate fatty acid aldehydes from relatively small amounts of tissues, using p-carboxyphenylhydrazine or carboxymethoxylamine as the condensing agents. Their yield was 30 mg. of aldehyde per 100 gm. of muscle. The proportion of palmitic and stearic aldehydes was found to differ in plasmals from different organs and species. It is certain that all plasmals contain, besides palmitic and stearic aldehydes, at least one other fatty aldehyde which so far has not been identified due to the solubility of its semicarbazide.

The isolation of plasmalogen was accomplished by Feulgen and Bersin (1939) on the basis of its relative stability against alkali. When the phosphatide emulsion, obtained from beef muscle, was treated with sodium hydroxide, a considerable part of the plasmalogen remained intact while the contaminating phosphatides were saponified. From the reaction mixture, plasmalogen could be precipitated, together with the fatty acids, by converting them into the brucine salts. After extraction with acetone, plasmalogen could be isolated by repeated treatment of the acetone-insoluble residue with benzene in which pure plasmalogen is not soluble. The substance could then be crystallized from alcohol at room temperature.

By treatment with mercuric chloride, plasmalogen is split into a higher aldehyde and colamine glycerophosphate, which crystallizes in fine needles from alcohol and is very soluble in water, but insoluble in cold alcohol. Its melting point is 86 to 87°C. When heated with 3.3% NaOH for 6 hours, plasmalogen is split into colamine and plasmalogenic acid which can be isolated as the lithium salt. Feulgen and Bersin (1939) were able to demonstrate that this split-product is composed of equivalent amounts of glycerophosphate and a fatty aldehyde.

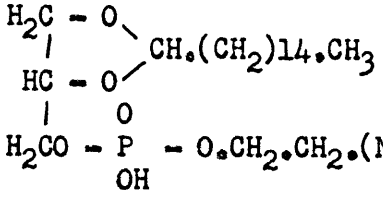


α-plasmalogenic acid

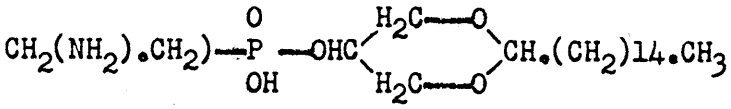


β-plasmalogenic acid

Feulgen and Bersin concluded from their observations that plasmalogens are acetals of fatty aldehydes with colamine glycerophosphate. Neuman (Quoted from Hayes, 1947) has adopted the view that colamine is not the only base to be found in plasmalogen. The structure of palmital plasmalogen, according to Feulgen and Bersin, may be represented by the following formulae:



α-palmital plasmalogen



β-palmital plasmalogen

The assumption of an acetal linkage is supported by the stability of the nitrogen-free group against alkali, and its sensitivity to acids and mercuric chloride.

Plasmalogens have no definite melting point but become soft at 50°C and decompose above 150°C. They are insoluble in acetone and water, incompletely soluble in alcohol, ether, petroleum ether, benzene, dioxane and methanol, and completely soluble in dilute aqueous potassium hydroxide, petroleum ether:chloroform (6:1) and chloroform alone. They are practically miscible with chloroform:methanol (1:1) (Neuman, 1944).

Recently Bersin, Moldtmann, Nafziger, Marchand and Leopold (1941) have succeeded in synthesizing acetal phosphatides from glycerin, acetaldehyde-diethylacetal and the silver salt of colamine phosphoric acid.

The Phenylhydrazine Reaction

Chemical Basis

Both aldehydes (1) and ketones (2) react with phenylhydrazine and its 2,4-dinitro derivatives according to the reactions:

(1) $R.CHO + H_2N.NH.C_6H_5 \longrightarrow R.CH{:}N.NH.C_6H_5 + H_2O$

(2) $R.C{:}O + H_2N.NH.C_6H_5 \longrightarrow R.C{:}N.NH.C_6H_5 + H_2O$

The mechanism of these condensation reactions has been shown to involve addition of the reagent and subsequent elimination of water. The behavior of phenylhydrazine towards α,β-unsaturated aldehydes or ketones. depends upon the conditions under which the reactions are carried out, as well as the substituents present in the carbonyl compound. In an acidic medium phenylhydrazones are usually formed, while in an alkaline medium the reactions are much more complex, and products have been obtained corresponding to reactions with both the ethylenic linkage and the carbonyl group.

Histochemical Application

Bennett (1940) introduced phenylhydrazine hydrochloride as a reagent for the histochemical study of the adrenal cortex. A yellow reaction was observed, which he attributed to the presence of ketonic lipids, the ketosteroids elaborated by this gland. Since this report, the phenylhydrazine reaction has been widely used, mainly in the histochemical study of the endocrine organs.

Bennett's finding has been confirmed by many workers (Aboim, 1943; Yoffey and Baxter, 1947; Rogers and Williams, 1947) but his interpretation has often been criticized. Aboim made the interesting observation that phenylhydrazones were present in the zona fasciculata of the adrenals of all mammals examined, and while specie differences existed in the

staining intensity, the pattern was always the same. Phenylhydrazine, like the "plasmal" reaction, has been employed in the study of the lipid shift in the adrenal cortex under various experimental conditions. Popjak (1944) observed that the yellow reaction was increased in the adrenal cortices of rats shocked by experimental crushing of one hind limb. This phenomenon was also demonstrated in the human (Rogers and Williams, 1947). Deane and her associates observed that the phenylhydrazine reaction, similar to the "plasmal" reaction, could be diminished in the adrenal cortex by hypophysectomy (1946a), thiamine (1947a) and pantothenic acid deficiency (1946b) and hypo- and hyperthyroidism (1947b; Greep and Deane, 1947). The staining reaction of the zona glomerulosa could be suppressed by treatment with desoxycorticosterone acetate in both intact and hypophysectomized rats (Greep and Deane, 1947). While investigating the effect of gonadectomy and substitution therapy on the adrenal cortex of the fowl, Kar (1947) found an increase in phenylhydrazones in the cortex following castration, while testosterone propionate or diethylstilbesterol returned the intensity to normal.

A positive reaction with phenylhydrazine also has been found in the syncytial layer of the placenta (Dempsey and Wislocki, 1944; Wislocki and associates, 1946a, 1946b, 1947), in the interstitial cells of the testis (Pollock, 1942) and in the interstitial cells, thecal and luteal cells of the ovary (Dempsey and Bassett, 1943; Claesson and Hillarp, 1947a, 1947b; McKay and Robinson, 1947). McKay and Robinson were able to show that yellow hydrazones first appeared in the human corpus luteum at 17 days and could be observed in the peripheral region of the cytoplasm of the granulosa lutein cells until the 22nd day, after which time the yellow "peripheral" rim disappeared and large extracellular oil droplets were to be seen. Some of these droplets reacted to give yellow phenylhydrazones

and some did not. This reactive material proved to be acetone soluble.

In general, with the dinitrophenylhydrazine reaction, the lipid material demonstrated in the endocrine glands was of the same intensity and distribution as the "plasmal" reaction previously described and seemed to be due to the same causal agent.

Significance

Bennett's claim, that the phenylhydrazine reactions would demonstrate the presence of ketosteroids in the adrenal, was based on the assumption that "no aldehydes with the solubility properties of corticosterone have been detected in the adrenal cortex" and therefore, any lipids having a carbonyl group found in the adrenal cortex must be ketosteroids. That this assumption was ill-founded can be seen from the fact that the carbonyl reagent, fuchsin-sulphurous acid, under the conditions of the "plasmal" reaction, also gives a strongly positive reaction in this locale. This interpretation of the phenylhydrazine reaction, supported by Wislocki and his associates, was extended to include the testis, ovary and placenta. Gomori (1942) criticized the specificity of Bennett's reaction and showed that the "plasmal" reaction had an identical distribution in the adrenal and suggested that plasmalogen was the causal agent. This observation was confirmed by Aboim (1943).

Evidence that both the "plasmal" and phenylhydrazine reactions stained one and the same substance had been obtained indirectly many years before by Feulgen, Immhauser and Behrens (1929) and Verne (1928b). These investigators demonstrated that if tissue sections were treated with phenylhydrazine, the "plasmal" reaction could no longer be obtained. In view of the fact that plasmalogens exist in far greater quantities in organs than ketosteroids, it would seem logical to assume that it is the causal agent. Such a clearcut decision, however, could not be made from the

available literature and therefore the investigations to be reported were undertaken.

Other "Ketosteroid" Reactions

In 1940, Bennett submitted evidence that the zones of the adrenal cortex reacting with phenylhydrazine also exhibited other reactions characteristic of steroids, for instance, a faint yellow tinge with semicarbazide, a black coloration with ammoniacal silver nitrate and a white precipitate with digitonine. Like fuchsin-sulphurous acid and phenylhydrazine, semicarbazide is only specific for the carbonyl group, while digitonine demonstrates the presence of a β-OH in a steroid molecule. These reactions in themselves did not prove that the causal agent was a ketosteroid, since plasmalogen and cholesterol could easily account for the reactions observed.

Realizing these difficulties, the reactions of steroid producing organs were further investigated by Dempsey and Bassett (1943) and Dempsey and Wislocki (1944). These workers showed that the lipid droplets present in these organs exhibited a battery of responses when submitted to certain histochemical procedures. A greenish-yellow fluorescence was observed, at the level of the intracellular lipid droplets, when these organs were irradiated with ultra-violet light, while, when examined under the polarizing microscope they exhibited a birefringence which alternately appeared and disappeared as the specimen was rotated. Furthermore, when sulphuric acid, the Liebermann-Burchardt reaction, was added to sections of placenta and ovary, the lipid droplets exhibited color reactions characteristic of unsaturated conjugated ring structures. These reactions in conjunction with positive fuchsin-sulphurous acid and phenylhydrazine reactions and the lipid solubility of the reactant droplets indicated to these investigators that although each reaction in itself was not specific, any compound giving all of these reactions would have to be lipid, polycyclic, unsaturated and possess a carbonyl group. The ketosteroids possess all these chemical features and were therefore assumed to be demonstrated by all these reactions.

Experimental[1]

Staining Techniques

In all the experiments to be described, tissues were removed immediately following the death of the animal and fixed for 48 hours at room temperature in 10 per cent formalin neutralized with magnesium carbonate. Following fixation they were placed in cheesecloth bags and washed in cold running water for 24 hours to remove as much of the formalin as possible. The washed tissue blocks were then sectioned at 15 microns on the freezing microtome and stored 12 to 24 hours in containers filled with distilled water. The sections were then ready for staining with either fuchsin-sulphurous acid or 2,4-dinitrophenylhydrazine. All tissue sections were treated in exactly the same manner to ensure as much uniformity as possible.

Fuchsin-Sulphurous Acid (Plasmal) Stain

The technique employed in the staining of tissues with fuchsin-sulphurous acid was essentially the same as that described by Lison (1936).

One gram of crystalline basic fuchsin (Anachemia, C.I. No. 677) was dissolved in 200 cc. of boiling water, cooled to 50°C and 20 cc. of N-HCl were added. The whole mixture was then cooled to 25°C. One gram of anhydrous sodium bisulphite was added and the mixture placed in a tightly stoppered brown bottle which was stored in a dark place for at least 24 hours, by which time bleaching (no trace of red in the solution) was complete. The reaction mixture now exhibited a straw color and was ready for use. Aliquots of reagent were removed from the container with a pipette as required.

1 - The writer wishes to acknowledge the aid of Miss C. Stevens and more recently that of Miss W. Storey of the Department of Histology, in the preparation and staining of the tissues. Photographic preparations were made up with the help of Miss R. Bogoroch and Mr. J. Isaac.

The tissue sections were removed from the container in which they had been stored and placed in a solution of saturated mercuric chloride for about 3 minutes. After being rinsed in distilled water for about 3 minutes they were transferred to fuchsin-sulphurous acid where they were left for 15 minutes. The sections were then carried through 3 separate rinses of sulphurous acid, remaining in each for 3 minutes, in order to remove the excess stain, and then transferred to dishes containing distilled water and finally mounted in glycerine-gelatine.

A positive reaction was indicated by a purple color which was found to be very stable and has persisted for more than a year in our material.

In order to eliminate the patchy staining observed in our preliminary tests a number of experiments were carried out to determine the effect of duration of fixation, length of washing and oxidation on the stainability of tissues with this reagent. It was first found that varying the duration of fixation from two to four, twenty-four or forty-eight hours had no influence on the patchiness, and that when sections were cut immediately after formalin fixation, without washing, no reaction at all was obtained.

On the other hand, a uniform staining was usually obtained if the sections were washed by letting them stand in water for 12 to 24 hours in shallow open dishes prior to staining. This suggested that oxidation by the air may have played a role in the reaction. Indeed, if the washing of the whole tissue and tissue sections as well as fixation were carried out in oxygen-free distilled water in completely filled, tightly stoppered bottles, very little or no reaction was observed. Conversely, the addition of 1-2 drops of 3 per cent hydrogen peroxide (Merck, U.S.P.) per cc. of fluid to the distilled water in which the sections were kept after sectioning

produced an intense and regular stain. Under these conditions washing for ½ hour instead of 12 to 24 hours was sufficient to induce a good stain. It was concluded that the reactions were made more definite by either contact with the oxygen of the air or with hydrogen peroxide treatment, but no modification of the pattern was observed. It was also noted that tissue sections stained, even if washing in mercuric chloride were omitted; however, this compound intensified, but in no way altered, the distribution of the fuchsin-sulphurous acid reaction.

As a result of these investigations special care was always taken that fixation, washing and the leaving of the sections 12 to 24 hours in distilled water, was done simultaneously for all experimental groups, thus ensuring a uniform technical procedure.

2,4-Dinitrophenylhydrazine Stain[1]

The same precautions with regard to fixation and washing described for the fuchsin-sulphurous stain were adhered to in the execution of this stain.

It was possible to improve Bennett's technique and obtain a more intense color in thinner sections, thus resulting in a better cellular localization. This was accomplished by using Eastman Kodak 2,4-dinitrophenylhydrazine instead of phenylhydrazine hydrochloride as used originally by Bennett, and by clearing the sections in 17 per cent alcohol. In our first experiments the sections were treated with iodine and thiosulphate with the intention of removing ascorbic acid (Bennett, 1940). In subsequent experiments it was found that the omission of this procedure did not affect the intensity or appearance of the hydrazone; the ascorbic acid presumably having dissolved out during fixation and subsequent washings.

1 - 2,4-dinitrophenylhydrazine was tried following the suggestion of Dr. H. Sobel, formerly of the Department of Biochemistry, McGill University. Dempsey and Wislocki (1946) mentioned that this substance was used in their laboratory as an unpublished modification of Bennett's reaction by Pechet.

Therefore, this step was eliminated. After washing the sections for 12 to 24 hours in distilled water, they were cleared for 4 hours in 17 per cent alcohol. This concentration of alcohol did not dissolve out the substances reacting with the 2,4-dinitrophenylhydrazine, which occurred with higher concentrations. It had been noted in preliminary experiments that the ketosteroid, testosterone, was practically insoluble in 17 per cent alcohol. The tissue sections were then carried to a saturated solution of 2,4-dinitrophenylhydrazine in 30 per cent alcohol to which a sufficient amount of 0.2N sodium acetate had been added to raise the pH to neutrality; this decreased the alcohol content to about 17 per cent. The sections were left in this solution overnight, washed in 17 per cent alcohol for 20 minutes to remove any excess stain, carried into distilled water and mounted in glycerine-gelatine. A positive reaction was indicated by a yellow color.

Sulphuric Acid Reaction

In the few cases where this reaction was employed, the technique consisted of simply placing a drop of concentrated sulphuric acid on a coverslip and then covering the tissue section. Usually the red-brown color denoting a positive reaction developed in about 1 to 2 hours.

Evaluation of the Results

The intensity of the staining reactions was graded in pluses on a scale of 0 to 4+. In the description of the results obtained the adjective "intense" refers to 4+, "fairly intense" to 3+, "moderate" to 2+, and "slight" to 1+.

The slides were not counterstained and were photographed by using the same exposure for the sections to be compared. In printing the positives a poorly staining area was picked out and brought to the same intensity in the photographs to be compared. For instance, in the adrenal,

the non-staining medulla was brought to the same intensity in comparable groups; in the seminal vesicles muscle was used as a guide. A Wratten purple filter was used to accentuate the yellow dinitrophenylhydrazones. Some unstained details may be apparent in the photographs due to lowering of the microscope condenser.

The Distribution of the "Plasmal" and 2,4-Dinitrophenylhydrazine Reactions In the Rat and the Mouse[1]

The histochemical evidence for the demonstration of ketosteroids in organs and tissues obtained by the Boston group (Dempsey and his associates) under normal physiological conditions was inconclusive, and did not exclude the possibility of plasmalogen as the causal agent. It was felt, therefore, that a more complete examination, embracing all the organs and tissues of the body, of the "plasmal" and 2,4-dinitrophenylhydrazine reactions under normal, experimental and pathological conditions might throw some light on the true meaning of these reactions.

Under Normal Conditions

For this purpose 6 male albino rats weighing 154 ± 10 gm. and 4 mice, comprising 2 males and 2 females about 3 to 4 months old, were examined. The animals had been given water and purina fox chow ad lib. They were sacrificed by means of chloroform, and the tissues removed at autopsy were immediately placed in 10 per cent neutral formalin.

The general distribution of the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions was similar in the rats and mice examined. The staining intensity of the adrenal cortex, corpus luteum and interstitial cells of the testis and ovary, however, was greater in the mice.

The distribution of the purple reaction, revealed by the fuchsin-sulphurous acid reagent, and of the yellow hydrazones, revealed by dinitrophenylhydrazine was very widespread extending to almost all organs and tissues. As far as could be ascertained the general distribution of the two reactions was identical. Cytological details stood out more clearly in the slides stained with dinitrophenylhydrazine as cellular damage was produced by the drastic reagents of the "plasmal" reaction. On the other hand, the staining with the latter was much more intense. At any rate, since the re-

1 - The mice were a gift from Dr. C. Fraser of the Department of Genetics, McGill University

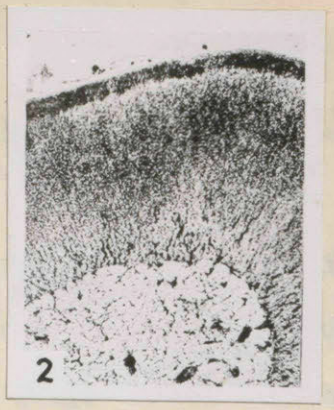
sults of both reactions were identical in mice and rats, they will be reported as one.

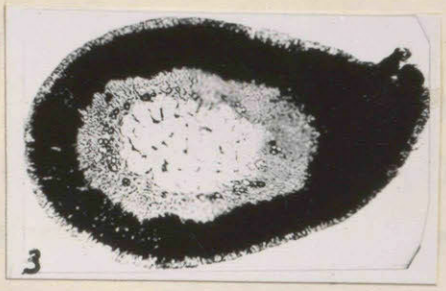
In the endocrine glands the most intense reaction was given by the adrenal cortex. In the rat (Fig. 2) the reaction varied in intensity being maximal in the zona fasciculata and glomerulosa with usually an unstained band between the two; while in the mouse (Figs. 3 and 4) the fasciculata stained most intensely and the thin glomerulosa, lightly. In both, the reticularis stained less intensely, but a definite reaction was present in all the cells, sharply demarcating them from the colorless cells of the medulla. Within the cells the reaction was present in the lipid globules.

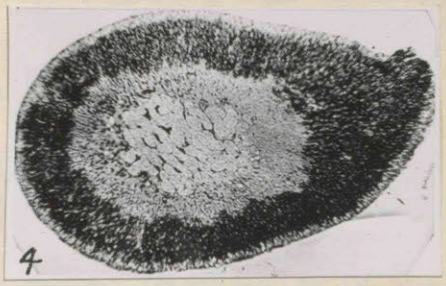
The anterior lobe of the rat hypophysis showed a slight reaction located in the basophilic and possibly in the acidophilic cells. The intermediary lobe did not react. The posterior lobe showed clumps of brightly staining globules in a few scattered cells.

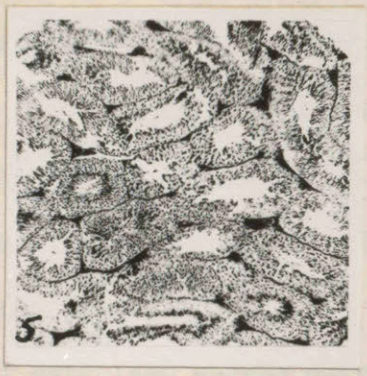
The thyroid of the rat showed a moderate reaction in the follicular epithelium but none or a slight one in the colloid (Figs. 12 and 13), while the parathyroid cells and the islets of Langerhans did not react or stained lightly.

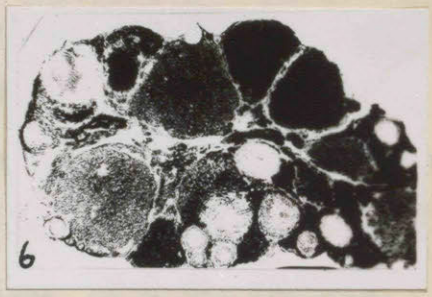
In the testis of the rat a slight reaction was found in the interstitial cells, while these cells stained most intensely in the mouse (Fig. 5). Pollock (1942) reported a marked reaction with phenylhydrazine in the interstitial cells of mice, rats, guinea-pigs, rabbits and cats. We were able to confirm his results in the mouse but not in the rat. Furthermore, single bright shining globules were occasionally seen in the periphery of the seminiferous tubules, presumably in the Sertoli cells. In addition, the tubules containing sperm nearing maturity showed reactions on round or oval structures adjacent to sperm heads; these structures











The grey to black color represents either the yellow of the dinitrophenylhydrazine or the purple of the fuchsin-sulphurous acid. The black spots are usually due to blood in the blood vessels.

Fig. 2 (same as Fig. 21N). Normal rat adrenal stained with dinitrophenylhydrazine. Zona glomerulosa stains intensely; fasciculata, moderately, with a colorless band between the two; reticularis, slightly and medulla not at all.

Fig. 3. Adrenal of 3 month old mouse stained with fuchsin-sulphurous acid. Zona glomerulosa stains slightly; fascicularis, intensely; the wide reticularis, slightly and the medulla, not at all.

Fig. 4. Same organ as in Fig. 3 stained with dinitrophenylhydrazine and showing the same pattern of staining. Photograph taken with a purple filter on panchromatic plate.

Fig. 5 (same as Fig. 49). 3 month old male mouse testis stained with fuchsin-sulphurous acid. The interstitial cells stain intensely and the spermatogenic tubules slightly or not at all, with a few dark staining granules about the heads of the spermatids.

Fig. 6 (same as Fig. 36). 3 month old female mouse ovary stained with fuchsin-sulphurous acid. The interstitial and thecal cells stain moderately to intensely; the corpora lutea, slightly to intensely and the granulosa cells slightly or not at all.

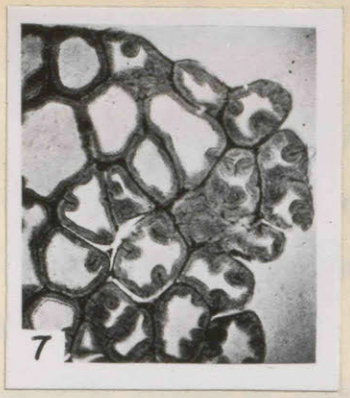
appeared to be the cytoplasm eliminated from the spermia during sperm formation.

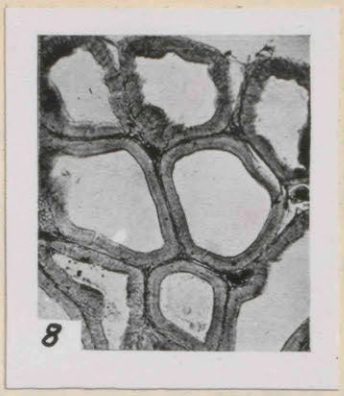
The ovaries of both species exhibited an intense reaction in the cells of the corpus luteum and in the thecal and interstitial cells, but none in the granulosa cells (Fig. 6). Within the cell the reaction was located in the lipid globules.

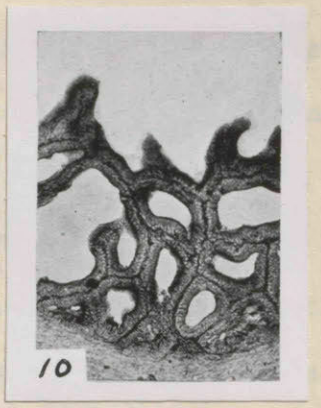
In male rats and mice an intense reaction was found in the prostatic epithelium (Figs. 7 and 8), where it extended throughout the cytoplasm and appeared accentuated at the level of the supra-nuclear Golgi apparatus. No reaction was found either in the secretion or in the interstitial tissue. In the seminal vesicles (Figs. 9 and 10) there was a fairly intense reaction located in the epithelium; this reaction was present in the vacuoles of the cell apex described by Moore, Hughes and Gallagher (1930). These so-called vacuoles are formed of a light zone with a dark central corpuscle. The stain seemed to locate in the light zone. The basal cytoplasm showed only a slight reaction. As in the prostate, the secretion present in the lumen did not stain. The epithelium of the epididymis showed a fairly intense reaction. No reaction was found in the tubular contents (sperm and secretion). The preputial glands showed an intense reaction in cells and sebum (Fig. 11).

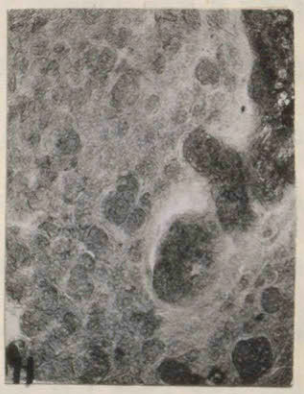
In the female rat and mouse the epithelium of the mammary gland showed a slight reaction, seemingly located in the intracellular globules.

The liver, in both species, showed a fairly intense reaction, which under high magnification appeared present throughout the cell. In the kidney (Figs. 16 and 19) an intense reaction was given by the proximal convoluted tubules; a moderate one, by the distal convoluted tubules and Henle's loop; none, by the tubes of Bellini and slight or none at all by the glomeruli. This pattern clearly emphasized the subdivision of the kidney









The grey to black color represents the yellow of the dinitrophenylhydrazine or the purple of the fuchsin-sulphurous acid.

Fig. 7 (same as Fig. 20N). Normal rat prostate stained with dinitrophenylhydrazine. Epithelium succulent and stains intensely. Secretion does not stain.

Fig. 8 (same as Fig. 24N). Normal rat prostate stained with fuchsin-sulphurous acid. Reaction same as in Fig. 7.

Fig. 9 (same as Fig. 22N). Normal rat seminal vesicle stained with dinitrophenylhydrazine. Epithelium succulent and stains fairly intensely. Concentration of the color in the apical portion of the cells.

Fig. 10 (same as Fig. 25N). Normal rat seminal vesicle stained with fuchsin-sulphurous acid. Same staining pattern as in Fig. 9.

Fig. 11. Rat preputial gland stained with fuchsin-sulphurous acid. The cells and sebum stain intensely.

into three zones: cortex intensely stained on account of the proximal convoluted tubules; outer medulla moderately stained on account of Henle's loops and distal convoluted tubules, and the inner medulla colorless on account of the tubes of Bellini.

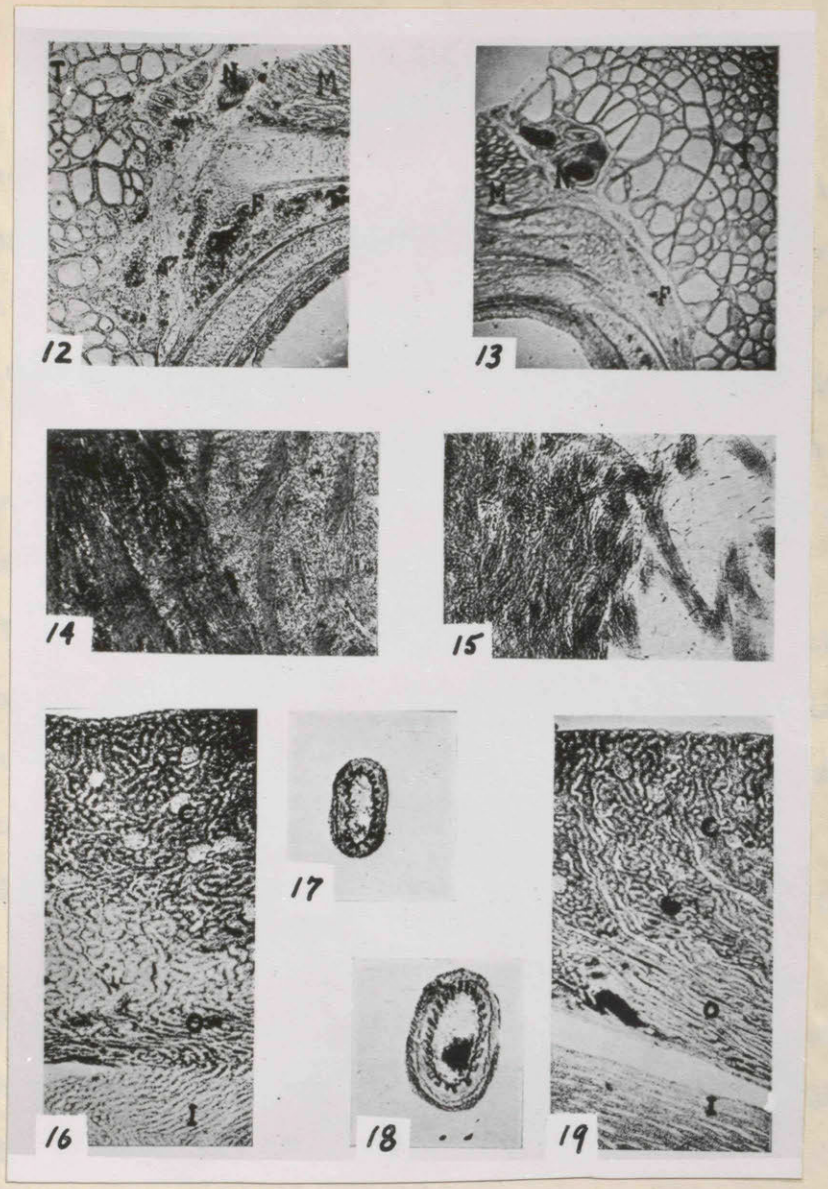
In the nervous system of both mice and rats an intense reaction was given by myelin sheaths. This was found in the peripheral nerves (Figs. 12 and 13N) as well as in the nerve tracts of the central nervous system (Figs. 14 and 15).

The gastro-intestinal tract of the rat displayed only a slight reaction or none at all. The intestinal epithelium reacted slightly. The gastric glands and epithelium did not react appreciably. In the salivary glands the reactions were absent; except for a moderate reaction in the excretory ducts of the submaxilllary. The pancreatic acini showed a moderate reaction. In general, mucus tissue (goblet cells of the intestine, mucus acini of the sublingual salivary gland) did not stain.

In rat lung no reaction was evident. The epithelium of the trachea and bronchi, however, showed a moderate reaction.

In the skin of both mice and rats the layers of keratin showed a slight reaction. The hard keratin of the hair did not react. A slight or moderate reaction was found in the sebaceous glands.

In the spleen of the mouse and rat, the red pulp was negative. The lymphatic nodules showed a slight reaction as far as lymphocytes were concerned. Some cells in the reticulum were laden with clumps of brightly staining globules. Similarly, in the thymus, a slight reaction was usually encountered while an intense one was found in some of the reticular cells. It may be noted that identical cells with irregular, deeply staining globules were also found in the posterior hypophysis. All these cells should be considered as part of the reticulo-endothelial system.



Stain—Fuchsin-sulphurous acid—Figs. 12, 14, 16, 17.
Dinitrophenylhydrazine—Figs. 13, 15, 18, 19.
The grey to black color represents either the purple of the fuchsin-sulphurous acid reaction or the yellow of the dinitrophenylhydrazine as seen in the tissue sections.

Figs. 12 and 13. Normal thyroid (T), Nerve (N), Muscle (M), and Fat (F).

Figs. 14 and 15. Normal brain. The myelin of the nerve tracts stains intensely. The grey matter, slightly.

Figs. 16 and 19. Normal kidney showing the intensely staining cortex (C) with the non-staining glomeruli and the poorly staining outer (O) and inner (I) medulla.

Figs. 17 and 18. Arteriole showing the darkly staining internal elastic membrane.

Connective tissue cells and collagenous fibers did not react in either species. But a fairly intense reaction was observed in the elastic tissue; this could be seen scattered throughout the connective tissue, as well as in the internal elastic membrane of arterial walls (Figs. 17 and 18). Fat cells (Figs. 12 and 13F) showed all types of reactions from intense to none at all; intensely staining globules were seen close to non-reacting ones. In cartilage cells sudanophilic globules were found which reacted with both reagents.

The reaction of muscle tissue varied according to the type. The smooth muscle fibers stained slightly or not at all; the striated muscle fibers of the skeleton stained moderately (Figs. 12 and 13M)and cardiac fibers, fairly intensely.

Generally speaking, the reactions while similar in the rat and mouse were more intense in the latter.

Finally, it must be emphasized that in all organs and tissues examined the nuclei never reacted with either the fuchsin-sulphurous acid or the dinitrophenylhydrazine stain.

Effect of Biological Interventions

In these experiments the distribution of the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions was examined in rats deprived of the two known sources of ketosteroids, namely testes and adrenals as well as in hypophysectomized animals.

All animals were sacrificed by means of chloroform. The tissues removed at autopsy were fixed and stained in the usual manner.

Three series of albino rats were investigated.

The first series comprised 4 groups of 5 or 6 mature rats weighing 154 ± 10 gm . The animals in the first group were normal controls; in the second, castrated for 7 days; in the third, castrated for 21 days; in the

fourth, castrated and given subcutaneous injections of 1 mg. of testosterone in 0.1 cc. of Mazola oil twice daily starting from the day of operation up to the seventh day, when they were sacrificed.

The second series consisted of 6 groups of 4 to 8 immature male albino rats weighing from 60 to 65 gm. The animals in the first group were normal controls; in the second, castrated for 21 days; in the third, adrenalectomized for 14 days; in the fourth, castrated for 21 days and adrenalectomized for 14 days; in the fifth, castrated for 21 days, adrenalectomized for 14 days and given subcutaneous injections of 1 mg. of testosterone suspended in 0.1 cc. of water twice daily for 7 days; in the sixth, castrated for 21 days, adrenalectomized for 14 days, and given subcutaneous injections of 1 mg. of desoxycorticosterone acetate (Percorten Ciba) in 0.2 cc. of oil twice daily for 7 days. All animals were sacrificed at the same time.

The animals of the first series were kept on water and purina fox chow ad lib., and were fasted for 14 hours before being sacrificed. The second series received purina fox chow and pablum ad lib.; the animals which had been adrenalectomized were given 1 per cent saline to drink; the other animals had access to water.

The organs examined in the third series were obtained from hypophysectomized animals belonging to experiments conducted by Drs. C. P. Leblond, D. Findlay and J. Gross. Four groups of animals comprising 6 to 10 mature male and female rats were investigated. All animals were kept on a low iodine diet and were given water ad lib. Those in the first and second groups, the latter receiving from 2 to 20 micrograms of iodine daily either in the drinking water or by subcutaneous injection, served as controls. The animals in the third and fourth groups were hypophysectomized from 6 to 16 weeks, two in the latter group received about 20 micrograms of iodine per day

TABLE 1

EFFECT OF VARIOUS INTERVENTIONS IN MATURE MALE RATS ON THE STAINING OF MALE ACCESSORIES WITH DINITROPHENYLHYDRAZINE AND FUCHSIN-SULPHUROUS ACID REAGENTS

Group	No. of Animals	Initial Body Weight (gm.)	Final Body Weight (gm.)	Seminal Vesicles Weight (mg.)	Intensity of Reaction		
					Seminal Vesicles	Ventral Prostate	Preputial Gland
Normal Controls	6	154 (154-160)	213 (200-225)	480 (320-605)	+++	++++	++++
7-day Castrates	6	155 (145-160)	159 (150-165)	85 (67-138)	++	+	++++
21-day Castrates Non-injected	5	154 (145-160)	203 (190-215)	47 (35-56)	±	±	++++
7-day Castrates Testosterone	5	155 (145-160)	164 (150-175)	791 (595-943)	++	++++	++++

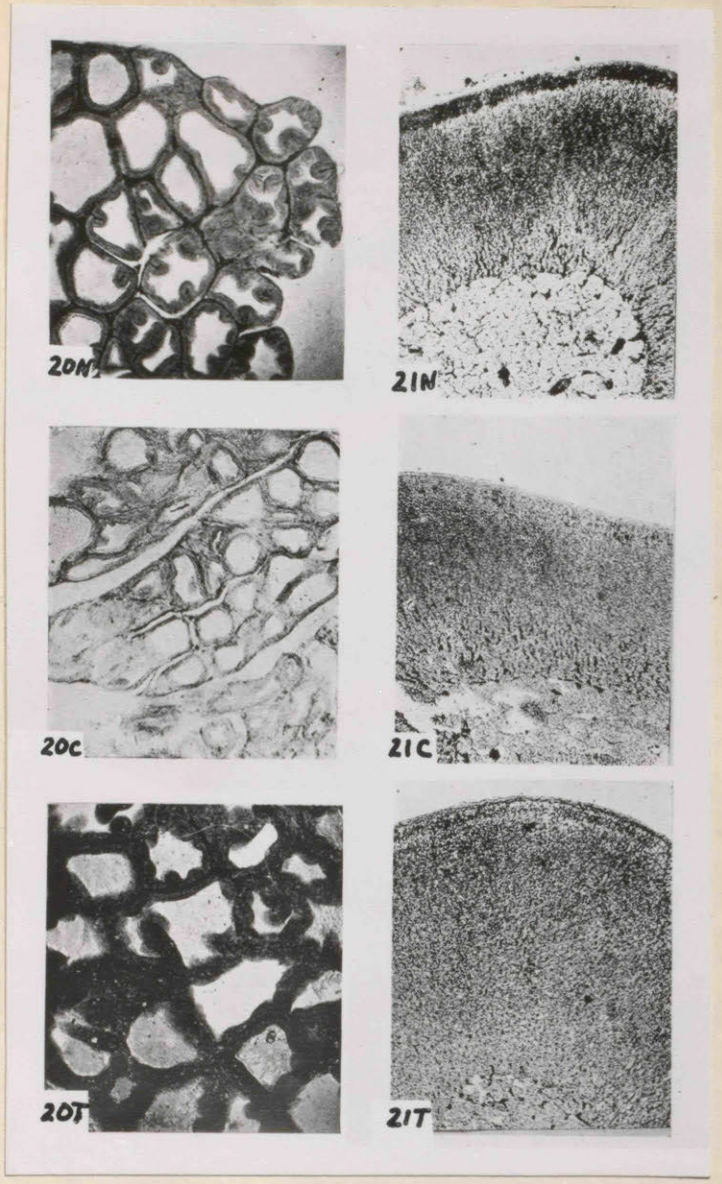
in their drinking water. At autopsy heart, liver, kidneys, ovaries and testes were taken for histochemical studies.

The tissues were fixed and stained in the usual manner.

Castration was performed by the ventral abdominal route, both testes and epididymis being removed. The bilateral adrenalectomies were performed through the dorsal route, while hypophysectomy was performed by the parapharyngeal approach. The suprarenal and pituitary regions were carefully checked at autopsy for completeness.

From the first series of experiments (Table I) it appeared that the prostate and seminal vesicles lost, to a considerable extent and some even completely their ability to react with either dinitrophenylhydrazine (Figs. 20C and 22C) or fuchsin-sulphurous acid (Figs. 24C and 25C). At 7 days after castration the reaction was moderate and at 21 days, slight. In the case of the seminal vesicles it is known that the apical vacuoles of Moore disappear after castration. The two reactions decreased gradually as these vacuoles disappeared. In addition, rare, scattered, small globules of sudanophilic material may appear in the cells of the castrates; these globules stained intensely with both reagents.

Injection of testosterone restored the reaction in prostate and seminal vesicles of castrated animals (Table I). In the prostate, this restoration was well marked, since the reaction after the hormone treatment may be more intense than in normal controls (Figs. 20T and 24T). In the seminal vesicles, on the other hand, the reaction was somewhat less intense in treated castrates than in normal controls (Fig. 22T and 25T). On the whole, it may be stated that testosterone restored staining ability to within the normal range. The reaction in the preputial glands was not appreciably affected by either castration or treatment with testosterone.



Stain--Dinitrophenylhydrazine. The grey to black color represents the yellow seen in the tissue sections. The black spots are usually due to blood in the blood vessels.

Fig. 20N. Normal rat prostate. Epithelium succulent and stains intensely. Secretion does not stain.

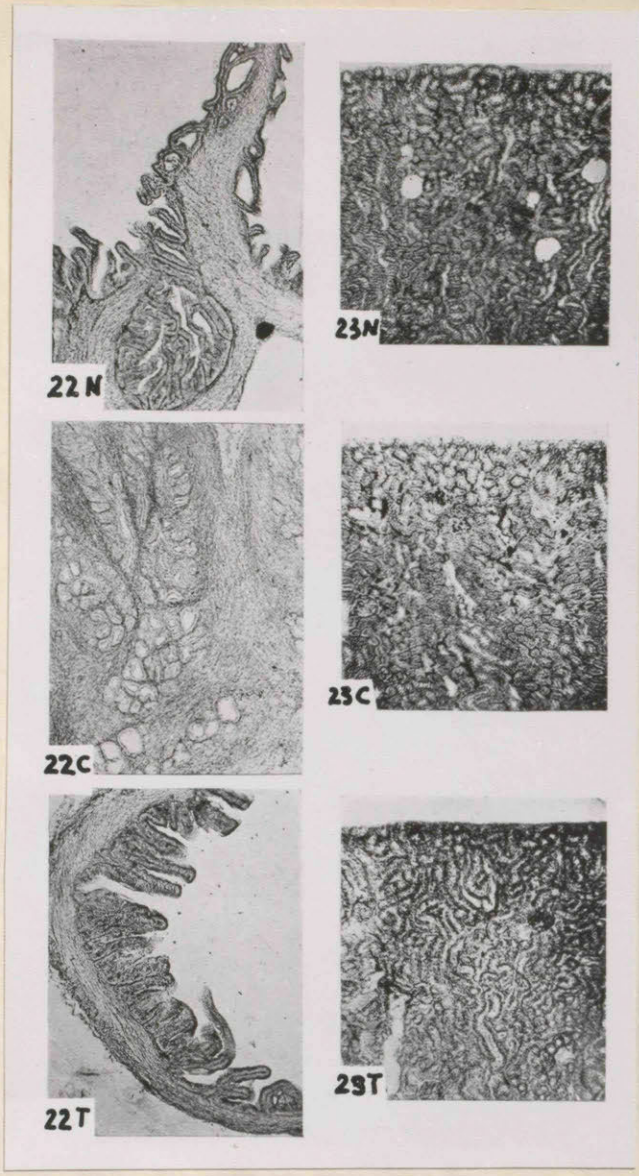
Fig. 20C. 21 day castrate rat prostate. Epithelium atrophic and stains slightly.

Fig. 20T. Testosterone treated, 7 day castrate rat prostate. Epithelium succulent and stains very intensely. Secretion does not stain.

Fig. 21N. Normal rat adrenal. Zona glomerulosa stains intensely; fasciculata, moderately, with a colorless band between the two; reticularis, slightly and medulla not at all.

Fig. 21C. 21 day castrate rat adrenal. Diminution in staining intensity of the zona glomerulosa and fasciculata. No change in the reticularis and medulla.

Fig. 21T. Testosterone treated, 7 day castrate rat adrenal. Same as in Fig. 21C.



Stain--Dinitrophenylhydrazine. The grey to black color represents the yellow seen in the tissue sections. The black spots are usually due to blood in the blood vessels.

Fig. 22N. Normal rat seminal vesicle. Epithelium succulent and stains fairly intensely. Concentration of the color in the apical portion of the cells.

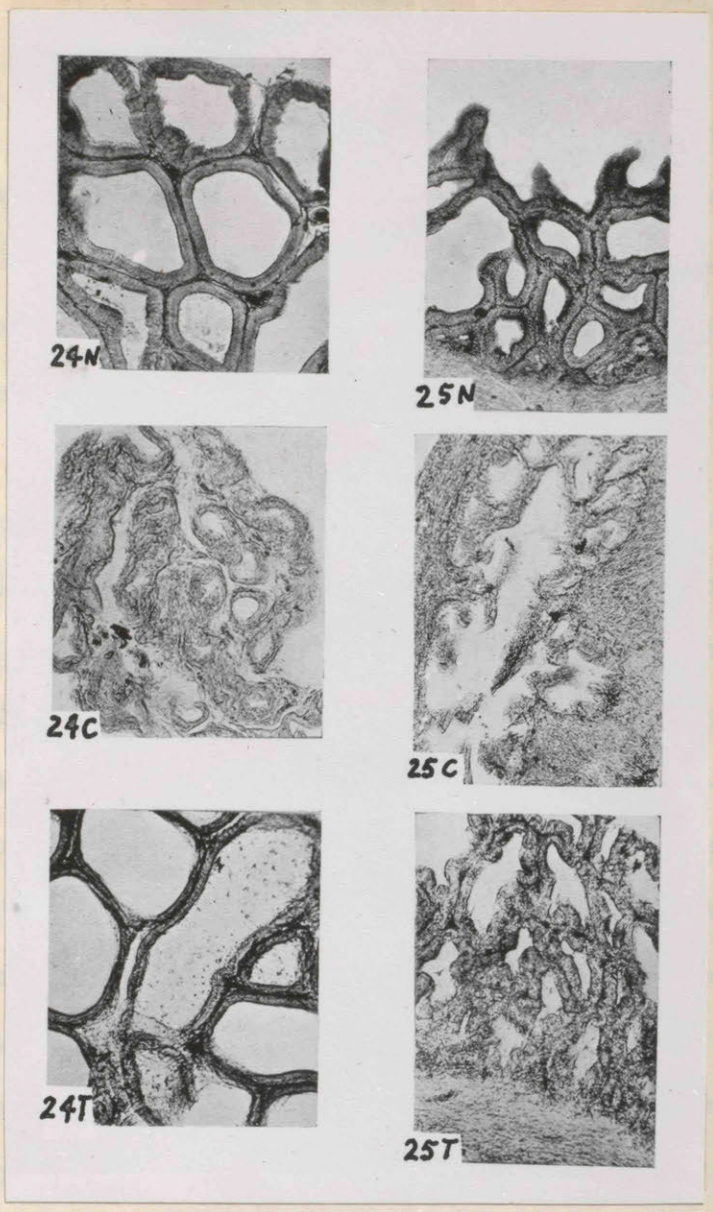
Fig. 22C. 21 day castrate rat seminal vesicle. Epithelium atrophic and stains slightly. Muscle as in Fig. 22N.

Fig. 22T. Testosterone treated, 7 day castrate rat seminal vesicle. Epithelium succulent and stains moderately. Color concentrated in the apical portion of the cell.

Fig. 23N. Normal rat kidney cortex. Intense reaction in the proximal convoluted tubules.

Fig. 23C. 21 day castrate rat kidney cortex. No change from Fig. 23N.

Fig. 23T. Testosterone treated, 7 day castrate rat kidney cortex. No change from Figs. 23N and 23C.



Stain—Fuchsin-sulphurous acid. The grey to black color represents the purple of the tissue sections.

Fig. 24N. Normal rat prostate. Epithelium succulent and stains fairly intensely. Secretion unstained.

Fig. 24C. 21 day castrate rat prostate. Epithelium atrophic and stains slightly.

Fig. 24T. Testosterone treated, adrenalectomized, castrate rat prostate. Epithelium succulent and stains intensely. Secretion unstained.

Fig. 25N. Normal rat seminal vesicle. Epithelium succulent and stains fairly intensely. Concentration of the color in the apical portion of the cells.

Fig. 25C. 21 day castrate rat seminal vesicle. Epithelium atrophic and stains slightly. Muscle as in Fig. 25N.

Fig. 25T. Testosterone treated, adrenalectomized, castrate rat seminal vesicle. Epithelium succulent and stains moderately. The color concentrated in the apical portion of the cell. Muscle as in Figs. 25N and 25C.

None of the other organs were influenced by either castration or testosterone therapy in so far as color intensity and distribution was concerned (Figs. 23N,C,T). The only exception was the adrenal cortex. In the present series it was found that castration produced a decrease in the color intensity in the zona glomerulosa and fasciculata (Fig. 21C). Examination under high power showed that the globules stained in the normal adrenal cortex were reduced in size in the castrates. Testosterone at the present dose level did not appear to restore the normal picture in the adrenal cortex (Fig. 21T). It must be remembered that in this series all animals had been fasted for fourteen hours prior to being sacrificed and this may have played a part in the changes of the staining intensity.

In the second series of experiments, performed on immature male rats (Table 2), it was also found that castration markedly decreased and testosterone restored the reactions observed in the prostate and seminal vesicles. The staining intensity in the normal controls, however, was less than in the more mature animals of the first series. The decrease in the staining intensity of the adrenals following castration was not clearcut in this series.

As far as the reactions in the other organs were concerned, it was not possible to detect any consistent action of castration or testosterone treatment.

Adrenalectomy and desoxycorticosterone acetate treatment caused no variation.

Following hypophysectomy there was no or only a slight decrease in the staining intensity of heart, liver and kidneys; while marked changes were found in the adrenals, ovaries and testes.

In the adrenals, in addition to the marked atrophy of the cortex, the reactions in the zona fasciculata were markedly altered. The normal

TABLE 2

EFFECT OF VARIOUS INTERVENTIONS IN IMMATURE MALE RATS ON THE STAINING OF MALE ACCESSORIES WITH DINITROPHENYLHYDRAZINE AND FUCHSIN-SULPHUROUS ACID REAGENTS

Group	No. of Animals	Initial Body Weight (gm.)	Final Body Weight (gm.)	Seminal Vesicles Weight (mg.)	Ventral Prostate Weight (mg.)	Preputial Gland Weight (mg.)	Intensity of Reaction		
							Seminal Vesicles	Ventral Prostate	Preputial Gland
Normal Controls	4	70 (60-85)	150 (135-170)	134.5 (64-197)	118 (118-128)	62.5 (38-87)	++	+++	++
21-day Castrates	4	70 (60-80)	130 (100-150)	7 (5-9)	9.7 (5-14)	45 (29-41)	+	+	+++
14-day Adrenalectomized	3	70 (60-80)	113 (110-120)	69 (45-95)	77.5 (62-94)	36 (22-52)	++	+++	+++
21-day Castrates 14-day Adrenalectomized	4	68 (60-80)	130 (120-150)	5 (4-6)	12.5 (8-16)	23 (16-34)	+	+	+++
21-day Castrates 14-day Adrenalectomized Testosterone	3	69 (60-85)	123 (100-145)	126 (104-155)	106 (96-119)	53 (52-64)	+	+++	++
21-day Castrates 14-day Adrenalectomized Desoxycorticosterone	6	72 (60-85)	128 (115-140)	8.5 (5-15)	9.5 (6-11)	28.5 (24-36)	+	0	+++

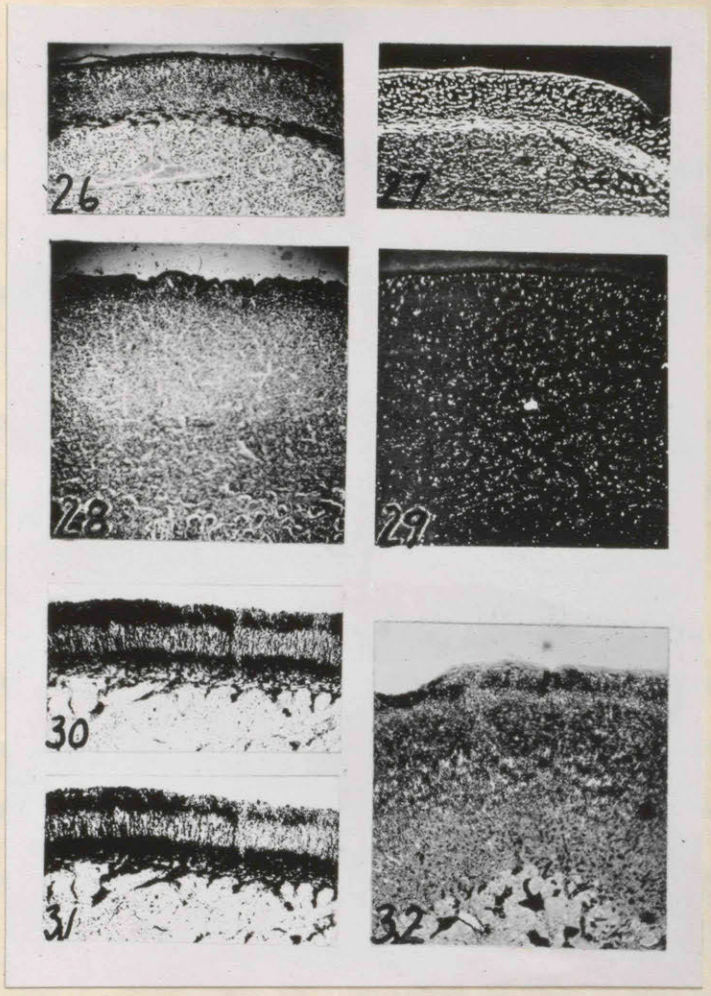
intense reaction in the outer fasciculata (Fig. 32) was completely suppressed (Fig. 30), while the poor reaction present in the inner fasciculata in the intact animal (Fig. 32) was replaced by an intense coloration (Fig. 30). On the other hand, the staining response of the zona glomerulosa was not affected (Figs. 30 and 32). An accumulation of brown "wear and tear" pigment was observed in the zona reticularis (Figs. 30 and 31) of the hypophysectomized animals but not in the controls. Since this pigment is rich in iron it can be demonstrated in slides stained with Prussian blue (Figs. 26 and 28) and by microincineration (Figs. 27 and 29).

A marked diminution in the staining reaction was found to occur in the interstitial cells of the testes and in interstitial, thecal and luteal cells of the ovary following hypophysectomy, accompanied by the deposition of iron containing brown pigment.

It must be emphasized that in all the experimental series none of the hormonal interventions affected the general pattern of distribution and intensity of the reaction, except in the seminal vesicles, prostate, adrenals, ovaries and testes. Furthermore, both stains ran parallel throughout all organs and tissues.

Effect of Age

Since it was found in the preceding experiments that the seminal vesicles of the immature male rats stained less intensely than those of the more mature animals, it appeared that the age of the animals was an important factor in the response of some tissues to staining with fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine. Indeed, previous experiments revealed that the age changes in staining pattern, especially in the case of the endocrine glands, resembled those after hypophysectomy. Therefore, it was decided to systematically investigate the staining intensity of these two reactions in animals of various age groups.



Histochemical reactions in adrenals of hypophysectomized animals. x 50.
Fig. 26. Cortex (above) and medulla (below) of adrenal from a hypophysectomized rat, showing the brown pigment in the reticular zone. The pigment, appearing black in the photograph, was stained with Prussian blue. Hematoxylin-eosin counterstain.
Fig. 27. Other section from same animal after micro-incineration. The pigment appears as a band of ashes. These ashes were red, and therefore due to the presence of iron.
Fig. 28. Control rat. No Prussian blue reaction in reticular zone.
Fig. 29. Control rat. No ash accumulation after micro-incineration.
Fig. 30. Hypophysectomized rat showing the fuchsin-sulphurous acid reactions in the zona glomerulosa and inner fasciculatis. (Green filter, panchromatic plate). The reticular zone photographs light.
Fig. 31. Same slide photographed on contrast plate with daylight filters, to show the pigment in the reticular zone.
Fig. 32. Fuchsin-sulphurous acid reaction in control rat. Moderate reaction in the zona glomerulosa and outer fasciculatis. No reaction in medulla. (Green filter, panchromatic plate).

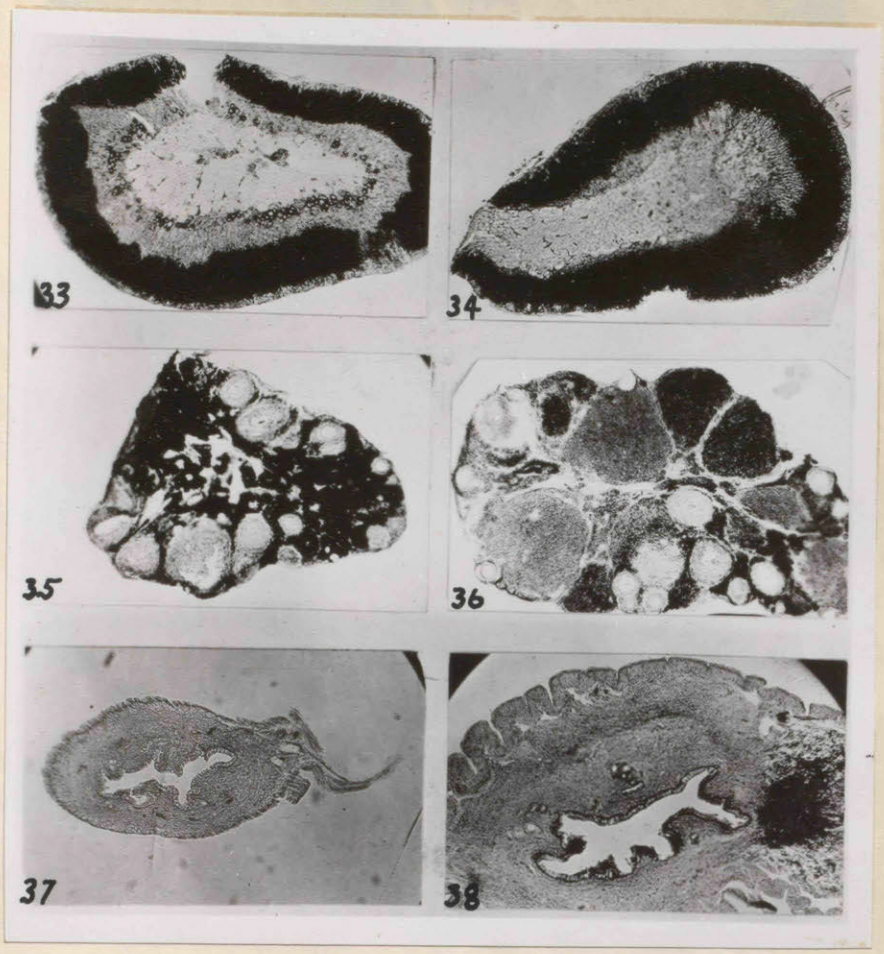
The mouse was chosen because in this animal the staining reactions were more intense than in the rat. Cancer-resistant "C57" and cancer-susceptible "A" strains were selected as the experimental animals. The latter strain was chosen because it was desired to see if susceptibility to spontaneous mammary cancer affected the staining intensity and because the virgin females of the "A" strain have a lower incidence of tumors than the breeders. It was hoped that this difference, presumably on an endocrine basis, might accentuate any age differences in response to the two reactions.

Six series of mice of the "C57" and "A" strains were investigated. These consisted of 1 day, 1 month, 3 to 4 month, 8 to 9 month, 11 month and 15 month old animals. Four mice of each strain were used per group, including 2 males and 2 females. In the 15 month old group only "A" mice were available. The animals were taken from breeding cages as required and kept on a diet of purina fox chow and water ad lib. The 1 day old mice were sacrificed by freezing and fixed in toto; all other animals were sacrificed by means of chloroform, and the organs fixed and stained in the usual manner.

Since the distribution of the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions was again found to be identical, wherever examined, they will be reported as one.

In day old mice of the "A" and "C57" strains the adrenal cortex stained intensely and in a rather uniform fashion. There was no division of the cortex into the three zones normally found in older animals; the only suggestion of zoning being that a few superficial cells stained less intensely. The medulla stained slightly or not at all as was observed in animals of all ages. At 1 month the adrenal cortex in animals of both strains had become divided into 3 zones, of which the fasciculata stained

about as intensely as the cortex of the newborn. The reticularis was thin and stained lightly. At 3 months the staining intensity of the zona fasciculata reached a maximum. The thin glomerulosa and the thickened reticularis stained lightly although more intensely than the medulla (Figs. 33 and 34). In the "A" females of this age group there was a band of signet cells filled with fatty globules located between the zona reticularis and the medulla. The fatty content of these cells stained moderately, but usually fell out during the preparation of these sections (Fig. 33). The small ring of cytoplasm contained small granules staining intensely. These signet ring cells were not found in the adrenals of the "C57" females of corresponding age (Fig. 34); the reticular zone in this strain being uniformly thinner than in the "A" animals. In the "C57" animals the general arrangement of the gland appeared less regular, thus the zona reticularis may be so small that it is hard to distinguish. At any rate, the limit between the zona fasciculata and reticularis was irregular with typical fascicular cells extending into the reticularis. In the older age groups of both strains there was a gradual decrease in the regularity of the cellular arrangement. For instance, the zona glomerulosa lost its distinctness, so that frequently in the oldest animals it became difficult to identify any glomerular cells at all. In the zona fasciculata the cell cords stained less intensely, became less regular and the outer and inner region reacted differently, as evidenced by a fairly intense reaction in the inner half, and a poor one in the outer half of the fasciculata (Fig. 39). The extreme case was the breaking of the inner fascicular cords into irregular deeply staining masses and granules (Fig. 40). This was observed in 2 females. The reticular zone was less regularly arranged than at 3 months and its vascularization was increased especially at the medullary limit. With age, fatty globules described at the limit of the medulla disappear in the "A" strain. Pigment was found in the zona



Comparison of the fuchsin-sulphurous acid reaction in organs of 3 month old "A" female mouse (left) and 3 month old female "C57" mouse (right). Photographs taken with daylight filter on panchromatic plates, the black color represents the purple reaction. x 40.

Fig. 33. "A" adrenal. The zona glomerulosa stains slightly; the fasciculata not at all. The darkly staining circles at the reticulo-medullary junction represent the signet cells described in the text.

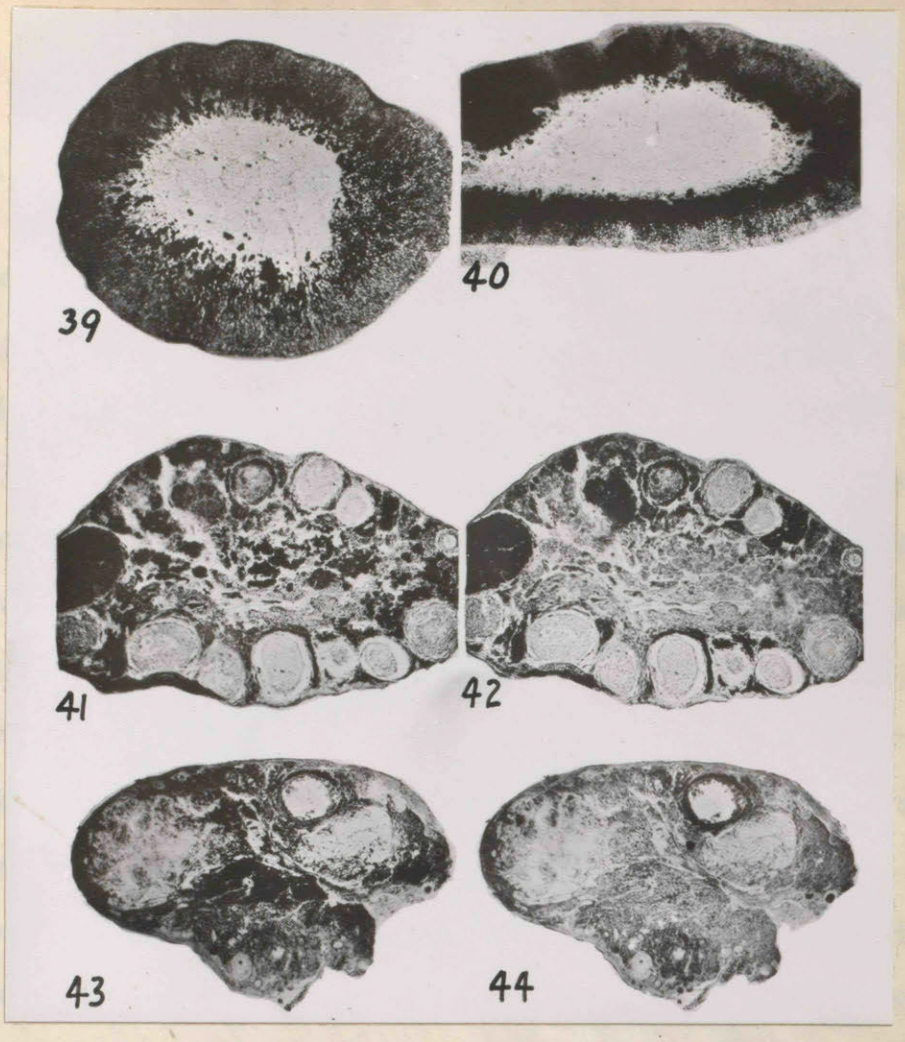
Fig. 34. "C57" adrenal. Same as Fig. 33. Note the absence of the darkly staining signet cells and the narrowing of the zona reticularis. Compare with Fig. 33.

Fig. 35. "A" ovary. The interstitial and thecal cells stain intensely; the granulosa cells slightly or not at all. Corpora lutea are absent.

Fig. 36. "C57" ovary. The interstitial and thecal cells stains moderately to intensely; the corpora lutea, slightly to intensely; the granulosa cells slightly or not at all.

Fig. 37. "A" uterus. The immature muscularis and epithelium stain slightly or not at all.

Fig. 38. "C57" uterus. The well developed muscularis stains slightly or not at all; the basal portions of the epithelial cells, intensely and the apex only slightly. A concentration of positively staining material may be seen in the mesometrium.



Fuchsin-sulphurous acid reaction in senile animals. In Figs. 39, 41 and 43 (daylight filter, contrast plates) the black color represents the purple staining reaction and brown pigment. In Figs. 40, 42 and 44 (green filter, panchromatic plates) show only the purple reaction. It is, therefore, possible to deduce the location of the brown pigment by comparing the same slide photographed both ways, i.e., 41 and 42 or 43 and 44.

Fig. 39. 16 month old female "A" mouse adrenal. The zona glomerulosa stains moderately; the outer half of the fasciculatis, moderately and the inner half, intensely; the indistinct reticularis, slightly and the medulla not at all. Throughout the reticularis abundant aggregations of darkly staining cells are found, probably representing the breaking down of the regular pattern of the inner fascicular cords. A small amount of pigment is present (greyish).

Fig. 40. 16 month old female "A" mouse adrenal. The zona glomerulosa stains slightly; the outer half of the fasciculatis, slight to moderate; and the inner half, intensely; the reticularis, slightly and the medulla, not at all. Throughout the reticularis are scattered intensely staining aggregations of cells.

Fig. 41. 8 month old female "C57" ovary. The interstitial, glomerulosa, thecal and corpus luteum cells stain as in Fig. 42. The deposition, however, of the non-staining but light absorbing brown pigment in the interstitial cells can be determined by comparing Figs. 41 and 42.

Fig. 42. 8 month old female "C57" mouse ovary. The interstitial and glomerulosa cells stain slightly or not at all, and the corpus luteum present, intensely.

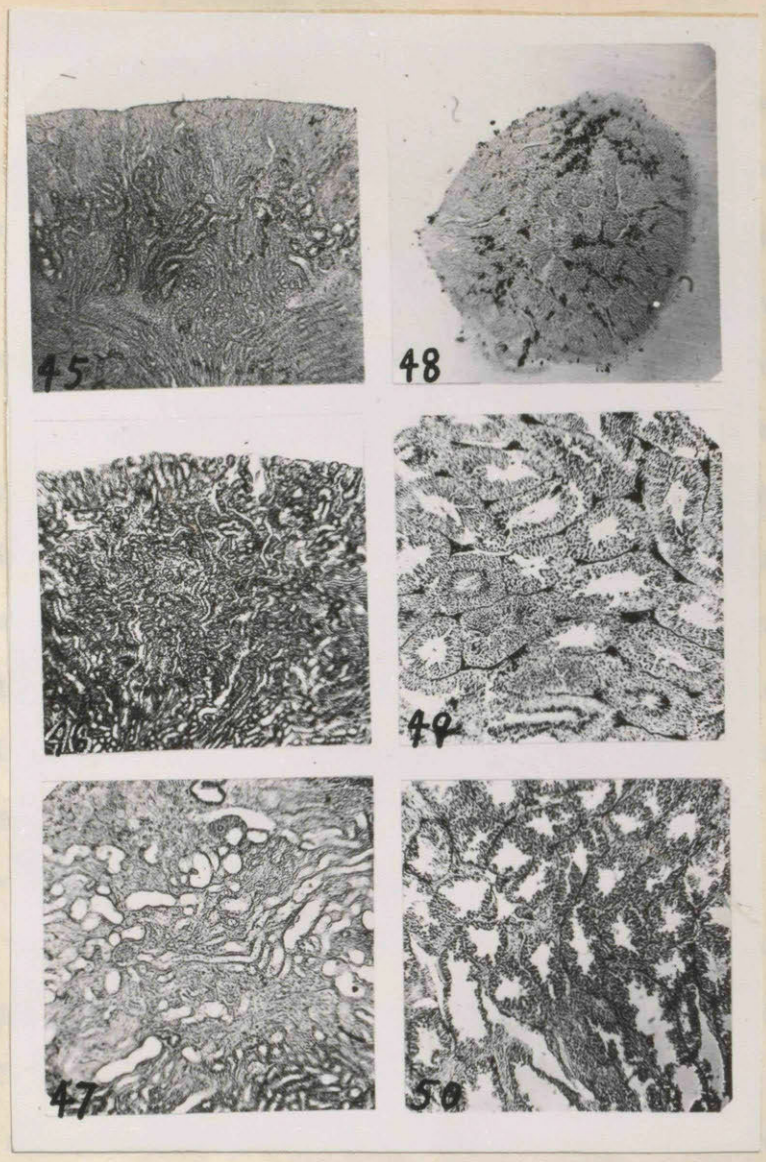
Fig. 43. 16 month old female "A" mouse ovary. By comparing Figs. 43 and 44, the extent of the deposition of the brown staining pigment can be determined.

Fig. 44. 16 month old female "A" mouse ovary. The entire ovary, with the exception of the cells lying in an ovarian cyst stains slightly or not at all. The hyalinized corpora lutea do not stain at all.

reticularis of mice of the later age groups in both sexes of both strains, especially in the "C57". These pigment masses appeared to be identical with the so-called pigment masses found in brown degeneration (Cramer and Horning, 1937; Leblond and Nelson, 1937; Cook and Kennaway, 1940; Gardner, 1941).

In the testis the slight reaction found in the tubules contrasted with the intensely staining interstitial cells. These cells showed a moderate reaction as early as the first day of life (Fig. 48). The reaction was fairly intense at one month. In the 15 month old mouse, however, there were only a few cells giving a definite purple reaction in the interstitial spaces; and even the staining intensity of these cells was much less than in the younger animals (Fig. 50). On the other hand, numerous irregular pigment granule masses were scattered throughout the interstitial spaces where the natural brown color of the pigment contrasted with the surrounding purple coloration in the fuchsin-sulphurous acid stained slides.

In the ovary of the immature animal the reactions were less intense than in the 3 month old animals (Figs. 35 and 36). The granulosa cells of the follicle gave slight or no reaction, the ovum showed fine, discrete granules with a fairly intense color, while the follicular fluid did not react. The thecal cells gave an intense color although in places their reaction was moderate (Figs. 35, 36 and 42). The interstitial cells showed a moderate reaction at 1 month, a very intense one at 3 months (Figs. 35 and 36) and a decreased reaction at the later age intervals especially at 15 months when they either stained slightly or not at all (Figs. 42 and 44). The degenerating follicles showed intensely staining patches corresponding to groups of lipid-laden cells. There was a wide range of staining intensity of the corpora lutea. In the 3 month old animal of the "C57"



Variation of Fuchsin-sulphurous acid reaction in kidney (left) and testis (right) with age. The black color represents the purple seen in the tissues. Photographs taken with a daylight filter on contrast plates. x 40.

Fig. 45. 1 day old mouse kidney. A few differentiated proximal tubules in the middle region stain slightly. No stain anywhere else.

Fig. 46. 3 month old mouse kidney. Fairly intense reaction in the proximal convoluted tubules.

Fig. 47. 16 month old mouse kidney. Slight or no staining throughout the organ.

Fig. 48. 1 day old male mouse testis. The interstitial cells stain fairly intensely and the poorly developed spermatogenic tubules slightly or not at all.

Fig. 49. 3 month old male mouse testis. The interstitial cells stain intensely and the spermatogenic tubules slightly or not all, with a few dark staining granules about the heads of the spermatids.

Fig. 50. 12 month old male mouse testis. The atrophied cells of the interstitial spaces and the disorganized spermatogenic tubules stain slightly or not at all.

strain for instance, intensely staining corpora lutea were seen next to moderately staining ones (Fig. 36). In the oldest animals corpora lutea were seen which stained very little or not at all (Fig. 44).

In general, as the animals grew older the following changes became apparent, starting at 8 months. Whole groups of interstitial cells and to a smaller extent, thecal cells lost more or less completely their ability to stain with fuchsin-sulphurous acid (Figs. 35, 36, 42 and 44). Next to and inside these non-staining interstitial cells, masses of pigment became visible and gradually increased with age (Figs. 41 and 43). Finally, large unstained areas with a glassy appearance, which were probably old corpora lutea, sometimes showed small groups of irregularly staining globules (Fig. 44). In the 15 month old animals, one showed a completely atrophic non-staining ovary.

In the male, a detailed study of the seminal vesicles was made at various ages. An intense reaction was present in the vacuoles of the cell apex. This apical reaction was slight to moderate at 1 month, most intense at 3 months when mice reach sexual maturity, moderate at 11 months and disappeared or was very slight at 15 months. The zone of the seminal vesicle that stained most intensely was essentially the epithelium; a slight reaction, however, was present in the smooth muscle and a slight to moderate reaction in the lamina propria, probably due to the presence of elastic fibers. The secretion did not react. In the epithelium, the moderate reaction observed in the bases of the cells contrasted with the intense reaction of the secretion vacuoles in the apex.

In the female, the uterus showed a slight reaction in the smooth muscle and, at least in some animals, a fairly intense reaction in the epithelial cells. The epithelial reactions were present only in the sexually mature animals, thus the 3 month old "C57" females showed a definite

reaction in the epithelium (Fig. 38); the apical and basal regions of the cells showing minute, brightly staining granules which were most intense in the basal part of the cell. In contrast, the uteri of the more slowly maturing "A" females showed no or only slight reactions at 3 months (Fig. 37). Older "A" strain females showed stainable granules in the epithelium. It may be noted that scattered, intensely staining cells could be found in the mesometrium (Fig. 38). Finally, brownish pigment was found to accumulate in the lamina propria and myometrium of the older animals.

The mammary glands showed irregularly occurring reactions in cells of the epithelium, apparently located on the fatty inclusions. Brightly staining fat globules were also found free in the lumen as described by Dempsey, Bunting and Wislocki (1947).

The liver showed a fairly intense reaction in the cells throughout life. The most pronounced reaction was observed in the youngest animal groups with a tendency toward a slow decrease with ageing. Individual variations were considerable in this organ. Deeply staining globules were occasionally seen in the Kupfer cells. The occurrence of intense reactions in cells of the reticular-endothelial system was fairly common in all locations and at all ages.

In the kidney, the greatest variations with age are encountered in the proximal convoluted tubules. In the newborn animals (Fig. 45), the outer part of the organ, where the proliferation of new tubules is actively taking place, did not react. In the middle region, where a few differentiated proximal tubules were apparent, a slight to moderate reaction was found. Finally, in the innermost part, where collecting tubules are located, no staining occurred. An intense uniform staining of the cortex was observed in 1 month and 3 month old animals (Fig. 46). As the animals aged, the staining of the cortex became less regular and showed

greater individual variations. Frequently groups of proximal convoluted tubules resisted staining. This was most marked in the senile animals (Fig. 47).

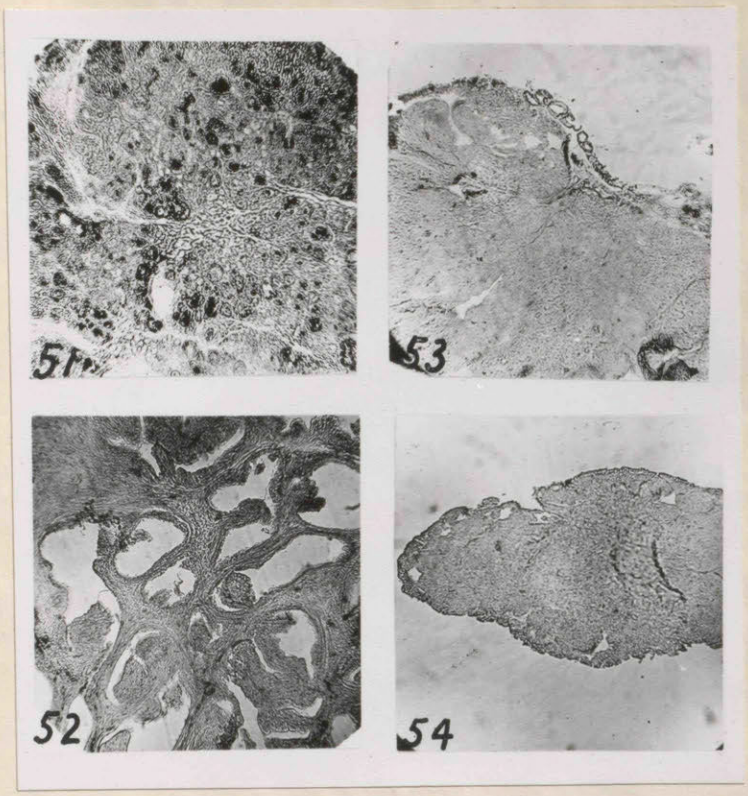
In cardiac muscle, the reaction was poor in the newborn animals, but fairly intense in all the other age groups examined.

In general, the staining intensity or distribution of the reactions did not differ in the cancer-resistant "C57" and cancer-susceptible "A" strains. The only exceptions were the differences, already described, found in the ovaries and uteri of the slowly maturing female mice of the "A" strain.

Effect of the Presence of a Mammary Cancer

In this series of experiments the distribution and staining intensity of the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions was examined in 4 mammary-cancer bearing, 8 to 9 month old, female mice of the cancer-susceptible "C_3H" strain. These mice were obtained from the colonies of the Roscoe B. Jackson Memorial Laboratory. They were sacrificed by means of chloroform, and the heart, liver, kidneys, adrenals, ovaries and uterus were removed, placed in fixative and stained in the usual manner.

On the whole, the distribution and staining intensity of the two reactions was quite similar to that previously found in "A" and "C57" strain female mice. The uteri of the "C_3H" mice seemed to stain a little more intensely than those of the other two strains, however, since the phase of the estrus cycle had not been rigorously controlled, any interpretation of this result would be hazardous. Of more interest was the fact that, while a moderate to intense reaction was observed in the non-cancerous mammary gland epithelium (Fig. 51), no reaction or only a slight one was present in the cystadenocarcinoma (Fig. 52) and in the adenocarcinomata



Fuchsin-sulphurous acid reaction in normal mammary glands and in mammary cancer. The black color represents the purple seen in the tissue sections. The photographs were taken with a daylight filter on contrast plates. x 40.

Fig. 51. 9 month old female "C_3H" mouse mammary gland. The lumen and cell of the alveoli contain intensely staining droplets, while the ducts and stroma stain slightly or not at all.

Fig. 52. Cyst-adenocarcinoma from a 9 month old female "C_3H" mouse. The epithelial and stromal elements stain slightly or not at all.

Figs. 53 and 54. Adenocarcinomata from two 9 month old female "C_3H" mice. The epithelial and stromal elements stain slightly or not at all.

(Figs. 53 and 54) of the mammary gland in the mice examined.

From the small series studied there seemed to be no doubt that the presence of a mammary tumor in no way modified the staining pattern of various organs.

Discussion

Four main conclusions may be drawn from these experiments in which two carbonyl reagents (fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine) were employed in an attempt to locate ketosteroids. First, both reactions are widespread throughout the tissues of the animal body; second, a strict parallelism exists between the fuchsin-sulphurous acid and dinitrophenylhydrazine reactions in all tissues examined; third, certain endocrine organs (adrenal cortex, ovary, interstitial cells of the testis) stain more intensely than any other tissue in the body; fourth, in most organs the intensity of the staining reactions varied with age.

Since the distribution and staining intensity of the fuchsin-sulphurous acid and dinitrophenylhydrazine were always identical in the rat and mouse, under normal, experimental and pathological conditions, it seemed that only one agent was responsible. Indeed, Feulgen, Immhauser and Behrens (1929) and Verne (1928b) showed that if tissue sections were treated with phenylhydrazine, the fuchsin-sulphurous acid reaction could no longer be obtained. This clearly demonstrated that the substance responsible for the fuchsin-sulphurous acid reaction was also able to react with phenylhydrazine. Therefore, it seemed quite reasonable to assume that there was only one causal agent.

Unfortunately, as yet, no decisive argument has been brought forth to clarify the nature of this substance. Ketosteroids, plasmalogens and fatty oxidative products of an aldehydic nature (Gerard, 1935), have been suggested.

In the following discussion the evidence for the histochemical demonstration of ketosteroids will be analyzed and the nature of the agents responsible for the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine

reactions, whether ketosteroids or plasmalogen, will be discussed. Our own results will be interpreted either for or against these two theories and an attempt made to arrive at the true meaning of these reactions.

Chemical Nature of the Compounds Reacting with Fuchsin-Sulphurous Acid and 2,4-Dinitrophenylhydrazine

The histochemical reaction proposed by Bennett (1940), utilizing phenylhydrazine hydrochloride for the demonstration of ketosteroids in the adrenal cortex and later by many other workers, was severely criticized by Gormori (1942). He emphasized that this reaction was not specific, since it merely indicated the presence of lipid bodies having aldehyde or keto-groups. Furthermore, lipid aldehydes, not related in any way to ketosteroids, are present in large amounts in the adrenal cortex. In addition, sections from different kinds of necrobiotic tissues (tubercles, infarcts, tumors) as well as the adrenal cortex reacted not only with phenylhydrazine, but also with fuchsin-sulphurous acid. Gormori pointed out that both the fuchsin-sulphurous acid and phenylhydrazine reactions necessitated a previous oxidation, since none of these reactions took place in unfixed tissue, which argued against the existence of free keto-groups. On the other hand, if frozen sections of tissues were exposed to aeration in alkaline buffer solution, or to oxidation with iodine as described by Bennett, both reactions became positive. According to Gomori, the acetal linkages of the acetal phosphatides break up during this procedure, and plasmal is liberated. He therefore concluded that plasmalogen was responsible. Gomori's experience with the effect of oxidation on the tissue response was confirmed by our experiments, on the enhancement of both reactions by exposure of tissue sections to air or hydrogen peroxide.

Realizing these difficulties, Dempsey and his associates later introduced a battery of six reactions which, when positive, were interpreted as indicating the presence of ketosteroids. These consisted of fluorescence, birefringence, Liebermann-Burchardt response, lipid solubility

and phenylhydrazine and/or fuchsin-sulphurous acid reactions. No one of these was considered specific for ketosteroids, however, any one tissue substance giving all reactions must be a polycyclic molecule with a carbonyl group and a double bond somewhere in its structure. The ketosteroids have such a structure and, therefore, could give all these reactions.

Fluorescence

Autofluorescent material has been described in the ovary (Dempsey and Bassett, 1943), placenta (Dempsey and Wislocki, 1944) and adrenal (Deane and Greep, 1946a), and was thought to be ketosteroid in nature when taken in conjunction with the other non-specific histochemical reactions above outlined.

While it is true that any sterol possessing a conjugated double bond or a benzene ring should fluoresce, and, in fact certain ones do (Ruzicka and Stepp, 1938; Reichstein and Shoppee, 1943), this fluorescence does not occur until the steroids have been treated with sulphuric or phosphoric acid. Recently, Finkelstein, Hestrin and Koch (1947), employing pure solutions of steroids in phosphoric acid, found that 0.5 micrograms of α-estradiol, 1 of estrone or estriol, 3 of desoxycorticosterone, 10 of testosterone and 100 of progesterone were detectable by fluorimetry. Cholesterol, androsterone, androstanediol, pregnanediol and pregnenolone were non-fluorescent. These observations indicate that it seemed possible to detect the minute amounts of ketosteroids present in tissue sections providing a proper medium, sulphuric or phosphoric acid, was employed. Dempsey and his associates, however, in their studies on autofluorescence employed sections mounted in glycerine-gelatin, thus making it improbable that ketosteroids were the source of the fluorescence observed. This criticism was supported by the fact that it is generally accepted that hormones

are not usually fluorescent in an aqueous medium unless protein in nature (Harvey, 1944). The one exception is α-estradiol, since Harvey (1944) observed that crystals of this estrogen (supplied by Ciba Pharmaceutical Products Inc.) were markedly fluorescent in the near ultra-violet light. Dempsey and Bassett (1943) observed this type of fluorescence in the ovary, but, since the causal agent would have to be some ketonic estrogen (to fit the other criteria for the demonstration of ketosteroids), it does not seem probable that the ketosteroids in this organ are responsible.

The presence of a greenish fluorescence in the adrenal cortex and testis had been demonstrated by Popper (1941) and Sjostrand (1944). These workers observed that this fluorescence was soon destroyed by irradiation with ultra-violet and behaved in a manner similar to that found with vitamin A. Dempsey and his associates (1946) pointed out that two types of fluorescence could be observed in certain endocrine organs, one being labile and the other stable to ultra-violet. Claesson and Hillarp (1947c) were unable to confirm these findings; in fact, they observed that the fluorescence, exhibited by the interstitial cells, theca interna and corpora lutea of rat ovaries, was ultra-violet labile, thus confirming the earlier findings of Popper and Sjostrand.

It is well established that organic compounds with unsaturated linkages exhibit fluorescence in solution. It seems probable that some of the more abundant compounds found in tissues such as vitamin A, non-steroid benzenoid structures, or unsaturated lipid complexes, which are known to fluoresce readily, would account for this phenomenon rather than the minute amounts of ketosteroids present.

Birefringence

The appearance of birefringence in tissue sections, examined under

the polarizing microscope, has long been known to histologists (Lison, 1936). Birefringence, when applied to the analysis of tissue lipids, rules out the presence of glycerides and fatty acids and indicates the presence of cholesterol esters, phospholipids or galactolipids, differentiation between these three being impossible (Lison, 1936). Recently, the Boston group (Dempsey and associates) have attempted to utilize this phenomenon as one of their tests for the demonstration of ketosteroids in the ovary, testis, adrenal cortex and placenta, since birefringence was found to be most marked in these organs which are known to elaborate ketosteroids.

Before birefringence can be ascribed to the presence of ketosteroids, cholesterol as a cause must be ruled out. Claesson and Hillarp (1947a) demonstrated that the birefringent substance found in ovarian sections gave a positive Schultz reaction and, when extracted, typical Liebermann-Burchardt and acetyl chloride responses, which are used for the detection and estimation of cholesterol. They also observed that, in keeping with the intense birefringence found in pregnant rabbit ovaries, up to 40 mg. of cholesterol per gm. of ovarian tissue was present.

More recently, Yoffey and Baxter (1947), employing X-ray powder photographs in an attempt to elucidate the nature of the birefringent material in the adrenal cortex, failed to establish their identity with crystals of desoxycorticosterone, androsterone, androstane, desoxycorticosterone acetate, ascorbic acid, tripalmitin, tristearin, or with the crystalline form of cholesterol employed.

While it is not possible to definitely ascribe birefringence in these lipid laden organs to cholesterol, it seems most likely that this sterol or its derivatives could better account for the magnitude of the

birefringence found, than the minute amounts of ketosteroids stored in these organs at any one time.

Liebermann-Burchardt Reaction

For the most part this reaction and its modifications (Schultz reaction, etc.) are based on the treatment of tissue sections with concentrated sulphuric acid with the subsequent development of a play of colors. In its simplest form, a drop of acid is placed on the tissue section leading to the development of a red-brown color. Although originally regarded as specific for cholesterol (Lison, 1936), other related compounds including a few biologically active steroid hormones, mostly adrenal cortical in organ, react positively (Schoenheimer and Evans, 1936; Reichstein and Shoppee, 1943). On this basis, Dempsey and Bassett (1943) proposed that sulphuric acid in addition to carbonyl reagents, fluorescence and birefringence be used for the demonstration of ketosteroids in endocrine organs known to produce them.

In actual fact, the use of sulphuric acid as a histochemical reagent is fraught with hazard, since at the present time many substances, in addition to steroids, are known to react. Miescher (1946) has recently demonstrated that aliphatic systems with conjugated ethylenic bonds have a tendency to form colored substances with this reagent. The inability of many steroids to react with sulphuric acid has been shown by Everett (1947), who observed that the Schultz test, a modification of the Liebermann-Burchardt reaction, is quite specific for the demonstration of diols such as those formed from cholesterol by mild oxidation. On the other hand, estrone, α-estradiol, progesterone, testosterone propionate, Δ^4-androstenedione-3, 17, Δ^4-cholestenone-3, dehydroisoandrosterone and pregnenolone, when tested under conditions closely simulating those found in tissue sections, gave no

color development which could be confused with that obtained with cholesterol.

In our own experiments with sulphuric acid, using as a test object rats deprived of the two known sources of ketosteroids (testes and adrenals), the reaction was found to be widely distributed. Nearly all tissues examined gave a red-brown coloration in the cellular cytoplasm which was unaffected by any of the experimental interventions.

It seems apparent from the foregoing analysis of the "ketosteroid" reactions that, while each in itself is not specific for this type of compound, the summation of their effects is still very inconclusive. Birefringence and the Liebermann-Burchardt reaction probably demonstrate the presence of cholesterol, while fluorescence indicates the presence of unsaturated organic compounds. Even if ketonic steroids could give rise to these reactions, the preponderance of other reactant material would only serve to mask the ketosteroid effect. The fact that these reactions are negative in sections extracted with lipid solvents merely indicates the fatty nature of the reactant material.

The introduction of the carbonyl reagents, fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine, while eliminating cholesterol and other non-carbonyl lipid compounds, retains the possibility that ketosteroids are the causal agent but introduces the acetal phosphatide, plasmalogen, as the substance responsible.

The ensuing discussion will be devoted to the interpretation of the nature of this causal agent in the light of our experimental findings.

Fuchsin-Sulphurous Acid and 2,4-Dinitrophenylhydrazine Reactions

The fact that in our experiments the most intense reactions were found in cellular structures known to elaborate ketosteroids (adrenal cortex,

corpus luteum, interstitial cells of the testis) seemed to add support to the idea that these reactions indicated their presence in these organs. Furthermore, since steroids have been reported to occur in the seminal vesicles of the rat (Delor, 1942), our finding of positive reactions in this organ appeared to support the opinion that these reactions were due to steroids. This point of view was further strengthened by the observation that castration decreased, and testosterone restored the staining intensity of the seminal vesicles and prostate (Table I). It must be borne in mind, however, that these tissues, atrophying after castration, might not only lose any steroids present but also a number of other biologically active compounds. Therefore, these experiments do not necessarily strengthen the case for steroids.

On the other hand, a slight reaction usually remained in the seminal vesicles and prostate in animals castrated for 21 days. It was thought at first that this slight reaction might be due to a small amount of adrenal cortical ketosteroid. It was therefore decided to carry out a second series of experiments in which the animals would be deprived of both testes and adrenals (Table 2). Again a slight reaction was found in the seminal vesicles and prostate, suggesting that a substance other than that of testicular or adrenal cortical origin was involved. Furthermore, the removal of both adrenals and testes in no way affected the reactions present in the other organs and tissues of the body. The stability of these reactions was emphasized by the persistence of the reactions in the heart, liver and kidneys, 16 weeks after the ablation of the hypophysis at which time the gonads and adrenal cortex were completely atrophic. This again eliminated the role of ketosteroids. Any remaining doubt was removed when experiments were performed in vitro with testosterone, progesterone and brei of kidney,

according to the method of Feulgen and Gruenberg (1938-39), to evaluate the reactivity of these substances with fuchsin-sulphurous acid. In contrast to plasmalogen, which was shown by Feulgen to produce an intense purple color in minute doses, testosterone and progesterone, in doses of 30 mg, were only slightly reactive. By comparing the color intensities, it could be calculated that progesterone or testosterone would have to be present in tissue concentrations of 1 in 10 or 1 in 2 respectively, to account for the intensity developed in the tissue brei. It is obviously impossible to find such concentrations of steroids in tissues; for instance, Allen and Goetsch (1936) found that the concentration of progesterone in sow's ovaries was of the order of 1 part in 40,000.

The ability of steroids to react with fuchsin-sulphurous acid and phenylhydrazine has been investigated by many workers. Oster (1946) subjected an alcoholic solution of testosterone-propionate, methyl testosterone, dehydroandrosterone acetate, androstenediol, androstenedione, pregnenolone, 21-acetoxy-pregnenolone and many other ketonic and non-ketonic steroids to an in vitro test with fuchsin-sulphurous acid, and found that none of them reacted. Hayes (1947) was also unable to obtain a positive reaction with desoxycorticosterone acetate, however, he found that this compound gave a positive reaction with phenylhydrazine under the conditions used in the histochemical application of this stain. There is no doubt that α,β-unsaturated ketones or aldehydes can react with phenylhydrazine to form hydrazones. A review of the literature indicates that either a very acid medium or boiling for a long period are required for the reaction. In our own experiments and those reported by Bennett, the reaction was carried out at neutrality thus making hydrazone formation with ketosteroids rather improbable.

It seems, therefore, necessary to look for substances other than ketosteroids to account for the reactions described above; plasmalogen seems the most likely substance.

For many years fuchsin-sulphurous acid has been used by chemists as a reagent for the in vitro demonstration of aldehydes, and there can be little doubt that in tissue sections this substance also demonstrates their presence.

The ability of fuchsin-sulphurous acid to produce the same kind of color complex with ketones as with aldehydes is very doubtful. Karrer (1938) in his text book on organic chemistry states that, although the fuchsin-sulphurous acid provides a very sensitive test for aldehydes, it is not specific for them reacting with certain ketones as well as oxidizing reagents, e.g., cupric salts. He does not mention the ketones involved, however, nor the type of color produced, nor the conditions under which the reaction takes place. Lison (1932) claimed that certain ketones and unsaturated compounds, such as oleic and cinnamic acid, gave a positive reaction with fuchsin-sulphurous acid. Oster (1946) in an attempt to evaluate Lison's work found that oleic acid could react only after mild oxidation. Among the ketones claimed by Lison to be able to react with decolorized fuchsin were acetone, methyl ethyl ketone, methyl isobutyl ketone and acetophenone. While the first three compounds produced a red color this reaction in no way resembled the characteristic purple color obtained with aldehydes. Furthermore, the color complex derived from the aldehyde-fuchsin-sulphurous acid reaction could be decolorized by alkali and recolorized by acids. This phenomenon was not shown by the red color complex derived from ketone-fuchsin-sulphurous acid reaction. Acetophenone gave no reaction whatsoever

while phenylacetaldehyde, heptaldehyde and formaldehyde as representatives of aromatic and aliphatic aldehydes, gave the typical purple reaction. From our own experiments and those of Oster (1946) it seems unlikely that ketonic steroids are involved. Aldehydic steroids, if present, in sufficient quantities, could contribute to the color developed.

On the whole, the available results make it unlikely that tissue ketones are responsible, although this possibility has not been eliminated. If aldehydes are thus considered as the reacting agents, their most plausible source is the group of acetal phosphatides described under the name plasmalogens. Other lipid or non lipid complexes that might contribute these aldehydes, however, must also be borne in mind. Stepp, Feulgen and Voit (1927) brought up the point that in the higher animals acetaldehyde might also react. This metabolite, however, is not present in any great quantity and gives a red-violet instead of the typical blue-violet color when treated. It was isolated in the blood and urine by these workers, but existed in such small quantities that it was not demonstrable. They also suggested that the aldehydes of unsaturated fatty acids might be present in tissues and cause a positive reaction (Feulgen, 1929). Oster (1942) found that para-hydroxy-phenylacetaldehyde, which can be produced in the kidney of some species under special conditions, also might give a blue color and suggested that aromatic aldehydes, which may be stored as tissue aldehydes, are formed in the process of amino acid metabolism and may contribute to the production of the blue color found. Gerard (1935) found that the reaction was not inhibited by treatment with dimethylhydroresorcine and therefore suggested that the reaction with fuchsin-sulphurous acid was not due to aldehydes, but to some oxidative power of fatty inclusions. It must be noted, however, that dimethylhydroresorcine may not affect the bound aldehydes present, for instance, in plasmalogens. These might be liberated

after treatment and then react with fuchsin-sulphurous acid.

Recently, Neuman (1944) has presented evidence that tissue aldehydes may also occur in combination with some polyalcohol, other than glycerol.

Thus, while there are indications that aldehyde-lipids do not occur solely as phospholipids, the weight of evidence is in favor of the theory that the aldehydic lipids responsible for the fuchsin-sulphurous acid and the dinitrophenylhydrazine reactions are derived, for the most part, from acetal phosphatides. This idea has been indirectly strengthened by the finding of Claude (1940, 1941) that cellular components 50-200 mμ in size, obtained by the ultracentrifugation of tissues, give a positive reaction with fuchsin-sulphuric acid. On chemical analysis, the particles were found to be composed essentially of two main portions, one lipid (40 to 50 per cent) the other largely protein in nature. About 75 per cent of this lipid material is represented by phospholipid-like compounds as shown by phosphorus and nitrogen analysis. A variety of tissues were investigated including liver, Brown Pearce tumor, lymphoid cells and chicken tumor 1.

In the reactions obtained in our experiments with fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reagents on formalin-fixed tissues, the distribution observed was, for the most part, in keeping with what has been generally reported by Feulgen, Verne and others using tissues either unfixed or fixed in mercuric chloride. There were several exceptions, however, the most notable being the fact that we found reactions of varying intensity in the hepatic cells, whereas other workers have reported that only the cells of the bile canals stain (Verne, 1929). Hayes (1947) has objected to the use of formalin fixation and to the ensuing oxidation which takes place in our tissue sections, claiming that the fatty aldehydes obtained under such conditions should not come from acetal phosphatides. He suggested the possibility

that unsaturated fatty acids (e.g., oleic acid) are formed into peroxides which later break down to aldehydes, and that such compounds produce the reactions under the conditions employed in our experiments. Such a possibility exists and might account for the discrepancies found in the distribution of the purple reaction. The fact, however, that mercuric chloride intensifies both the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions, even after formalin fixation, suggests the probability that, even in our experiments, formalin fixation and oxidation have still not unmasked all the aldehydes present, and that some acetal phosphatide still remains in which the acetal bond is loosened by mercuric chloride treatment. Furthermore, the reactions unmasked by mercuric chloride differ in intensity, but not in distribution from those found without mercuric chloride. The possibility therefore exists that during the process of formol fixation, the chemistry of which is poorly understood, some of the acetal phosphatides are disrupted; the released fatty aldehydes are reduced; and, during the process of subsequent oxidation are reconstituted into an active form. This hypothesis would account for the experiments reported under the heading "Staining Techniques: Fuchsin-Sulphurous Acid Stain".

Significance of the Fuchsin-Sulphurous Acid and 2,4-Dinitrophenylhydrazine Reactions

The results of the age experiments revealed that the staining of most organs with fuchsin-sulphurous acid was markedly influenced by growth and senility consisting of a gradual overall decrease with age. The results were most striking in the gonads and sex accessories, where the reactions were slight or absent at birth, increased rapidly to reach a maximum at sexual maturity and remained at this level for a number of months during the active sexual life of the animal. With advancing age the reactions decreased and became slight or completely disappeared. Thus, for instance, in the epithelium of the seminal vesicle, the reaction became intense at puberty and disappeared in senile animals. This cycle may be compared to what occurs after either castration, which almost completely suppresses the reaction of seminal vesicles and prostate, or testosterone treatment of castrates, which restores the staining reaction. There is obviously a marked parallelism between functional activity of these organs and their staining reaction. The parallelism in the testis between function and interstitial cell staining, which was moderate at birth, most intense at puberty and slight in old age, was also quite apparent. Similar examples may be found in the kidney, ovaries and to a lesser extent in the adrenal.

It is of interest to note that hypophysectomy produced some decrease in the intensity of the staining reaction only in those endocrine organs under its direct control (adrenals, testis, ovary). The decrease and shift in reaction in the adrenals has been carefully studied by Deane and Greep (1946a) and resembles that found in the mouse during the ageing process. Here again the decrease in function paralleled the weaker reaction.

In the light of the hypothesis that the intensity of the reaction indicates an actively functioning tissue, the lack of reaction in the carcinomas would suggest a lack of differentiation.

There is one exception, however, to the theory that staining is indicative of functional activity. Frequently cells with signs of fatty degeneration at the level of their fat globules, stain with both these reagents. For instance, intensely staining fatty globules could be observed in degenerating follicles and old corpora lutea of the ovary, in the reticular zone of the adrenal, occasionally in the collecting tubules of the kidney and fairly often in the cells of the reticulo-endothelial system.

An interesting side-observation was made in the old animals, in whom it is well known that brownish "wear and tear" pigment is deposited in many organs. There was an inverse relationship between the staining intensity of the steroid secreting cells (adrenal cortex, interstitial cells of the testis, theca and interstitial cells of the ovary, corpus luteum) and the occurrence of large irregular cells laden with brown pigment. In other words, as the animals aged, the fuchsin-sulphurous acid and dinitrophenylhydrazine reactions decreased in intensity in these tissues and the number of stainable cells diminished, while the cells themselves and surrounding reticular cells gradually accumulated more and more pigment. Hooker and Pfeiffer (1942) noted that after estrogen treatment the interstitial cells of the testis were phagocytosed by large cells in which brown pigment accumulated. The present results indicate that a similar process is occurring during the disappearance of all steroid secreting cells when their secretory role is reduced or terminated. In the experiments using tissues from hypophysectomized rats, pigment

also was found to occur in the zona reticularis of the adrenal cortex and in the interstitial spaces of the gonads. This transformation had been previously noted in the hypophysectomized mouse by Leblond and Nelson (1937). From these observations it would appear that the large phagocytic pigment cells in the interstitial spaces of the gonads, as in the zona reticularis of the adrenal, have disposed of the senile steroid secreting cells.

The thesis that these aldehydic substances stained with the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions reflect the functional activity of steroid secreting and some of their target organs seems to suggest a role played by plasmalogen. Normally the staining intensity characteristic of an organ indicates the balance resulting between the quantity of the compound elaborated or incorporated by the organ and the quantity used up or excreted by it. The presence of enough compound in the cells for a staining reaction to be obtained indicates some dominance of production over elimination of the compound. Consequently cells like the interstitial cells of the testis which stain evenly should have a steady secretion; while a cyclic secretion would be the lot of cells that do not stain regularly, for instance, those of the corpus luteum or interstitial cells of the ovary.

Resume

These experiments have demonstrated that there is a strict parallelism between the histochemical reactions produced by the fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine reactions in rat and mouse tissues. The substances producing these reactions are very widespread throughout the body and are especially abundant in lipid rich tissues, such as the adrenal cortex, corpus luteum, mammary gland, preputial glands, myelin sheaths of nerves, and fat cells.

Castration, in addition to producing the well recognized atrophy of seminal vesicles and prostate, causes a considerable decrease in the reaction of these organs; and testosterone restores it to an approximately normal level. Neither castration nor testosterone affect the intensity or distribution of the reaction in any organ or tissues, with the exception of the adrenal. Adrenalectomy, in addition to castration does not modify the reaction anywhere. Hypophysectomy does not affect the staining intensity of most organs with the exception of the adrenal, ovary and testis.

Two types of substances have been considered as the causal agents; ketosteroids and plasmalogens. The persistence of the reactions after ablation of the two known sources of ketosteroids in the male rat, namely testes and adrenals, denies the first hypothesis; so does the inability of progesterone and testosterone to markedly react with fuchsin-sulphurous acid in vitro. On the other hand, the increase of both reactions with mercuric chloride suggests that "plasmalogens" are the effective agents.

The staining ability of most organs, especially the endocrines and sex accessories varies with the age of the animals, increasing at puberty and decreasing with advancing age.

It is suggested that the aldehydic lipids, derived for the most part from the acetal phosphatides (plasmalogens), reflect the functional activity of steroid secreting organs and some of their targets and therefore take part in their functional activity.

As the staining intensity and number of the steroid secreting cells decrease in the endocrine organs of senile animals, cells laden with "wear and tear" pigment appear in their neighborhood.

PART III

RADIOACTIVE TRACER INVESTIGATION

In our previous experiments, using various histochemical reagents, intense reactions were obtained in some endocrine organs. These were at first attributed to the presence of endogenously produced steroids, but were later explained on the basis of aldehydes, liberated from acetal phosphatides, of the plasmalogen type. These latter compounds were shown to be intimately related to the functional state of the steroid producing organs and though these unexpected findings opened up a new field of research, they did not supply any information concerning the tissue localization of steroids.

In the meantime, experiments were in progress designed to trace exogenous steroids in various organs and tissues of the body by labeling them with radioactive isotopes. It was realized from the outset that C_{14} was the isotope of choice, for by its use, steroids could be prepared in no way different from the naturally occurring ones. In fact, Turner had synthesized cholestenone and testosterone containing C_{14} in the C_3 position (1947a, 1947b). Because of the non-availability of this isotope in Canada, at the time this project was undertaken, it was decided to use the readily available isotope of iodine, I^{131}. Alpha-estradiol was selected since it possessed a phenolic hydroxyl in the C_3 position. By the analogy of the iodination of tyrosine, it was hoped that the phenolic estrogen might react to form ortho and/or para mono- or di-iodo-estradiol.

Berger (1946) had already prepared and investigated the distribution of triphenylethylene labeled with radio-bromine in mice. Despite

this lead it was thought that the investigation of labeled naturally occurring α-estradiol would be of more interest than the study of such an artificial estrogen.

Methods

The Preparation and Separation of Radioactive Iodine (I^{131})

Throughout all experiments to be reported the radioactive, 8 day isotope of iodine, I^{131}, was used. This isotope decays to Xenon emitting negative beta, internal conversion electrons and nuclear gamma rays. The particulate radiation has a maximum energy of 0.595 and an average energy of 0.205 MEV while that of the gamma rays varies from 0.080 to 0.367 MEV.

Radioactive iodine was obtained either as a deuteron-bombarded tellurium target from the cyclotrons of Washington University (St. Louis, Mo.) or the Institute for Terrestrial Magnetism (Washington, D.C.) or as iodide from the cyclotron of the Massachusetts Institue of Technology (Boston, Mass.) and recently from the National Research Council of Canada.

In those instances where tellurium targets were received, radio-iodine was prepared in the following manner: The tellurium metal was placed in a 500 cc. round boiling flask connected to a ground glass joint, incorporating a dropping funnel, and to which was fused a U-shaped glass tube. The other end of the U-tube dipped into a 25 cc. Erlenmeyer flask containing about 5 cc. of 60 per cent chromic acid to trap any fumes evolved, thus providing salvage of a moderate amount of radio-iodine. Twenty-five cc. of concentrated sulphuric acid, previously purified by boiling with hydrochloric acid to remove any contaminating iodine, per gram of tellurium were added through the dropping funnel. The flask was then vigorously heated; the tellurium dissolved giving a reddish-purple color which disappeared on further boiling. A fine white precipitate now appeared. The chromic acid in the trap was washed into the flask,

5 cc. of 60 per cent chromic acid and 50 cc. of doubly distilled water per gram of tellurium were added in order, and then the material was ready for distillation according to the method of Matthews, Curtis and Brode (1938) which will be described below.

In the case of the material received from the National Research Council, the radioactive iodine had to be extracted to eliminate the sodium bisulphite vehicle which was injurious to the iodination reaction later performed. As in the case of the tellurium, the Matthews, Curtis and Brode method and apparatus was used.

The iodide to be distilled was placed in a 500 cc. boiling flask and 19 cc. of 60 per cent chromic acid were run in. 15 cc. of purified sulphuric acid were added slowly while the flask was rotated. After the mixture no longer boiled vigorously, the procedure was repeated. Finally, 65 cc. of sulphuric acid were added to make up the required amount. The mixture was now heated rapidly, in a hood, up to 215°C. and maintained at that temperature for 5 minutes. When the flask had cooled to below 100°C. a volume of doubly distilled water, equivalent to 25 cc. in excess of the sulphuric acid employed, was added. Two anti-bumps (Fig. 55-111) were inserted and the mixture stirred thoroughly by rotating at a 45 degree angle. The flask was then connected to the distillation apparatus (Fig. 55 -1) which had been thoroughly washed. A 250 cc. Erlenmeyer flask containing 1 cc. of 1 per cent NaOH and an anti-bump was placed under the condenser stem so that the tip dipped into the solution. The flask was then heated and as soon as the distillation began, 10 cc. of 50 per cent phosphoric acid, and 0.5 cc. of Merck's Superoxol (30 per cent hydrogen peroxide) were added through the entry tube. The distillation was made at the rate of approximately

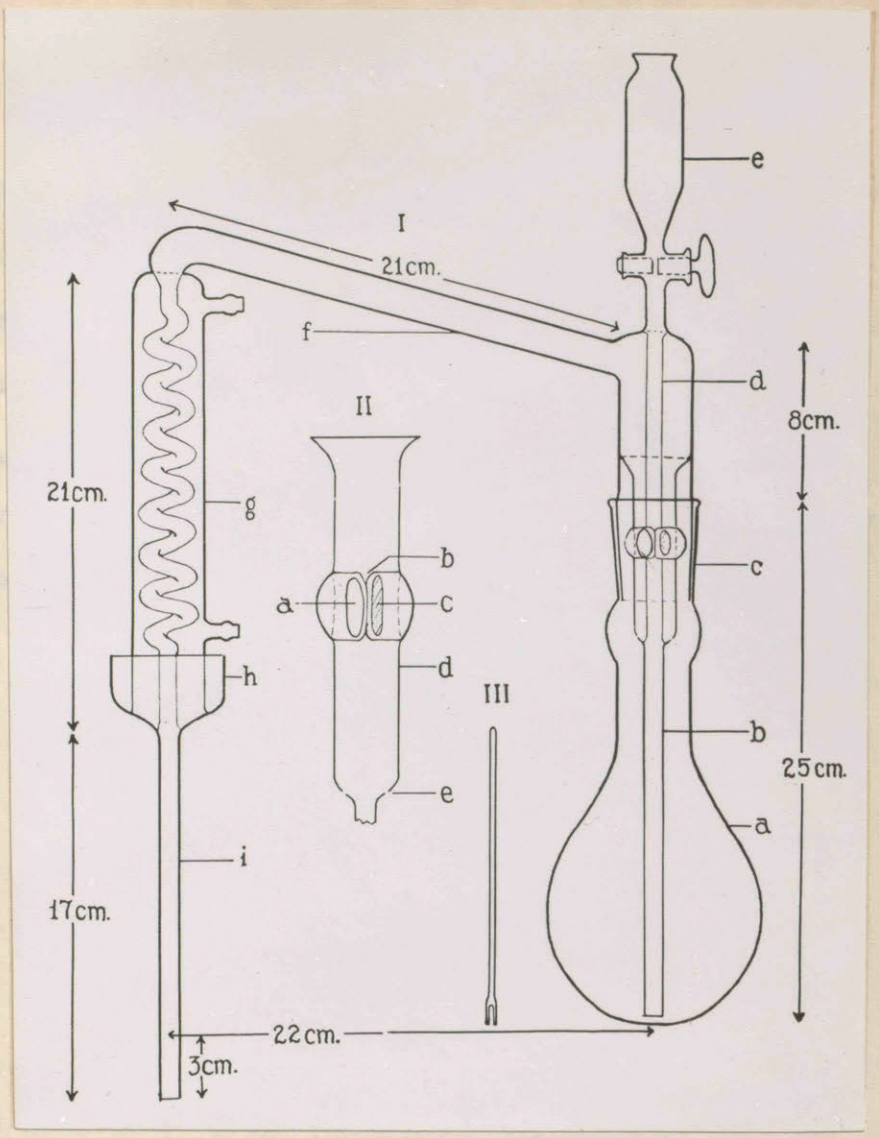


Fig. 55. Iodine Distillation Apparatus (Matthew, Curtis and Brode, 1938)

The digestion and distillation flask, 1-a, is a 500 cc. round bottom boiling flask, while 1-c is a standard joint of 30 mm. The coil condenser, 1-g, may or may not have a dew-collecting cup, 1-h, attached. Tubes have the following respective inside and outside diameters; 1-b, 3 and 9 mm; 1-d, 3 and 6 mm; 1-f, 11 and 15 mm and 11-d, 13 and 15 mm.

11- represents the entrainment trap. Vapors on leaving the flask enter at 11-a, follow the concentric tube around 11-d, and enter 11-d through 11-c. The concentric tube is closed off at 11-b, between 11-a and 11-c. Three small holes, 0.2 mm in diameter, are located at 11-e, the bottom of 11-d, in order to permit the condensed liquid to flow back into the flask. The inside cross-section area of the concentric tube is approximately 30 sq. mm. In some apparatus the concentric tube is eliminated and is replaced by a longitudinal slot in tube 11-d.

111- represents the type of anti-bump used.

100 cc. per 15 to 20 minutes. A volume equivalent to 25 cc. plus one-half the volume of acid was distilled. The pH of the distillate was frequently checked and maintained at higher than 8 to 9 by the addition of 1 per cent NaOH, if necessary. After completion of distillation, the distillate, containing the radioactive iodine as iodide, was evaporated to about 5 cc. and then made up to the required volume, usually about 10 cc. with doubly distilled water.

The Estimation of Iodine[1]

The I^{127} content of samples was estimated, by the thiosulphate titration method, in the following manner: To a 2 cc. sample, containing from 1 to 100 micrograms of iodine, 1 or more drops of 1 N potassium permanganate were added from the pipette of a dropping bottle until a purple color was obtained. The flask was then rotated gently upon its side and placed in a boiling water bath for 5 minutes. Three to six drops of 85 per cent phosphoric acid were added and, after 2 minutes, 1 or more drops of 0.5M sodium nitrite were run in until the permanganate color disappeared. The solution was again heated in the boiling water bath for 30 seconds; 3 to 4 drops of 40 per cent urea were added and the flask replaced in the water bath for 1 minute. Finally the flask and contents were cooled over ice, and 0.1 to 1 cc. of a 1 per cent potassium iodide solution, depending on the estimated iodine present, were added. The yellow solution was then titrated against 0.001N thiosulphate and 1 drop of a 2 per cent starch solution was added just before the end point and the titration continued until the blue color disappeared.

In a few cases, when iodine estimations were performed on oily samples, a wetting agent, duponol c (sodium lauryl sulphate), was added to the reaction mixture in the boiling flask in order to facilitate a complete oxidation of the organically bound iodine. It was found that this procedure gave a satisfactory iodine recovery.

1 - Iodine estimations were carried out with the aid of Miss J. Cambron, Department of Histology, McGill University.

Preparation and Radioactive Assay of Samples

Prior to the injection of animals with iodinated estradiol, several 0.05 cc. aliquots were removed from the syringe and suitably diluted, in a volumetric flask, with 2N NaOH. A 1 cc. aliquot was placed on a watch glass and treated in the manner described below. These various fractions served as standards for the experiment and permitted the calculation of the total of radioactivity administered to each animal.

At autopsy, the organs were removed, weighed, and separately homogenized by heating in 2N NaOH (1 cc. per 100 mg.). That alkaline digestion did not cause disintegration of the iodinated steroid had been previously determined. In cases where organs weighed less than 100 mg., 1 cc. of 2N NaOH was used. A 1 cc. aliquot of the homogenate was evaporated to dryness on a 40 mm. watch-glass in a constant temperature oven maintained at 70°C. Whole oxalated blood was centrifuged and the volume of cells and plasma measured. The blood cells were then washed three times with saline, and cells and plasma treated in the manner described above.

All radioactive assays were made by means of a bell type β-ray Geiger-Müller tube fitted with either a duraluminum or mica window. The tube was connected to a "64" scaling instrument, which recorded one out of every 64 disintegrations occurring in the radioactive sample. For convenience specimens were counted in multiples of 6.4 minutes; the most active for 1.6 minutes, the less active for 3.2 and the least active for 6.4. All background[1] counts were made for 6.4 minutes. As an indication of the stability of the tube, and to provide a standard for comparison of

1 - The background consists of the number of counts per minute registered by the shielded Geiger tube in the absence of a radioactivity source, and reflects the cosmic and other radiations present in the atmosphere. In actual practice, this value is determined many times during the counting of a series, by placing an empty watch glass below the window of the tube and counting for 6.4 minutes.

results when different Geiger tubes were employed, a uranium standard was counted with each series. A count of 10 above the background was taken as significant, since this number has been calculated to be approximately three times the standard error of the background. After subtraction of the average background, the count for each sample was corrected and expressed as number of counts per minute.

Fractionation of Organs and Excreta[1]

Prior to establishing a definite routine for the fractionation of samples, the number of ether washings required to extract the organically bound radio-iodine from organs and excreta had to be determined. This was done in the following manner: A small amount of crude radio-iodo-estradiol was added to liver, entire gastro-intestinal tract and feces and extracted before and after acid hydrolysis for 5 minutes.

	Per cent ether soluble radioactivity	
	First 3 extractions	Second 3 extractions
Liver (non-hydrolysed)	75	3
Entire gastro-intestinal tract (non-hydrolysed)	82	2
Entire gastro-intestinal tract (hydrolysed)	82	0
Feces (non-hydrolysed)	71	11

Since very small amounts of activity remained after the first 3 extractions, a standard technique of 4 was adopted.

The effect of acid hydrolysis on crude radio-iodo-estradiol was then investigated. It was found that without hydrolysis, 88 to 97 per cent could be recovered as ether-soluble material, while, after 5, 10, 20 and 30 minutes of vigorous heating in an acidic medium 77, 72, 40 and 44 per cent was recovered. Since the greatest destruction occurred after 10 minutes, it was decided to use the 10 minute hydrolysis to avoid excessive destruction and ensure dissolution of the conjugated material

1 - All fractionations were performed by Mrs. J. Saffran, Department of Biochemistry, McGill University, under the direction of Dr. R.D.H. Heard.

present in the samples (Smith, Smith and Pincus, 1938; Smith, Smith and Schiller, 1939; Pincus and Pearlman, 1942). Even so, it was quite probable that some conjugated material present was unaffected.

The following routine was then established: The alkaline digests of organs and excreta were acidified to a 10 per cent excess with 50 per cent HCl (about pH 1), and extracted 4 times with 10 cc. portions of freshly distilled ether. In cases where it was desired to hydrolyze the samples before extraction, the acidified digests were heated vigorously for 10 minutes and, after rapid cooling, were extracted as described above. The combined ether extracts were washed with two 10 cc. portions of water, evaporated to dryness, dissolved in 2 cc. of 1N NaOH and plated for counting. The aqueous phases were alkalinized with pellets of NaOH, diluted with doubly distilled water so that the resulting solution was 2N, and a 1 cc. aliquot was plated and prepared for counting. In all material plated, care was taken that the amount of NaOH on the plate was always equivalent to the amount present in 1 cc. of 2N NaOH.

In a few instances the ether-soluble material was further fractionated into neutral, acid and phenol fractions in the following manner: A 2 cc. aliquot of the alkaline digest was acidified to a 10 per cent excess with 50 per cent HCl and extracted with four 10 cc. portions of freshly distilled ether. The residue constituted the aqueous phase. The ether portions were combined and extracted with four 10 cc. portions of 10 per cent sodium carbonate. The carbonate portions were combined and constituted the acid fraction. The remaining ether fraction was then extracted with four 10 cc. portions of N NaOH. The ether fraction contained the neutrals. The alkaline fractions were combined, acidified with 50 per cent HCl and re-extracted with four 10 cc. portions of ether. The combined ether frac-

tions comprised the phenolic fraction, while the aqueous residue was discarded since only traces of radioactivity were present.

The Preparation of Crude Radio-Iodo-Estradiol[1]

Model experiments with I^{127} were first conducted in order to establish the optimum conditons for the iodination of α-estradiol with radioactive iodine. Two types of iodinations were investigated, namely iodination with I_2 in alkaline medium and iodination with N-iodo-acetamide. Both methods yielded a yellow oil which could not be crystallized. In both cases a chromatographic purification of the oil on alumina with benzene and crystallization from benzene and petroleum ether yielded a micro-crystalline material which gave a positive copper wire test for halogens. Its melting point, 173-174.5°C. with decomposition, was depressed on admixture with pure α-estradiol (M.P. 176-177°C.).

The results of the model experiments indicated that the best yield of crystalline material could be obtained by reaction of the α-estradiol with N-iodoacetamide. The aqueous nature of the radioactive iodine available, however, made the iodination in an alkaline medium the method of choice. This was carried out as follows:

To the tracer iodine I^{131} present as iodide in 1 cc. of 1 per cent NaOH, were added 103.25 micrograms of carrier potassium iodide and 26.62 micrograms of potassium iodate, and the mixture was acidified with 0.2 cc. of 2.5N sulphuric acid. At this point the yellow color of iodine became visible. 100 micrograms of α-estradiol dissolved in 1 cc. of methanol and 0.2 cc. of concentrated ammonia were added. The pH of the resulting solution was approximately 9-10. The reaction mixture was neutralized with glacial acetic acid, diluted to 10 volumes with distilled water, and extracted with three 15 cc. portions of freshly distilled ether.

1 - The preparation of crude radio-iodo-estradiol and di-iodo-estradiol, and chromatographic analyses were performed by Mrs. J. Saffran, Department of Biochemistry, McGill University, under the direction of Dr. R.D.H. Heard.

The combined ether extracts were twice washed with water and then evaporated to dryness. The resulting oil was then taken up in 0.1 cc. of ethanol.

TABLE 3

CHROMATOGRAPHIC ANALYSIS OF CRUDE-IODO-ESTRADIOL

	Eluate	Weight in mg.	I content in µg per mg. of fraction	Specific activity (Counts per µg. I)	Calculated content in mg. of Estradiol	Mono-iodo-estradiol	Di-iodo-estradiol
Total oil used		340.6	260	3.7			
Crystals 1 (M.P. 176-177°C)	Benzene + 4-8% acetone	48.2	0	0	48.2		
Crystals 2 (M.P. 173-174.5°C with decomposition)	Benzene and benzene + 1% acetone	38.5	480	1.2	0	0	38.5
Crystals 3 (M.P. 163-169.5°C with decomposition)	Benzene-ether (50:1)	2.4	310	—	0	2.4	0
Fractions 1 and 2	Benzene	41.6	396	2	3.5	12.4	25.7
Fractions 3 and 4	Benzene-acetone	12.5	340	4	1.3	11.2	0
Fraction 5	Benzene	4.9	50	5	4.2	0.7	0
Fraction 6	Benzene-ether	57.8	317	5	0	57.8	0
Fraction 7	Benzene-ether	21.9	245	4.5	5.2	16.7	0
Fraction 8	Benzene-ether	3.3	150	11	2	1.3	0
Totals					64.4	102.5	64.2

The Composition of Crude Radio-Iodo-Estradiol

The nature of the injection material was determined indirectly by the use of the isotope dilution technique. For this purpose, it was first mixed with a large amount of non-radioactive iodo-estradiol and chromatographed. A determination of the iodine content and specific activity (amount of radioactivity per unit of iodine) of the various fractions from the chromatographed material was carried out.

The product of the reaction of 225 mg. of α-estradiol with one equivalent of N-iodoacetamide (155.7 mg. containing 106.6 mg. I) was mixed with a small fraction of radio-iodo-estradiol (prepared by the method outlined above). The mixture was then separated chromatographically on alumina. Prior to chromatography, the iodine and the radioactivity contents of the crude oil were determined by the methods outlined above. Following chromatography, all crystalline and oily material were similarly examined.

The results (Table 3) showed that three types of crystals were obtained. Crystals 1 had the same melting point as pure α-estradiol and were taken to consist of α-estradiol that had not reacted with iodine.

Crystals 2 were shown by mixed melting point determinations to be identical with the halogenated crystals isolated in the pilot experiments described previously. Their iodine content was close to the theoretical for a di-iodo-estradiol, which is 485 micrograms of I per mg. These crystals were therefore considered to be di-iodo-estradiol (Fig. 56); and their purity was indicated by the constancy of the specific activity following recrystallization.

Crystals 3 were long, fine, and needle shaped (Fig. 57), depressing the melting point of di-iodo-estradiol and containing an amount

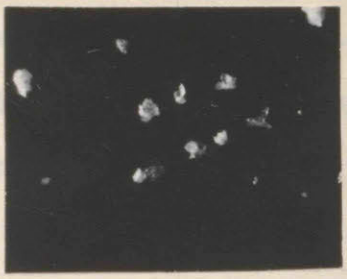


Fig. 56. Di-iodo-estradiol crystals

Fig. 57. Mono-iodo-estradiol crystals

of iodine close to the theoretical for mono-iodo-estradiol, namely 322 micrograms of I per mg. Their specific activity could not be measured because they were obtained at a time when the radioactivity of the material was almost exhausted. It may be noted, however, that fraction 6, which immediately followed crystals 3 in the chromatographic column and had a similar iodine content may be considered to consist of mono-iodo-estradiol. Its specific activity, namely 5, was taken as representative of mono-iodo-estradiol.

In order to calculate the content of each chromatographic fraction it was assumed that only three substances were present, namely estradiol, mono-iodo-estradiol and di-iodo-estradiol. For each fraction it was possible to set three equations, one based on the total weight, another based on the iodine content and a third based on the specific activity. Thus, the three unknowns, namely the weights of estradiol, mono-iodo-estradiol and di-iodo-estradiol in each fraction could be calculated (Table 3).

A total of 102.5 mg. of mono-iodo-estradiol and 64.2 mg. of di-iodo-estradiol was thus obtained. It is apparent that the small amount of crystalline mono-iodo-estradiol obtained (crystals 3) is not due to a low yield but to difficulty of purification. Another fraction not reported on the table and weighing 39.6 mg. was not examined. In conclusion, the injection mixture consisted of estradiol, mono-iodo-estradiol and di-iodo-estradiol in quantities roughly proportional to the totals listed at the bottom of Table 3.

The Preparation of Di-Iodo-Estradiol

In the most recent experiments, pure di-iodo-estradiol was used in a few animals to determine whether its distribution and excretion would differ from that of the crude material. By the use of this pure material dosage could be adequately controlled.

Radio-di-iodo-estradiol was prepared in the following manner: 4 mg. of unlabeled di-iodo-estradiol crystals were mixed with a batch of radio-iodo-estradiol dissolved in benzene. About 2.7 mg. of radio-active di-iodo-estradiol were obtained by slow crystallization out of the mixture. The crystals were dissolved in 0.1 cc. of ethanol.

Experimental

The crude radio-iodo-estradiol and the pure di-iodo-estradiol were prepared for injection, as a coarse suspension, in a vehicle of 10 per cent ethanol and 90 per cent physiological saline. In one of the experiments employing radio-di-iodo-estradiol 2 drops of a wetting agent, duponol c (sodium lauryl sulphate), were added to the injection mixture, producing a very fine, uniform colloidal suspension, which was well tolerated by the experimental animals.

At the time of autopsy, the animals were anesthetized with ether, blood was removed as completely as possible from the inferior vena cava, and the animals allowed to die. The site of injection was discarded and the organs were removed and handled in the manner described under Methods.

In calculating the results it was assumed that muscle comprised 50 per cent, and blood, 5 per cent of the body weight; half the blood was taken as plasma and half as cells.

Biological Assay of Mono- and Di-Iodo-Estradiol[1]

In order to determine the estrogenic activity of mono- and di-iodo-estradiol, they were assayed on spayed rats. Five test animals were employed for each test. 1.14 mg. of mono- and 1.83 mg. of di-iodo-estradiol were taken up in 0.72 and 1 cc. of ethanol respectively, and diluted 10 times with water (solution 1). 1 cc. of the di-iodo-estradiol solution was rediluted to 10 cc. with 10 per cent ethanol (solution 2). Three subcutaneous injections comprising 0.2 cc. of the solution to be tested were administered to each test animal.

	Mono-Iodo-Estradiol	Di-Iodo-Estradiol	Response
Solution 1	0.6 cc. (96 micrograms)	0.6 cc. (110 micrograms)	0
Solution 2		0.6 cc. (11 ")	0

Since these bioassays revealed that the iodinated estradiol was physiologically inert, it was thought that this compound might inhibit the action of α-estradiol. It was found, however, that the estrogenic activity of a solution of α-estradiol containing up to 1000 times as much iodinated estradiol was in no way impeded.

1 - These bioassays were carried out by Ayerst, McKenna and Harrison, Limited, of Montreal, through the courtesy of Dr. D. Beall.

Distribution of Labeled Estradiol

The distribution of the iodinated estradiol was examined in over 40 organs and tissues of seven diestrous female mice of the cancer-susceptible "C3H" strain and one diestrous female mouse of the cancer-resistant "C57" strain. All animals were sacrificed 10 to 12 hours after a single subcutaneous injection.

Five were normal, non-tumor bearing animals; animal (a), a 4 month old, 30 gm. "C3H" mouse, received 18 micrograms and animal (b), a 6 month old, 26 gm. "C3H" mouse, received 15 micrograms of crude radio-iodo-estradiol in ethanol-saline in the dorsal region; animal (f), a 3 month old, 11 gm. "C3H" mouse, received 2 mg. of radio-di-iodo-estradiol in ethanol-saline in the right thigh; animal (g), a 3 month old, 20 gm. "C3H" mouse and animal (h), a 3.5 month old, 20 gm. "C57" mouse, received 0.54 mg. of radio-di-iodo-estradiol in ethanol-saline and 1 per cent duponol c (sodium lauryl sulphate) in the right thigh. Three of the cancer-susceptible animals bore mammary tumors. Of these, two, (c) and (d), were in good health and the third, (e), was apparently in poor condition; animal (c), a 9 month old, 32 gm. mouse, received 3 micrograms; animal (d), a 9 month old, 26 gm. mouse, received 16 micrograms and animal (e), a 10 month old, 25 gm. mouse, received 11.2 micrograms of crude radio-iodo-estradiol in ethanol-saline in the dorsal region. Animals (a), (b), (c), (d), and (e) were kept in individual mouse cages lined with filter paper for the collection of urine and feces. Animals (f), (g), and (h) were kept individually in specially constructed metabolism cages[1] which provided good separation of

1 - The cages were constructed in the following manner: The removable lid and floor, the latter containing the centrally placed food bin, and the circular walls were made of wire mesh. The bottom of the cage was welded to the base of one of two cones attached at their apices, and the whole rested on a shallow cup with a centrally placed inner receptacle. The latter was fitted with a circle of wire mesh, which could be raised or lowered as desired. The urine trickled down the sides of the upper cone, followed the lower cone and was trapped on filter paper lining the outer third of the shallow base. The fecal pellets fell through the mesh of the floor directly onto filter paper lining the inner receptacle of the shallow base.

urine and feces and prevented the contamination of the animal's fur and skin usually occurring in normal mouse cages. In all experiments food and water were given ad lib.

The results were expressed as percentage of the injected dose and as concentration (the number of counts per mg. of organ weight over the number of counts recovered in the animal's body per mg. of body weight, after exclusion of the site of injection, gastro-intestinal tract, feces and urine).

In order to explain more clearly the calculations involved in the determination of the injected dose and expression of the results, animal (c) will be taken as an example.

100 micrograms of α-estradiol were iodinated with a solution containing 18 million counts of I^{131} per minute. Following ether extraction, the iodinated steroid was found to contain about 600,000 counts per minute, therefore, the yield was about 3 per cent and indicated that 3 micrograms of radio-iodo-estradiol had been produced.

At the time of injection 0.05 cc. of the iodinated steroid were removed from the syringe for a standard, and the remainder (0.5 cc.) injected subcutaneously into the animal.

The standard was diluted up to 10 cc. with 2N NaOH, 1 cc. of which was plated and subsequently counted. It was found that this aliquot, when counted for 1.6 minutes, gave a figure of 150.50 on the mechanical counter of the scaler. This was multiplied by 64, the scaler recording 1 out of every 64 disintegrations, and divided by 1.6 yielding a value of 6020 counts per minute. The average background in this series was 20, and when subtracted from the standard gave the corrected figure of 6000 disintegrations per minute. When this value was corrected for the dilution (10), it

was found that the standard (0.05 cc.) contained 60,000 counts per minute, and since 0.5 cc. were administered to the animal the total injected dose was about 600,000 counts.

The results were expressed as percentage injected dose and as concentration and, using liver as an example, were calculated in the following manner:

Percentage injected dose.- The liver contained a total of 3216 counts per minute, which was 0.536 per cent of the total injected dose (3216 multiplied by 100 and divided by 600,000).

Concentration.- A total of 15,119 counts were recovered in the animal's body (excluding the gastro-intestinal tract, feces and urine). Since the animal weighed 32 gms., assuming an even distribution of the radioactivity, each mg. of body weight contained 0.47 counts per minute. The liver possessed 3216 counts and weighed 2996 mg., thus yielding 1.2 counts per mg. When this latter figure was divided by 0.47 (the number of counts per mg. of body weight) the concentration figure of 2.553 for this organ was obtained.

The distribution of radio-iodine after injection of crude iodinated α-estradiol (Table 4) revealed, despite individual variations, a similar pattern in normal (a and b) and in tumor-bearing (c and d) animals. The distribution, however, was somewhat different in the animal with the advanced mammary tumor (e). On the whole, a similar distribution was observed with the crude iodo compound (a-e) as with the pure di-iodo-estradiol (f-h).

Less than 1 per cent of the injected dose was recovered in the blood plasma, while a smaller, but appreciable amount was found to have entered the cellular elements. The occurrence of such minute amounts of

TABLE 4

DISTRIBUTION OF RADIO-IODO-ESTRADIOL

	Crude iodo-estradiol in normal (non-tumor bearing) mice		Crude iodo-estradiol in mammary tumor bearing mice		Di-iodo-estradiol in normal (non-tumor bearing mice	
	% of injected dose	concentration	% of injected dose	concentration	% of injected dose	concentration
Blood						
Plasma	a. (0.091	(0.478	c. (0.061	(1.289	f. (0.037	(0.244
	b. (0.115	(0.615	d. (0.100	(0.276	h. (0.166	(2.825
			e. (0.450	(2.079		
Cells	a. (0.036	(0.188	c. (0.024	(0.377	f. (0.015	(0.090
	b. (0.008	(0.044	d. (0.009	(0.026	h. (0.057	(0.971
			e. (0.091	(0.422		
Gastro-intestinal Tract						
Stomach and Contents	a. (0.787		c. (0.240		f. (0.541	
	b. (0.620		d. (1.483		g. (0.278	
			e. (0.786		h. (0.904	
Duodenum and Contents	a. (0.124		c. (0.216		f. (1.091	
	b. (0.574		d. (0.172		g. (0.202	
			e. (0.761		h. (0.275	
Jejunum-Ileum and Contents	a. (1.771		c. (4.964		f. (2.945	
	b. (6.461		d. (3.514		g. (2.412	
			e. (16.158		h. (3.249	
Colon-Caecum	a. (2.411		c. (3.209		f. (3.300	
	b. (15.037		d. (2.311		g. (4.380	
			e. (2.946		h. (3.006	
Faeces	a. (30.419		c. (72.457		f. (21.995	
	b. (17.590		d. (30.456		g. (30.765	
			e. (2.125		h. (24.271	
Glands Related to Gastro-Intestinal Tract						
Liver	a. (0.555	(1.042	c. (0.536	(2.553	f. (0.859	(1.959
	b. (0.754	(1.956	d. (0.861	(0.867	g. (0.570	(4.063
			e. (2.593	(0.416	h. (0.673	(4.756
Pancreas	a. (0.027	(0.393	c. (0.015	(0.489	f. (0.028	(0.961
	b. (0.020	(0.420	d. (0.037	(0.227	g. (0.012	(0.863
			e. (0.127	(0.168	h. (0.009	(0.413
Gall Bladder (with some bile)	a. (0.002	(1.709	c. (0.004	(18.298	f. (0.599	(21.594
	b. (0.005	(0.544	d. (0.014	(7.908		
			e. (0.018	(1.764		
Submaxillary	a. (0.032	(1.225	c. (0.010	(5.319	f. (0.015	(0.770
	b. (0.069	(2.835	d. (0.011	(0.249	g. (0.013	(1.379
			e. (0.181	(0.124	h. (0.024	(2.501
Sublingual	a. (0.001	(0.177	c. (<0.002	(<0.318	f. (0.010	(0.568
	b. (0.004	(0.402	d. (0.004	(0.159	g. (0.002	(0.764
			e. (0.006	(0.712	h. (0.003	(1.447
Parotid	a. (0.001	(0.161	c. (0.002	(0.425	f. (0.007	(0.325
	b. (0.010	(0.561	d. (0.006	(0.223	g. (0.005	(0.788
			e. (0.016	(1.909	h. (0.009	(1.711
Respiratory System						
Lungs	a. (0.015	(0.380	c. (0.017	(1.085	f. (0.013	(0.220
	b. (0.027	(0.531	d. (0.028	(0.318	g. (0.013	(1.009
			e. (0.095	(0.153	h. (0.024	(1.673
Urinary System						
Kidneys	a. (0.041	(0.404	c. (0.025	(0.681	f. (0.046	(0.407
	b. (0.052	(0.562	d. (0.061	(0.282	g. (0.031	(0.899
			e. (0.123	(1.156	h. (0.042	(1.579
Bladder	a. (0.007	(0.729	c. (0.025	(0.681	f. (0.004	(0.445
	b. (0.007	(1.000	d. (0.061	(0.279	g. (0.0008	(0.107
			e. (0.123	(1.455	h. (0.003	(1.851
Urine	a. (3.766		c. (24.789		f. (5.925	
	b. (13.477		d. (3.469		g. (9.265	
			e. (8.695		h. (4.948	
Endocrine Glands						
Thyroids	a. (0.121	(107.157	c. (0.039	(72.128	f. (0.121	((80.723
	b. (0.145	(91.162	d. (0.289	(163.340	g. (0.026	(115.284
					h. (0.019	(108.229
Adrenals	a. (0.0003	(0.244	d. (<0.002	(<2.553	f. (<0.001	(<0.465
	b. (0.001	(0.646	d. (0.001	(0.405	g. (0.002	(1.125
			e. (0.003	(1.277	h. (0.001	(1.481
Ovaries	a. (0.0008	(0.231	c. (<0.002	(<3.404	f. (0.002	(0.611
	b. (0.002	(0.544	d. (0.002	(0.286	g. (<0.001	(<0.830
			e. (0.005	(1.223	h. (0.0006	(0.705
Pituitary	a. (0.0002	(0.172	c. (<0.002	(<4.893	f. (<0.001	(<4.652
					g. (<0.001	(<2.296
					h. (<0.0007	(<2.865
Lymphatic System						
Spleen	a. (0.007	(0.136	c. (0.003	(0.234	f. (0.005	(0.143
	b. (0.018	(0.266	d. (0.032	(0.191	g. (0.004	(0.406
			e. (0.053	(0.709	h. (0.007	(0.682
Thymus	a. (0.0001	(0.106	c. (<0.002	(<2.978	f. (0.001	(0.100
	b. (0.003	(0.412	e. (0.002	(0.911	g. (0.001	(0.346
					h. (0.003	(0.499
Lymph Nodes	a. (0.011	(0.308	c. (0.025	(0.638	f. (0.018	(0.267
	b. (0.016	(0.434	d. (0.016	(0.190	g. (0.012	(1.331
					h. (0.008	(0.854
Accessory Sex Organs						
Uterus	a. (0.020	(0.345	c. (0.002	(0.596	f. (<0.001	(<0.237
	b. (0.017	(0.398	d. (0.022	(0.241	g. (0.007	(1.125
			e. (0.019	(1.273	h. (0.011	(1.186
Cervix	a. (0.004	(0.416	c. (<0.002	(<2.553	g. (0.002	(0.857
	b. (0.006	(0.792	d. (0.002	(0.183	h. (0.001	(0.854
			e. (0.019	(1.040		
Vagina	a. (0.006	(0.492	c. (0.006	(2.766	f. (0.005	(0.707
	b. (0.011	(1.163	d. (0.007	(0.237	g. (0.004	(0.937
			e. (0.012	(1.505	h. (0.007	(0.951
Mammary Gland	a. (0.442	(3.018	c. (0.741	(32.765	f. (0.344	(9.368
	b. (0.208	(2.525	d. (0.098	(0.606	g. (0.079	(2.045
			e. (0.142	(32.173	h. (0.061	(2.023
Mammary Tumor			c. (0.030	(0.383		
			d. (0.045	(0.212		
			e. (0.768	(1.440		
Preputial Glands	a. (0.0009	(0.257	c. (<0.002	(<7.021	f. (0.003	(0.402
	b. (0.001	(0.955	d. (0.001	(0.317	h. (0.001	(1.475
			e. (0.001	(0.720		
Varia						
Skeletal Muscle	a. (0.650	(0.225	c. (0.362	(0.362	f. (1.401	(0.754
	b. (1.182	(0.393	d. (0.777	(0.135	g. (0.718	(0.800
			e. (7.347	(2.120	h. (0.835	(0.882
Cardiac Muscle	a. (0.005	(0.134	c. (0.003	(0.255	f. (0.004	(0.103
	b. (0.014	(0.406	d. (0.013	(0.172	g. (0.005	(0.540
			e. (0.029	(0.762	h. (0.011	(0.871
Skin	a. (6.222	(5.531	c. (0.851	(2.340	f. (2.854	(2.483
	b. (7.819	(8.881	d. (11.157	(5.875	g. (0.481	(1.716
			e. (1.643	(2.039	h. (0.550	(2.100
Fat	a.	(0.614	c.	(2.191	f.	(1.349
	b.	(28.641	d.	(0.660	g.	(1.540
			e.	(2.004	h.	(1.201
Bone			c. (<0.002	(<0.020	f. (0.018	(0.076
	b. (0.104	(0.513	d. (0.047	(0.125	g. (0.009	(0.278
					h. (0.007	(0.132
Brain	a. (0.004	(0.042	c. (<0.002	(<0.064	f. (0.004	(0.025
	b. (0.006	(0.056	d. (0.006	(0.031	g. (0.004	(0.122
			e. (0.016	(0.016	h. (0.011	(0.885
Site of injection	a. (0.273		d. (0.354		f. (0.192	
	b. (0.161		e. (27.437		g. (15.674	
					h. (34.258	
Carcass	a. (0.588	(0.237	c. (0.279	(0.319	f. (0.980	(0.421
	b. (1.579	(0.256	d. (1.406	(0.256	g. (0.891	(1.301
			e. (2.086	(0.896	h. (0.630	(0.771

All G.M. counts below 10 (10 counts being approximately three times the Standard Error of the background) were made equal to 10 and after the proper calculations were reported in the table preceded with a <.

activity in this tissue indicated a rapid disappearance of the radioactivity from the blood.

The bulk of the injected activity was found in the various segments of the gastro-intestinal tract and feces, where an average of 30 per cent was present. The liver contained a moderate concentration, which was almost 10 times less than that of the bile. Of the glands related to the gastrointestinal tract, the submaxillary showed the highest concentration.

An average of about 9 per cent of the injected dose was excreted in the urine, while the values for the kidney itself were rather low.

A significantly high concentration was found in mammary glands, fat and skin. In the mammary tumors, the concentration approximated the lower limit found in normal mammary tissue. The concentration in other organs and tissues was low, being on the average, below 1, with the possible exception of vagina, uterus and adrenals. Other endocrine glands also showed similar low values. The thyroid, however, collected a large amount of radioactivity, namely, from 0.02 to 0.29 per cent of the injected dose.

The skin was found to contain a very high concentration. The figures may be too high in animals a, b and d, because of the possibility of contamination from urine and feces; but the presence of a fairly high concentration in the skin of the animals f, g and h, which were kept in special cages, seemed to confirm the ability of integument to collect activity. The localization of the radioactivity was determined by the method of radioactive autography, using frozen sections of the dorsal skin coated with photographic emulsion (Bélanger and Leblond, 1946). A large amount of water-insoluble radioactivity was observed in the derma, a

Fig. 58. Autograph of the skin of animal c. The integument from the back was fixed in neutral formalin, sectioned in the freezing microtome, coated with photographic emulsion and exposed in the dark. The black lines in the epidermis (top of photograph) are artifacts. The smooth darkening of the dermis indicates a high concentration of radioactivity, while the light graying in the hypodermis (lower part of photograph) points to a low concentration. x 58

small amount in the hypodermis, and none in the epidermis or hair follicles (Fig. 58).

The animal bearing the advanced tumor (e) showed more radioactivity than the other animals in most organs and tissues, especially in blood, jejunum-ileum and contents, liver, mammary glands, mammary tumor and skeletal muscle; while the amount of radioactivity excreted in the feces was much lower than in the other animals.

In most animals very little remained at the site of injection, following termination of the experiment. The animal, however, bearing the advanced mammary tumor (e) and the animals (g and h) receiving the iodinated estradiol in the ethanol-saline-duponol vehicle, still retained 27, 16 and 34 per cent of the injected dose respectively in this area, indicating a marked slowing of the rate of absorption.

The distribution studies revealed that in all groups of animals the skin, mammary glands, fat and liver had the ability to accumulate more radioactivity than any other organs. In two of the mammary tumor bearing animals (c and e), however, the submaxillary glands, vagina and blood plasma, while in animals (g) and (h) the blood plasma, salivary glands, preputial glands, adrenals, lungs and kidneys all had concentrations above one. Indeed, the animal with the advanced mammary tumor (e) had the highest concentrations. The high fat value (28) showed by animal (b), since it is far in excess of that found in any of the other animals, must be regarded as a contamination.

TABLE 5

THE INFLUENCE OF SEX ON THE RADIO-IODO-ESTRADIOL CONTENT OF LIVER, INTESTINE, FECES AND URINE

Organ	Males		Females	
	% Injected Dose	Concentration	% Injected Dose	Concentration
Jejunum, Ileum and Contents	4.810	0.683	0.627	0.067
	4.080	0.643	2.460	0.296
	1.424	0.237	2.896	0.255
Average	3.438	0.521	1.994	0.206
Colon, Cecum and Contents	5.835	1.369	1.751	0.350
	7.143	1.755	4.426	0.868
	2.892	0.653	2.552	0.499
Average	5.290	1.259	2.910	0.572
Feces	37.291	10.578	39.358	12.198
	40.695	14.195	36.514	19.155
	47.391	16.224	46.737	11.547
Average	41.792	13.666	40.870	14.300
Liver	0.821	0.140	0.428	0.070
	0.946	0.161	0.867	0.135
	0.586	0.092	0.642	0.083
Average	0.785	0.131	0.646	0.096
Urine	10.656		3.477	
	4.912		15.963	
	3.693		2.913	
Average	6.420		7.451	

Physiological Variation in Radio-Iodo-Estradiol Distribution

Effect of Sex

A second experimental series was devised to determine sex differences in the distribution of radio-iodo-estradiol. 1.7 micrograms of the iodinated estrogen was administered subcutaneously into the left thigh of each of three male "C3H" mice weighing about 28 grams and each of three female mice "C3H" weighing about 24 grams. The experimental procedure in this, and subsequent series was the same as in the preceding experiment. The concentration in this and following experiments was expressed as number of counts per mg. of organ weight over the injected number of counts per mg. of body weight. The radioactivity content of liver, intestine, feces, urine and mammary glands was similar in both sexes (Table 5). The amount of radioactive material found in the jejunum-ileum and contents, and colon-caecum and contents was somewhat lower in females than in the males, but such large individual variations normally exist that these differences are of doubtful significance.

In the males, the testes showed an average radioactivity concentration of 0.030 and the seminal vesicles, 0.139; the latter value varied as the weight of the glands, and presumably was dependent on the amount of secretion present. The prostate showed an average concentration of 0.152; but, since part of the urethra was included in the prostatic mass, the samples may have been contaminated with minute amounts of urine.

Effect of Pregnancy and Lactation

Because of the high concentration of radioactivity found in the mammary gland, it was decided to investigate the ability of these glands to collect the iodinated estradiol under various physiological conditions. 1.8 micrograms of radio-iodo-estradiol was administered subcutaneously in the left thigh

TABLE 6

THE INFLUENCE OF PREGNANCY AND LACTATION ON THE RADIO-IODO-ESTRADIOL UPTAKE OF MAMMARY GLAND, LIVER AND FAT

Organ	Normal Controls		Pregnant		Lactating	
	% Injected Dose	Concentration	% Injected Dose	Concentration	% Injected Dose	Concentration
Liver	1.029	0.102	1.309	0.234	1.145	0.230
	0.991	0.184	1.367	0.212	1.629	0.204
	0.687	0.134	1.369	0.158	1.146	0.123
	0.704	0.100	1.988	0.255	1.385	0.135
	1.221	0.155	1.855	0.242	0.615	0.084
	0.773	0.120	1.755	0.234	0.889	0.113
Average	0.901	0.133	1.607	0.223	1.135	0.148
Mammary Gland	0.106	0.210	0.258	0.210	0.032	0.032
	0.106	0.083	0.373	0.190	0.380	0.162
	0.087	0.078	0.440	0.205	0.535	0.132
	0.074	0.059	0.474	0.301	1.130	0.303
	0.126	0.166	0.304	0.279	0.052	0.046
	0.089	0.093	2.409	1.682	0.069	0.036
Average	0.098	0.115	0.710	0.478	0.366	0.119
Fat 1. (interscapular)	0.049		0.087		0.054	
	0.044		0.064		0.088	
	0.036		0.076		0.084	
	0.033		0.117		0.055	
	0.042		0.039		< 0.023	
	0.050		0.303		< 0.055	
Average	0.042		0.114		< 0.057	
Fat 2. (periuterine)	0.211		0.216		< 0.036	
	0.035		0.130		0.044	
	0.046		0.049		< 0.093	
	0.016		0.099		< 0.060	
	0.044		0.097		< 0.054	
	0.043		0.066		< 0.038	
Average	0.066		0.110		< 0.054	

All G. M. counts below 10 (10 counts being approximately three times the Standard Error of the background) were made equal to 10 and after the proper calculations were reported in the table preceded by <.

of eighteen hybrid, female albino mice divided into three groups, consisting of normal controls weighing 20-23 grams, of pregnant mice weighing 31-33 grams, and of lactating mice weighing 28-30 grams. All animals were sacrificed 10 to 12 hours after injection. The mammary glands were digested together, with the exception of the inguinal glands adjacent to the injection site. These discarded glands comprised less than one fourth of the total mammary tissue.

Pregnancy exerted a definite influence on the ability of liver, fat and especially the mammary glands to collect the administered radioactive material (Table 6). The mammary glands of pregnancy showed at least a two-fold increase in concentration over that found in the normal controls even if the high figure of 1.682 found in one animal was eliminated. Lactation on the other hand, tended to depress the amount of activity found in liver, mammary glands and fat to the control level.

Finally, in the pregnant females, the uterine contents showed a moderate amount of radioactivity, namely, an average concentration of 0.101 in the placentas and 0.090 in the foetuses.

TABLE 7

Distribution of Radio-Iodo-Estradiol after Common Bile Duct Ligation

The figures show the radio-iodine content of organs and excreta expressed as percentage of the injected dose

	Blood Plasma	Stomach and Contents	Duodenum and Contents	Jejunum, Ileum and Contents	Colon, Caecum and Contents	Faeces	Liver	Urine
Sham-operated Controls	0.26	2.11	1.09	7.45	8.21	18.67	0.89	1.93
	0.21	2.60	0.95	5.23	5.68	26.19	0.82	11.44
	1.38	3.45	1.09	7.16	11.39	34.60	0.99	3.24
	0.54	2.68	0.97	12.62	16.73	14.95	1.38	15.51
Average	0.35	2.71	1.03	8.12	10.50	23.60	1.02	8.03
Bile-duct Ligated Animals	0.94	5.32	0.45	2.44	0.47	0.02	4.91	30.74
	0.94	1.25	0.13	1.95	0.34	0.00	1.30	12.92
	1.52	1.42	0.79	2.38	0.60	0.01	2.87	25.62
	1.05	0.22	4.51	1.92	0.75	0.00	1.29	36.01
Average	1.11	2.05	1.47	2.17	0.54	0.01	2.59	26.32

Metabolism of Radio-Iodo-Estradiol

Effect of Common Bile Duct Ligation

The role of the liver and gastro-intestinal tract in the metabolism of iodinated estradiol, was examined after ligation of the common bile duct in four females of the cancer-susceptible "A" strain weighing 24-30 gm.; while 4 similar controls were sham operated. The common bile duct was exposed by a ventral incision and severed between two ligatures. Each animal received a subcutaneous injection of 3.5 micrograms of radio-iodo-estradiol.

Following ligation of the common bile duct (Table 7), there was an increase in the radioactivity of bile, liver, plasma and urine; a marked diminution of the activity in the jejunum-ileum and contents, in the colon-caecum and contents, and no activity in the feces. Furthermore, fractionation of the urine after hydrolysis revealed an increase of 30 per cent in the radioactivity of the ether fraction after bile duct ligation.

Effect of Intragastric Instillation

In order to determine whether the radio-iodo-estradiol would be absorbed from the gastro-intestinal tract, 7 micrograms were injected directly into the stomachs of 4 cancer-susceptible "A" strain female mice weighing 20 to 25 gm. The stomach was exposed by a ventral incision, and the material was injected with a 26 gauge needle as far from the site of entry into the stomach as possible. When the needle was withdrawn, the gastric wound was closed with a cotton ligature to prevent leakage.

The results (Table 8) showed that absorption of the material from the gastro-intestinal tract could take place, since plasma, thyroid, liver, gall bladder, uterus, submaxillary gland, mammary glands and urine showed an amount of activity comparable to, although lower than that found after subcutaneous injec-

TABLE 8

DISTRIBUTION OF RADIO-IODO-ESTRADIOL AFTER ITS INTRAGASTRIC INSTILLATION

The figures show the radio-iodine content of organs and excrete as percentage of the injected dose

	Blood Plasma	Stomach and Contents	Duodenum and Contents	Jejunum Ileum and Contents	Colon Caecum and Contents	Faeces	Liver	Sub-maxillary	Urine	Thyroid	Uterus	Mammary Glands
	0.28	1.19	0.51	1.34	2.71	7.62	0.39	0.10	8.20	1.30	0.01	0.06
	0.89	30.97	0.58	9.88	11.51	——	0.47	0.10	12.33	1.79	0.04	0.08
	0.15	1.93	0.13	1.98	4.94	16.24	0.35	0.05	1.26	2.23	0.01	0.09
	0.44	3.92	0.14	4.48	4.35	14.95	0.52	0.28	24.53	1.53	0.03	0.09
Average	0.44	9.50	0.34	4.42	5.88	12.94	0.43	0.13	11.58	1.71	0.02	0.08

tion (Table 4). Some of the material was still bound in organic combination, since the ether fractionation of the liver, for instance, contained 19 per cent of the radioactivity present in the organ. These values were, however, considerably lower than in normal animals (Table 9).

It is of interest to note, that the amount of radioactivity found in the thyroids of these animals, averaged 1.71 per cent of the injected dose (Table 8). This amount is considerably higher than what is usually found in these glands following subcutaneous injection (Table 4), and would indicate, that considerable breakdown of the radio-iodo-estradiol has occurred in the stomach with the subsequent release of iodide, which is selectively taken up by the thyroid glands.

Fractionation of Organs and Excreta

In order to obtain information concerning the nature of the radioactive material present in the injection material, tissues and excreta, they were fractionated with ether before and after acid hydrolysis according to the method previously described.

A simple extraction of the material as prepared for injection, revealed that on the average 93 per cent of the radioactivity was extractable with ether (Table 9, column 2). Further fractionation of the ether-soluble material into neutrals, acids and phenols revealed the following:

Substance	Neutral (per cent)	Acid (per cent)	Phenol (per cent)
Standard 1	19	9	60
Standard 2	21	5	61

The recovery of only 88 per cent for the total ether-soluble material probably represents some loss occurring during the various manipulations, since usually over 90 per cent is recoverable. As would be expected, the bulk of the

TABLE 9

PERCENTAGE OF RADIO-IODO-ESTRADIOL OF ORGANS AND EXCRETA PRESENT IN THE ETHER-SOLUBLE FRACTION

	In vitro experiments with radio-iodo-estradiol			Organs from mice treated with radio-iodo-estradiol	
	Hydrolysed	Non-hydrolysed		Hydrolysed	Non-hydrolysed
Sample of radio-iodo-estradiol as prepared for injection	77 53 71 80	92 88 97 95			
Same stomach and contents	90 76 65	96 87 91	Stomach and contents	d. (21 g. (11 h. (48 i. (39 j. (47	(19 (14 (44 (32 (41
Same duodenum and contents	82	84	Duodenum and contents	b. (85 g. (67 h. (54 i. (75 j. (83	(82 (64 (56 (57 (70
Same jejunum-ileum and contents	94	95	Jejunum-ileum and contents	b. (81 d. (78 g. (76 h. (71 i. (64 j. (61	(74 (70 (70 (70 (61 (57
Same colon-caecum and contents	91	92	Colon-caecum and contents	b. (71 d. (75 g. (62 h. (70 i. (46 j. (54	(57 (76 (65 (73 (43 (58
Same feces	87 82 81	82 84 81	Feces	b. (67 d. (50 g. (64 h. (57 i. (38 j. (32	(69 (50 (65 (60 (38 (34
Same liver	87 97 83	94 89 86	Liver	b. (61 d. (64 g. (68 h. (64 i. (53 j. (56	(51 (47 (63 (62 (55 (52
Same mammary glands	82	86	Mammary glands	g. (64 h. (62	(54 (52
Same fat	86	87	Fat	g. (43 h. (55	(39 (54
			Urine	b. (39 d. (44 g. (24 h. (31 i. (61 j. (53	(37 (31 (20 (24 (47 (38

of the animals receiving the radio-di-iodo-estradiol in ethanol-saline-duponol (g and h) were higher than, while the amount found in the same fraction of the urine was lower than, that found in any of the other animals. Otherwise there were no marked differences between the animals receiving radio-iodo-estradiol and those receiving radio-di-iodo-estradiol.

Further fractionation of the ether-soluble material from the feces of the animal which received the injection material analyzed above revealed the following:

Substance	Neutral (per cent)	Acid (per cent)	Phenol (per cent)
Feces	4	39	25

The 4 per cent in the neutral fraction is not significant and probably represents acidic or phenolic material that was carried over in the partition. The 25 per cent found in the phenol phase represents a marked drop from the 60 per cent found in the corresponding fraction of the standard, while the 39 per cent represents a marked increase.

Biological Assay of Gastro-Intestinal Tract and Feces Following the Administration of α-Estradiol

A preliminary attempt was made to determine, with the help of biological assays whether the intestinal excretion of pure α-estradiol is similar to that of its iodinated prototype. Ten female "A" mice received 100 micrograms of α-estradiol each by subcutaneous injection and were sacrificed 10 hours later. Three bio-assays were carried out with the jejuno-ileum and contents, the colon-caecum and contents, and the feces of the 10 animals. The material was digested in 2N NaOH extracted 5 times with ether, dried, taken up in ether again, washed with a 5 per cent solution of $NaHCO_3$ and then with water, taken to dryness and finally dissolved in corn oil. Five spayed, female rats were used for each

sample tested, and were given 6 per cent of the total. Negative results were obtained with jejuno-ileum and colon-caecum, while the feces gave a 100 per cent response. However, another group of 5 rats given 1.2 per cent of the feces samples showed uniformly negative results. The amount of biologically active estrogen excreted in the feces expressed as estradiol was calculated to be between 2.5 and 12.5 micrograms, that is to say between 0.25 and 1.25 per cent of the injected dose.

Discussion

The results of the isotope dilution indicated that the crude radioactive injection material consisted of mono- and di-iodo-estradiol. Furthermore, since this material was found to partition like phenolic estrogens, the fixation of either one or two atoms of iodine had presumably not affected the phenolic structure of ring A. Although no other evidence was available it was assumed that the iodine was fixed in the 2 and/or 4 position as in the case of other phenols.

Biological results indicated that the distribution of the iodo-estradiol was similar to that of natural estrogens, but different from that of the artificial ones.

The resorption of the iodinated estradiol from the site of injection must have been quite rapid, since little or no activity remained 10 to 12 hours after injection in animals (a), (b), (c), (d) and (f). Estradiol and estrone also have been found to be quickly resorbed from subcutaneous injection sites, while their esters and artificial estrogens are resorbed more slowly (Dingemanse and Laqueur, 1937; Zondek, 1941). It may be noted that suspension of the iodo-estradiol in a solution containing a wetting agent (animals g and h) slowed the resorption, as indicated by the presence of radioactivity at the injection site. A similar slowing of the rate of resorption of estrogens and testosterone, by the use of wetting agents, had been demonstrated by Bischoff and Pilhorn (1947). Finally, the slow resorption encountered in animal (e) could probably be accounted for by the advanced cancer present which might have produced the slow rate of metabolism generally associated with pronounced sicknesses.

The very rapid disappearance of radioactivity from the blood suggested a quick turnover of the iodinated estradiol, comparable to that noted with natural estrogens (Frank, Goldberger and Spielman, 1932; Dingemanse and Tyslowitz, 1941; Cantarow and associates, 1942, 1943; Szego and Roberts, 1946). In constrast, the artificial estrogens have been found to persist in the blood for rather longer periods (Dingemanse and Tyslowitz, 1941; Berger, 1946).

The withdrawal of the material from the blood was effected by three different routes, namely, liver, gastro-intestinal tract and kidney. The considerable amount of radioactivity in the lumen of the digestive tube and feces was found to have a two-fold origin, namely, bile and the gastro-intestinal tract itself. The bile was the main source, contributing 90 per cent of the radioactivity found in the digestive tract. This was demonstrated in bile duct ligated animals in whom 6.24 per cent of the radioactivity was found in the gastro-intestinal tract and feces (Table 7) as compared to 45.96 per cent in the normals. A recent report on radio-brom-equilin showed that this compound behaved like iodinated estradiol; since 35 per cent of the injected dose was found in the intestinal contents 6 hours after injection (Twombly, 1947). Similarly, in the case of natural estrogens, a large proportion of the injected dose was found to be excreted in the bile (Longwell and McKee, 1942; Cantarow and associates, 1943a; Pearlman and associates, 1947a).

On the other hand, the liver, through which large amounts of the labeled compound must have passed before being excreted into the bile, did not show a high concentration (Table 4). Similar low values following the administration of natural estrogens, had been obtained by Frank, Goldberger and Spielman(1932) and Dingemanse and Tyslowitz (1941). In constrast, following the administration of artificial estrogens, Tschopp (1946) and Berger (1946) found

that the liver concentration was very high.

In the bile duct ligated animals (Table 7), the labeled material was still excreted to some extent in the lumen of the stomach and intestines. In other words, the gastro-intestinal walls (and/or pancreas) have the ability to excrete steroids. The possibility of the elimination of natural estrogens through the walls of the gastro-intestinal tract was first demonstrated by Pearlman and associates (1947a) who found estrogenic activity in the feces of external bile-fistula dogs following the administration of estrone. In our experiments, only one-third of the radioactivity present in the stomach was ether-soluble and presumably steroidal in nature, while the greater proportion of that found in the intestines and feces was in the same state.

Finally, the large amounts found in the gastro-intestinal contents and feces did not give a true figure of the magnitude of excretion, since appreciable resorption from the digestive tube must have taken place. This was demonstrated by the entry of radioactivity into parenchymatous organs after the intragastric instillation of radio-iodo-estradiol (Table 8). It may be concluded that there is an interplay of elimination into, and resorption from, the intestinal lumen. That α-estradiol itself may also be resorbed from the intestine was shown by the presence of some estrogenic material in the bile after instillation of this estrogen into the duodenum (Cantarow and associates, 1943a).

Another role of the small intestine, and especially the duodenum, in the excretion of injected iodinated estradiol, and possibly of natural estrogens, was indicated by our fractionation experiments (Table 9). A small amount of conjugated material was found in the liver, duodenum and contents and jejunum-ileum and contents. Only one animal out of six showed any conjugated material in

the colon-caecum and contents, while none showed any in the feces. These findings suggested that the conjugated material, while being excreted through the intestine into the feces, must be completely hydrolysed; another possibility was that the conjugated material may have been resorbed and excreted through the urine, while the non-conjugated portion was for the most part excreted through the gastro-intestinal tract into the feces.

Both the liver and the duodenum have been suggested as the site of conjugation. Glass, Edmondson and Soll (1944) found that after the injection of estrone or estradiol into 4 men with cirrhosis of the liver, the bulk of the estrogenic material in the urine was in the combined form in three of the cases, while in the fourth, the estrogen was mainly in the free form. They interpreted their results to indicate that in the last case, because of the extreme liver damage, conjugation did not take place. While the liver seemed to be the likely site for the conjugation of a fraction of iodo-estradiol, the duodenum may also have taken part in this process, since the ether-soluble material present in the liver existed to a slight degree as a conjugate, while the conjugated fraction in the bile was smaller or even absent. The estrogenic material in the bile has been observed to be free in the dog and cow by Cantarow and associates (1942) and Pearlman and associates (1947b). These workers have also observed that, when α-estradiol was introduced into the duodenum of bile-fistula dogs, and bile collected, there was a considerable amount of conjugated estrogenic material present. When this estrogen was introduced into a jejunal loop, in a vehicle of bile, the free and total estrogen content of the blood collected from this loop were identical, demonstrating that resorption, but not conjugation, took place. The implication was that either the bile inhibited conjugation or that the duodenum was responsible. (Cantarow and associates, 1943a).

Very few workers have examined the excretion of estrogenic material in the feces under physiological conditions (Siebke and Schuschania, 1930; Kemp and Pedersen-Bjergaard, 1933-34; Dorfman and Gardner, 1944). The phenomenon is more apparent during pregnancy (Dohrn and Faure, 1928; Kemp and Pedersen-Bjergaard, 1933-34; Levin, 1945), as also after the administration of estrogens (Luchsinger and Voss, 1929; Siebke and Schuschania, 1930; Kemp and Pedersen-Bjergaard, 1933-34; Tschopp, 1946). However, in each case the amount recovered was rather small. In our experiments with pure α-estradiol, between 0.25 and 1.25 per cent of the injected dose was recovered by bioassay of the feces 10 hours after injection, while, in contrast, about 30 per cent of the injected radio-iodo-estradiol was found, of which at least more than half was in an ether-soluble form. Further fractionation revealed that 39 per cent of the ether-soluble material was present in the acid, 25 per cent in the phenol and 4 per cent in the neutral fraction. Since a sample of the injection material revealed traces in the acid fraction, and 60 per cent in the phenol, it seems reasonable to assume that the increase in the acidic fraction represents metabolic products derived from the injection material. If α-estradiol behaves like radio-iodo-estradiol, it would appear, that, in addition to the minute amounts bioassayable, there also exist phenolic and acidic metabolites of a non-bioassayable nature. However, the alternate possibility that, as far as the fecal excretion is concerned, the behaviour of radio-iodo-estradiol in the gastro-intestinal tract was different from that of natural estrogens must not be disregarded.

It would be interesting to speculate, that the body might employ a method of substitution of the reactive hydrogen, ortho and para to the phenolic hydroxyl in ring A of the estrogen molecule in a manner analogous to the inactivation of α-estradiol by iodine, and thus produce biologically inert

acidic and phenolic metabolites. Indeed, the hypothesis of ortho and para inactivation as been proposed by Heard and Hoffman (1941).

The third path for the elimination of radioactivity was via the urinary system, since 3.5 to 24.8 per cent of the injected radioactivity was found in the urine, a third of which was ether-soluble. Similar values, following the administration of natural estrogens, had been previously reported by many investigators (Part 1).

Despite the widespread distribution of radioactivity following the administration of radio-iodo-estradiol, it was found that, with the exception of the bile, gastro-intestinal tract and thyroid, only very few organs actually fixed radioactivity in excess of what would be expected from a purely random distribution (a concentration figure of one or less was assumed to represent such a distribution). This increased ability to fix radioactivity or "specific" localization was calculated on the assumption that all organs having a concentration[1] above one (Table 4) specifically concentrated the injected material, while those showing a figure below did not. It was found, on this basis, that, in all groups of the distribution experiment, the mammary glands, skin, submaxillary glands, liver and fat specifically fixed the administered radioactivity.

1 - In the distribution experiments, the concentration was calculated in the following manner: The total number of counts recovered in the bodies of the animals (excluding gastro-intestinal tract, feces, and urine) was divided by the weight of the animal, thus yielding the number of counts per mg. of body weight remaining in the animal at the end of 10 to 12 hours, providing the injected material had been uniformly distributed (random distribution). This factor was then divided into the number of counts per mg. of organ weight thus yielding concentration.

On the whole, the mammary glands showed a higher concentration than any other parenchymatous organ, and, that this uptake was due to mammary tissue was indicated by the lower figure obtained with surrounding fat (Table 6). Ether fractionation revealed the possibility that some of the labeled steroid was present in a conjugated form (Table 9).

Among the various groups studied, the highest mammary gland concentrations were found in two of the three tumor bearing animals. The presence of a mammary tumor (Table 4, animals c and e) and pregnancy (Table 6) seemed to increase the ability of mammary tissue to retain the injected material. These findings suggested the possibility that, concomitant with the presence of a tumor, in some animals, the mammae are in a similar physiological state to that found during pregnancy.

An interesting observation, made in the distribution studies, was the fact that the concentration of radioactivity in the mammary glands of the animals (Table 4 - a, b, f), possessing a low blood level of activity, was comparable to, or even higher than that of the animal (Table 4 - h), possessing a much higher blood level. Although great individual variations existed, this latter finding suggested that the concentration in the mammary glands was independent of the circulating blood level, and probably more dependent on the physiological state of the gland.

On the basis of concentration, it was found that the amount of radioactivity present in the skin of the animals receiving the wetting agent in their injection mixture was appreciably lower than that of any of the other experimental animals (Table 4 - animals g and h). It would appear that in these animals, in whom there was a slow resorption of the injected material, a steady blood level was maintained and therefore they were better able to handle the

injected material. On the other hand, in those animals, receiving their iodinated estradiol in ethanol-saline, there was a sudden flooding of the organism, and, quite possibly the skin acted as a storage site for the administered material. Two out of the three animals bearing mammary tumors showed the same trend (Table 4, animals c and e).

Among the endocrine organs, the pituitary, ovaries and adrenals fixed very little of the radioactive material. However the latter gland, in the animal with the advanced mammary tumor (Table 4 - e) and in the two animals receiving the wetting agent in their injection mixture (Table 4 - g and h), showed a slight degree of "specific" fixation of the injected radioactivity.

The low concentration of radioactivity found in the uterus, cervix and vagina indicated two possibilities; either the iodinated estradiol did not behave like natural estrogens, or the amount of hormone normally required to produce a biological effect is very small, reflecting the extreme sensitivity of these organs to estrogenic stimulation. Since it is well known that estrogens, especially α-estradiol, are active in minute doses, and since the general behaviour of the iodinated estradiol in these experiments seemed to resemble what has been found for natural estrogens, it seems more likely that the second postulation might be correct.

In most organs over one half of the radioactivity was present in an ether-soluble form. Furthermore, a 10 minute hydrolysis increased the yield moderately of ether-soluble compounds in mammary glands, liver, duodenum and contents, jejunum-ileum and contents and urine. Therefore the amount present in these organs and excreta in a conjugated form was rather small.

Since only a small fraction of radioactivity in the aqueous fraction

was conjugated, it was assumed that the remaining radio-iodine was in the form of iodide released from the labeled steroid. This assumption was supported by the presence of some radioactivity in the thyroid, since so far no other iodine compound, other than iodide, has been shown to enter this gland (Leblond, 1942). While throughout the body a number of reducing systems could split iodine from a phenolic ring, it may be speculated that the main site of breakdown of the labeled steroid to iodide was the gastro-intestinal tract, since there was a greater increase in iodide concentration in the thyroid following intragastric instillation of the iodinated estradiol (Table 8) than after subcutaneous administration (Table 4). Bearing in mind that the method for separation of ether-and water-soluble fractions gave results that were about 19 per cent too low, it was calculated from the overall average of the available figures (Table 9) that 32 per cent of the iodo-estradiol had been broken down to iodide within 10 to 12 hours after its administration.

The amount of radioactivity recovered in the excreta and total body of the animals was disappointingly low, varying from 43 to 112 per cent, and averaging 64 per cent. Several factors may have been responsible for these low recoveries. Firstly, due to the insolubility of the iodinated estradiol, a coarse suspension was produced when it was taken up in ethanol-saline, making it difficult to obtain accurate and reproducible standards, thus rendering the estimation of the total injected dose inaccurate. This was partially overcome by the use of the wetting agent, duponol c, by means of which a very fine and uniform colloidal suspension was obtained. Secondly, a great deal of activity probably was lost by absorption into the porous metal of the cages. Ideally, some non-porous material, such as glass or plastic, should be used in the construction of cages for this type of work. Lastly, due to the hard pellet nature of the feces homogenization may not have been complete, and aliquots taken for

plating not truly representative, thereby resulting in a further loss of activity.

Resume

Iodination of α-estradiol with I 131 resulted in the production of a mixture containing about half mono- and half di-iodo-estradiol. When bioassayed, iodo-estradiol proved to be physiologically inert.

The outstanding feature of the metabolism of iodinated estradiol in both male and female mice was the accumulation of the labeled material in the gastro-intestinal tract. The route of entry into the digestive tube was predominantly via the liver and bile. A large fraction of the material entering the gastro-intestinal tract was excreted in the feces. However, some of it must have been resorbed, since, following direct intragastric instillation, radioactivity was found in many organs and in the urine.

A large concentration was present in the derma and especially in the mammary glands, particularly during pregnancy and in the presence of a mammary tumor. Mammary tumors themselves were found to contain a smaller concentration of radioactivity than normal mammary tissue.

The presence of some iodinated estradiol in a conjugated form was found in small intestine, liver, mammary glands and urine.

Like natural estrogens, but unlike their esters or artificial ones, radio-iodo-estradiol was rapidly resorbed from the site of injection, swiftly cleared from the blood and largely excreted in bile, and to a lesser extent in the urine. However, the large fecal excretion of the labeled material has not been found with natural estrogens, possibly because the latter are excreted mostly in a biologically inactive form.

PART IV

SUMMARY AND CONCLUSIONS

Hormonal steroids have been isolated from testis, ovary, adrenal and placenta - presumably sites of their formation - but from no other tissues. These same organs displayed the most intense reactions in the body, when their sections were treated with the two reagents for carbonyl groups, fuchsin-sulphurous acid and 2,4-dinitrophenylhydrazine. Both these reagents are well known in chemistry; the former is employed in the detection of aldehydes, the latter, for both aldehydes and ketones. The questions to be decided were twofold: first, did these reagents demonstrate the steroids themselves as claimed by the Boston group (Wislocki, Bennett, Dempsey), or second, if they did identify some other substance, was it related to the elaboration of steroids?

In order to answer the first question, a series of experiments were designed to produce either a deficiency or a flooding of the body with steroids. Animals were rendered deficient in these hormones by either hypophysectomy or, in the case of the male, by castration and adrenalectomy. No decrease in the staining reaction was observed in most organs and tissues, with the exception of seminal vesicles and prostate. The decreased reaction found in these male accessories could be accounted for by their atrophy following the above interventions. It was concluded that on the whole the absence of steroids had not affected the intensity of the staining reactions throughout the body. Similarly, no intensification of the staining above normal was produced by flooding the animals with the ketosteroids, testosterone and desoxycorticosterone acetate.

Finally, in vitro experiments with ketosteroids, such as testosterone, showed no reaction with either histochemical reagent under conditions simulating those employed in the histochemical techniques. These findings definitely indicated that the intense reactions found in tissues with fuchsin-sulphurous acid and phenylhydrazine were not due to their ketosteroid content.

The answer to the second question was secured by investigating the two reactions in animals of various ages and under various experimental conditions. The evidence pointed to a close relationship between the activity of steroid producing organs and their response to the histochemical reagents. Thus, the staining of the adrenals, corpora lutea, interstitial cells of the testis and ovary was accentuated during functional activity (steroid production) and disappeared on the interruption of secretory activity. It was concluded, therefore, that the reactant compounds, aldehydic lipids, presumably derived from acetal phosphatides or plasmalogens, were closely connected with steroid elaboration. This relation, indicated by histological means, remains to be investigated biochemically.

Since our efforts to detect the minute amounts of circulating hormonal steroid by histochemical methods were unsuccessful, a new approach, the labeling of naturally occuring steroids with radioactive isotopes, was employed. It was found that estradiol could be iodinated with I^{131} and followed throughout organs, tissues, body fluids and excreta. Our own experiments have demonstrated the feasibility of the tracer technique in this type of problem and enabled us to piece together, in logical sequence, the fragmentary data found in the literature on the fate of estrogens and thus obtain an overall picture of their behavior in the body.

Our first expectation had been to find large amounts of steroid in the accessory sex organs, and indeed this was found to be the case in the mammary glands where, especially during pregnancy and in the presence of a mammary tumor, the labeled steroid accumulated in greater amounts than in any other organ. On the other hand, relatively minute amounts were found in the uterus, cervix and vagina, probably indicating that these organs are most sensitive to the hormonal effect.

The labeled iodinated estradiol was found to behave, in the body, like the natural estrogens, as far as could be judged from the scanty information available in the literature. It was rapidly resorbed from the site of injection, swiftly cleared from the blood, and excreted mostly through the liver into the bile and gastro-intestinal tract, from where it was partially resorbed. A relatively minor portion of the steroid was eliminated through the kidneys into the urine. Close to half of the administered material could be recovered in the feces. This finding is not in accordance with the previous demonstration by biological assay of only very minute amounts of natural estrogens in this excreta. It is quite possible, however, that the elimination of natural estrogens by this route also takes place, but in a biologically inactive form.

In a few cases the autographic technique was employed for the cytological localization of the labeled steroid in tissues. That this method was feasible was demonstrated by the precise localization found in the skin (Fig. 58). In the future, extension of this method to the tracing of estrogens labeled with C_{14} may provide an exact knowledge of their point of action within the cell.

Cancer-susceptibility had no effect either on the general staining

pattern of organs nor on their ability to collect the iodinated steroid. On the other hand, the neoplastic transformation of mammary tissue decreased its ability to stain with fuchsin-sulphurous acid and dinitrophenylhydrazine, and concentrate the iodinated estradiol.

B I B L I O G R A P H Y

Abderhalden, R.: Med. Klin.,38: 730, 1942.

Aboim, A. N.: Bull. Soc. Portuguise Sc. Nat., 114: 119, 1943.

Adler, A. A., P. de Fremery, and M. Tausk: Nature, London, 133: 293, 1934.

Allen, W. M., and G. Goetsch: J. Biol Chem., 116: 683, 1936.

Allende, I. L. C. de: Proc. Soc. Exper. Biol. & Med., 44: 534, 1940.

Anchel, M., and H. Waelsch: J. Biol. Chem., 116, 683, 1936.

Ascheim, S., and B. Zondek: Klin. Wchnschr., 78: 1322, 1927.

Beatson, G. T.: Lancet, 2: 104, 1896.

Becher, H.: Anat. Anz., 88: , 1939. (Quoted from Voss, H., Ztschr. f. Zellforsch. u. mikr. Anat. (Abt A), 31: 43, 1940).

Behrens, M.: Ztschr. f. physicl. Chem., 191; 183, 1930.

Bélanger, L. F., and C. P. Leblond: Endocrinology, 39: 8, 1946.

Bennett, H. S.: Am. J. Anat., 67: 151, 1940.

Berdnikoff, A.: Compt. rend. Soc. de biol., 122: 164, 1936.

Berger, M.: Semaine d. Hôp. de Paris, 30: 1416, 1946.

Bersin, T., H. G. Moldtmann, H. Nafziger, G. Marchand, and W. Leopold: Ztschr. f. physiol. Chem., 269: 241, 1941.

Bird, S., L. I. Pugsley, and M. O. Klotz: Endocrinology, 41: 282, 1947.

Bischoff, F., and H. R. Pilhorn: Am. J. Physiol., 150: 444, 1947.

Bouin, P., and P. Ancel: Compt. rend. Soc. de biol., 88: 758, 1923.

Bruhl, R.: Klin. Wchnschr., 8: 1766, 1929.

Bullough, W. L.: Philosoph. Tr. Roy. Soc. London, 231: 453, 1946.

Cantarow, A., A. E. Rakoff, K. E. Paschkis, L. P. Hansen, and A. A. Walkling: Endocrinology, 31: 515, 1942.

Cantarow, A., A. E. Rakoff, K. E. Paschkis, L. P. Hansen, and A. A. Walkling: Proc. Soc. Exper. Biol. & Med., 52: 256, 1943a.

Cantarow, A., K. E. Paschkis, A. E. Rakoff, and L. P. Hansen: Endocrinology, 33: 309, 1943b.

Claesson, L., and N. A. Hillarp: Acta physiol. Scandinav., 13: 115, 1947a.

Claesson, L., and N. A. Hillarp: Acta physiol. Scandinav., 14: 102, 1947b.

Claesson, L., and N. A. Hillarp: Acta anat., 3: 109, 1947c.

Claude, A.: Science, 91: 77, 1940.

Claude, A.: Cold Spring Harbor Symp. on Quant. Biol., 9: 263, 1941.

Cook, J. W., and E. L. Kennaway: Am. J. Cancer, 39: 521, 1940.

Cori, C. F.: J. Exper. Med., 45: 983, 1927.

Cramer, W., and E. S. Horning: J. Path. & Bact., 44: 633, 1937.

Cuyler, W. L., C. Ashley, and E. H. Hamblen: Endocrinology, 27: 177, 1940.

David, K., E. Dingemanse, J. Freud and E. Laqueur: Ztschr. f. physiol. Chem., 233: 281, 1935.

Deane, H. W., and R. O. Greep: Am. J. Anat., 79: 117, 1946a.

Deane, H. W., and J. M. McKibbin: Endocrinology, 38: 385, 1946b.

Deane, H. W., and J. H. Shaw: J. Nutrition, 34: 1, 1947a.

Deane, H. W., and R. O. Greep: Endocrinology, 41: 243, 1947b.

Deansley, R.: J. Physiol., 92: 34P, 1938.

Delor, J.: Compt. rend. Soc. de biol., 136: 787, 1942.

Dempsey, E. W., and D. L. Bassett: Endocrinology, 33: 384, 1943.

Dempsey, E. W., and G. B. Wislocki: Endocrinology, 35: 409, 1944.

Dempsey, E. W., and G. B. Wislocki: Physiol. Rev., 26: 1, 1946.

Dingemanse, E., and E. Laqueur: Am. J. Obst. & Gynec., 33: 1000, 1037.

Dingemanse, W., and R. Tyslowitz: Endocrinology, 28: 450, 1941.

Dohrn, M., and W. Faure: Klin. Wchnschr., 7: 943, 1928.

Dorfman, R. I., and J. B. Hamilton: Endocrinology, 25: 28, 1939.

Dorfman, R. I., and W. U. Gardner: Endocrinology, 34: 421, 1944.

Ehrenstein, M., and S. W. Britton: Am. J. Physiol., 119: 302, 1937.

Ehrhardt, K.: München. med. Wchnschr., 81: 869, 1934.

Everett, J. W.: Endocrinology, 41: 364, 1947.

Fels, E.: Klin. Wchnschr., 5: 2349, 1926.

Feulgen, R., and K. Voit: Arch. f. d. ges. Physiol., 206: 389, 1924.

Feulgen, R., K. Immhauser, and M. Behrens: Ztschr. f. physiol. Chem., 180: 161, 1929.

Feulgen, R.,and H. Gruenberg: Ztschr. f. physiol. Chem., 257: 161, 1938-39.

Feulgen, R., and T. Bersin: Ztschr. f. physiol. Chem., 260: 217, 1939.

Finkelstein, M., S. Hestrin, and W. Koch: Proc. Soc. Exper. Biol. & Med., 64: 64, 1947.

Fluhmann, C. F.: Endocrinology, 18: 705, 1934.

Forbes, T. R., and C. W. Hooker: Science, 107: 151, 1948.

Frank, R. T., M. A. Goldberger, and F. Spielman: Proc. Soc. Exper. Biol. & Med., 29: 1229, 1932.

Gardner, W. U.: Cancer Research, 1: 345, 1941.

Gardner, W. U.: Surgery, 16: 8, 1944.

Gérard, P.: Bull. d'histol. appliq., No. 7: 274, 1935.

Giroud, A., M. Martinet, and M. T. Bellon: Compt. rend. Soc. de biol., 134: 441, 1940.

Giroud, A., A. R. Ratsimamanga, and H. Chalopin: Comp. rend. Soc. de biol., 135: 725, 1941.

Glass, S. J., H. A. Edmondson, and S. N. Soll: J. Clin. Endocrinol., 4: 54, 1944.

Glandular Physiology and Therapy, A. M. A., 1942.

Goldberg, M. W.: Ergebn. d. Vitamin- u. Hormonforsch., 1: 371, 1938.

Gomori, G.: Proc. Soc. Exper. Biol. & Med., 51: 133, 1942.

Greep, R. O.: Bull. New England M. Centre, 8: 244, 1946.

Greep, R. O., and H. W. Deane: Endocrinology, 40: 417, 1947.

Gsell-Busse, M. A.: Arch. f. exper. Path. u. Pharm., 139: 328, 1929.

Gustavson, R. G.: J. Biol. Chem., 92: lxxi, 1931.

Hartwell, J. L.: Survey of Compounds which have been Tested for Carcinogenic Activity, National Cancer Institute, National Institute of Health, United States Public Health Service, 1941.

Harvey, E. N.: Medical Physics. Edited by O. Glasser, Year Book Publishers, Inc., Chicago, 1944.

Häussler, P. E.: Helvet. Chem. Acta, 17: 531, 1934.

Hayes, E. R.: A Histochemical Study of Carbonyl Lipids. A Dissertation, The Ohio State University, 1947.

Heard, R. D. H., and M. M. Hoffman: J. Biol. Chem., 141: 329, 1941.

Heard, R. D. H., W. S. Bauld, and M. M. Hoffman: J. Biol. Chem., 141: 709, 1941.

Hill, R. T.: Endocrinology, 21: 495, 1937.

Hirano, S.: J. Pharm. Soc. Japan, 56: 122, 1936.

Hoffman, F., and L. von Lam: Zentralbl. f. Gynäk., 66: 1145, 1942.

Hoffman, M. M., V. E. Kazmin, and J. S. L. Browne: J. Biol. Chem., 147: 259, 1943.

Hoffman, M. M., M. Sabin, and M. L. Desbarats: Proc. Canad. Physiol. Soc., p. 15, 1947.

Hooker, C. W., and C. A. Pfeiffer: Cancer Research, 1: 759, 1942.

Immhauser, K.: Biochem. Ztschr., 186: 360, 1927.

Immhauser, K.: Biochem. Ztschr., 193: 416, 1928.

Innes, J. R. M.: J. Path. & Bact., 54: 485, 1942.

Jayle, M. F., O. Crepy, O. Libert, S. Vandel, and O. Judas: Bull. Soc. de chim. biol., 28: 363, 1946.

Jayle, M. F., and O. Libert: Ann. de biol. Clin., No. 2: p. 1, 1947.

Joseph, S.: Monatschr. f. Geburtsh. u. Gynäk., 83: 219, 1929.

Kar, A. B.: Anat. Rec., 99: 177, 1947.

Karrer, P.: Organic Chemistry. Elsevier, N.Y., pp. 145-146, 1938.

Kemp, T., and K. Pedersen-Bjergaard: Compt. rend. Soc. de biol., 111: 329, 1932.

Kemp, T., and K. Pedersen-Bjergaard: Endokrinologie, 13: 156, 1933-34.

Krichesky, B., and S. J. Glass: Endocrinology, 40: 192, 1947a.

Krichesky, B., and S. J. Glass: Endocrinology, 41: 196, 1947b.

Lacassagne, A.: Compt. rend. Acad. d. sc., 195: 630, 1932.

Lacassagne, A.: Compt. rend. Soc. de biol., 114: 427, 1933.

Lacassagne, A.: Compt. rend. Soc. de biol., 126: 385, 1937.

Lacassagne, A.: Am. J. Cancer, 37: 414, 1939a.

Lacassagne, A.: Compt. rend. Soc. de biol., 132: 365, 1939b.

Lacassagne, A., and A. Raynaud: Compt. rend. Soc. de biol., 132: 431, 1939.

Laqueur, E., K. David, E. Dingemanse, and J. Freud: Acta brev. Neerland., 5: 84, 1935.

Leblond, C. P., and W. O. Nelson: Bull. d'histol. appliq., 14: 181, 1937.

Leblond, C. P.: Rev. canad. de biol., 1: 402, 1942.

Levin, L.: J. Biol. Chem., 157: 407, 1945.

Lipschutz, A.: Arch. f. path. Anat., 285: 35, 1932.

Lison, L.: Bull. d'histol. appliq., 9: 177, 1932.

Lison, L.: Histochimie Animale. Gauthier-Villars, Paris, 1936.

Loeb, L.: Univ. Pennsylvania M. Bull., 20: 2, 1907.

Longwell, B. B., and F. S. McKee: J. Biol. Chem., 142: 757, 1942.

Luchsinger, J., and H. E. Voss: Klin. Wchnschr., 8: 1577, 1929.

Matthews, N. L., G. M. Curtis, and W. R. Brode: Indust. & Engin. Chem., 10: 612, 1938.

McCullagh, D. R., and W. O. Osborn: J. Biol. Chem., 126: 299, 1938.

McCullagh, D. R.: Endocrinology, 24: 326, 1939.

McEuen, C. S.: Am. J. Cancer, 36: 551, 1939.

McKay, D. G., and D. Robinson: Endocrinology, 41: 378, 1947.

Miescher, K.: Experientia, 2: 237, 1946.

Möckel, G.: Ztschr. f. physiol. Chem., 277: 135, 1943.

Moore, C. R., W. Hughes, and T. F. Gallagher: Am. J. Anat., 45: 109, 1930.

Motta, G.: Folia Gynaec., 28: 519, 1931.

Murray, W. S.: J. Cancer Research, 12: 18, 1928.

Neuman, W. T.: Studies of the Acetal Phospholipids. A Dissertation, University of Rochester, 1944.

Oster, K. A., and N. C. Schlossman: J. Cell. & Comp. Physiol., 20: 373, 1942.

Oster, K. A., and M. G. Mulinos: J. Pharmacol. & Exper. Therap., 80: 132, 1944.

Oster, K. A., and J. G. Oster: J. Pharmacol. & Exper. Therap., 87: 306, 1946.

Oster, K. A.: J. Exper. Med. & Surg., 4: 20, 1946.

Parker, F., Jr., and B. Tenney, Jr.: Endocrinology, 23: 492, 1938.

Parkes, A.: Nature, London, 139: 965, 1937.

Pearlman, W. H., K. E. Paschkis, A. E. Rakoff, A. Cantarow, and A. A. Walkling: Federation Proc., 6: 283, 1947a.

Pearlman, W. H., A. E. Rakoff, A. Cantarow, and K. E. Paschkis: J. Biol. Chem., 170: 173, 1947b.

Philipp, E.: Zentralbl. f. Gynäk., 53: 2386, 1929.

Pincus, G., G. Wheeler, G. Young, and P. A. Zahl: J. Biol. Chem., 116: 253, 1936.

Pincus, G., and W. H. Pearlman: Endocrinology, 31: 507, 1942.

Pincus, G., and W. H. Pearlman: Vitamins & Hormones, 1: 293, 1943.

Pollock, W. F.: Anat. Rec., 84: 23, 1942.

Popjak, G.: J. Path. & Bact., 56: 485, 1944.

Popper, H.: Arch. Path., 31: 766, 1941.

Prelog, V., and H. C. Beyerman: Experientia, 1: 64, 1945.

Prelog, V., E. Tagmann, S. Lieberman, and L. Ruzicka: Helvet. Chim. Acta, 30: 1080, 1947.

Price, D.: Physiol. Zoöl., 20: 213, 1947.

Reichstein, T., and C. W. Shoppee: Vitamins & Hormones, 1: 345, 1943.

Reynolds, S. R. M., and N. Ginsberg: Endocrinology, 31: 147, 1942.

Roberts, S., and C. M. Szego: Endocrinology, 39: 183, 1946.

Rogers, W. F., Jr., and R. H. Williams: Arch. Path., 44: 126, 1947.

Ruzicka, L., and W. Stepp: Ergebn. Vitamin- u. Hormonforsch., 1: 419, 1938.

Ruzicka, L., and V. Prelog: Helvet. Chim. Acta, 26: 975, 1943.

Sacchi, U., and A. Fraschini: Arch. ital. di biol., 94: 14, 1935.

Samuels, L. T., and J. J. Bittner: 38th Meet. A. A. for Cancer Research, Chicago, 1947.

Schiff, H.: Ann. d. Chem., 144: 45, 1866.

Schinzinger: Quoted from Chase, H. C., Breast Cancer. Surg., Gynec. & Obst., 85: 712, 1947.

Schoenheimer, R., and E. A. Evans, Jr.: J. Biol. Chem., 114: 567, 1936.

Selye, H., J. B. Collip, and D. L. Thomson: Endocrinology, 19: 151, 1935.

Selye, H.: Encyclopedia of Endocrinology. Section 1: The Steroids. Vol. 2, p. 344. A. W. T. Franks Publ. Co., Montreal, 1943.

Selye, H.: Textbook of Endocrinology. Acta Endocrinol., Univ. de Montréal, 1947.

Shipley, E. G., R. K. Meyer, and C. Biddulph: Am. J. Physiol., 140: 230, 1943.

Siebke, H., and P. Schuschania: Zentralbl. f. Gynäk., 54: 1734, 1930.

Sjostrand, F.: Ueber die Eigenfluoreszenz tierischer Gewebe mit besonderer Beruchsicht gung der Saugetierniere. Stockholm, 1944. (Quoted from Claesson, L. and N. A. Hillarp: Acta anat., 3: 109, 1947c).

Smith, G. van S., O. W. Smith, and G. Pincus: Am. J. Physiol., 121: 98, 1938.

Smith, O. W., G. van S. Smith, and S. Schiller: Endocrinology, 25: 509, 1939.

Stamler, C. M.: Bull. biol. et méd. expér., 3: 31, 1937. (Quoted from Dingemanse, E. and R. Tyslowitz: Endocrinology, 28: 450, 1941).

Stepp, W., R. Feulgen, and K. Voit: Biochem. Ztschr., 181: 284, 1927.

Stoeltzner, W.: Biochem. Ztschr., 142: 380, 1923.

Symeonidis, A.: Fourth Internat. Cancer Research Cong., St. Louis, p. 103, 1947.

Szego, C. M., and S. Roberts: Proc. Soc. Exper. Biol. & Med., 61: 161, 1946.

Tagmann, E., V. Prelog, and L. Ruzicka: Helvet. Chim. Acta, 29: 440, 1946.

Tonutti, E.: Klin. Wchnschr., 20: 1196, 1941.

Tschopp, E., von: Helvet. physiol. Acta, 4: 401, 1946.

Turner, R.: J. Am. Chem. Soc., 69: 726, 1947a.

Turner, R.: Science, 106: 248, 1947b.

Twombly, G. H.: Fourth Internat. Cancer Research Cong., St. Louis, p. 121, 1947.

Venning, E. H., and J. S. L. Browne: Endocrinology, 21: 711, 1937.

Venning, E. H., and V. Kazmin: Endocrinology, 39: 131, 1946.

Verne, J.: Compt. rend. de l'Assoc. Anat., 23: 465, 1928a.

Verne, J.: Compt. rend. Soc. de biol., 99: 266, 1928b.

Verne, J.: Ann. de Physiol., 5: 245, 1929.

Vogt, M.: J. Physiol., 102: 341, 1943.

Voss, H.: Ztschr. f. mikr. Anat. Forsch., 10: 583, 1927.

Voss, H.: Ztschr. f. Zellforsch. u. mikr. Anat. (Abt. A), 31: 43, 1940.

Voss, H.: Deutsche med. Wchnschr., 67: 127, 1941.

Werner, S. C.: J. Clin. Investigation, 20: 21, 1941.

Wieland, H., and G. Scheuing: Ber. Chem. Ges., 54: 2527, 1921.

Winter, E. W.: Arch. f. Gynäk., 151: 201, 1932.

Wislocki, G. B., and E. W. Dempsey: Am. J. Anat., 77: 365, 1945.

Wislocki, G. B.,and E. W. Dempsey: Endocrinology, 38: 90, 1946a.

Wislocki, G. B., and E. W.Dempsey: Am. J. Anat., 78: 1, 1946b

Wislocki, G. B., and W. A. Wimsatt: Am. J. Anat., 81: 269, 1947.

Yoffey, J. M., and J. S. Baxter: J. Anat., 81: 335, 1947.

Zondek, B.: Nature, London, 133: 494, 1939.

Zondek, B.: Clinical and Experimental Investigations on the Genital Functions and their Hormonal Regulation. The Williams and Wilkins Co., Baltimore, 1941.

Zondek, B.: Acta radiol., 28: 434, 1947.