BIOLOGICAL ASSAYS OF CORTICAL HORMONE AND THEIR APPLICATION.

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CONTENTS

INTRODUCTION

Studies on the adrenal cortex began with the demonstration that it is essential for life. The next step was the demonstration that within the gland some substance or substances are present which can be extracted and used in substitution therapy.

This was followed by fractionation of adrenocortical extracts and the isolation of crystalline steroids. With the help of these extracts and steroids it became possible to establish a vast body of knowledge concerning the rate of secretion of cortical hormone, their metabolic effects, metabolism, urinary excretion and other physiological data concerning the adrenal cortex. In all these studies, especially in the earlier ones, biological assays, whether used in a qualitative or a quantitative manner, have played the most prominent, if not the only, role in obtaining such information.

Vogt (1947) has classified the several procedures that have been suggested for the assay of adrenal cortical/

cortical hormones. Her classification is presented below with modifications. They fall into three groups.

A - Maintenance or prolongation of life in adrenalectomised animals (dogs, cats, rats, rabbits, guinea-pigs, birds and frogs).

B - Tests on normal animals or tissues:

1. Augmented Potassium excretion (West, 1942).
2. Sodium retention (Hartman and Spoor, 1940).
3. Semi-contraction of melanophores of scales of carp (Giroud, Santa and Martinet, 1941).
4. Growth of ovipositor of bitterling (Duyvene de Wit, 1941).

C - Tests which measure the correction by cortical hormone of some deficiency due to adrenalectomy:

1. Growth (Grollman, 1941).
2. Non-protein - nitrogen in dog's blood, (Pfiffner, Swingle and Vars, 1934).
3. Performance of muscular work (Ingle, 1944; Everse and de Fremery, 1932).
4. Water diuresis (Petranyi, 1941).
5. Resistance to various agents:-
   (a) Resistance to water intoxication, (Eversole, Gaunt and Kendall, 1942).
   (b) Resistance to histamine poisoning, (Perla and Gottesman, 1931).
   (c) Resistance to Potassium poisoning, (Feil and Dorfman, 1945).
   (d) Resistance to shock, (Elmadjien and Pincus, 1944).
   (e) Resistance to typhoid vaccine, (Lewis and Page, 1946).
6. Glycogen deposition in Liver (Reinecke and Kendall, 1942); sensitive modifications (Eggleston, Johnston and Dobriner, 1946; Venning, Kazmin and Bell, 1946).
7. Survival on exposure to low temperature, (Selye and Schenker, 1938; Vogt, 1943).

8./

8. Fall in body temperature on exposure to low temperature (Tyslowitz and Astwood, 1942).

9. Sodium retention (Dorfman, 1947; Spencer, 1950; Tait, Simpson and Grundy, 1951; Lockett, 1951).

10. Effect on lymphocytes (Schrek, 1951).

11. Fall in circulating eosinophils (Speirs and Meyer, 1949 and 1951).

A. The first biological assays were based on the ability of adreno-cortical extracts to prolong life in adrenalectomised animals. This effect was used for many years in designing experiments to extract active principles from the adrenal cortex. Several species have been used.

1. Dog.

Rogoff and Stewart (1926, 1927 and 1928) made extensive studies on adrenalectomised dogs. If these dogs were not maintained with salt or adreno-cortical extract, they soon stopped eating, lost weight rapidly and died. At first, the survival period was found to be one week, but with experience, it was prolonged to ten days. An extract of the adrenal cortex given by subcutaneous injections daily, increased the average survival period to twelve days. But, as pointed out by Britton (1930) and by Swingle and/

and Pfiffner (1931), the differences in survival periods were not statistically significant, thus invalidating the claim made by Stewart and Rogoff to have extracted potent substances from the adrenal cortex.

2. Cat.

Cats apparently stand adrenalectomy somewhat better than dogs. Hartman et al., (1930a), and Swingle and Pfiffner (1930 and 1931) have used cats extensively in their biological assays. The cats were adrenalectomised and kept at a constant room temperature. The untreated controls were dead in one to five weeks. Those given a daily injection of a beef adrenal extract continued to live as long as they were treated. When treatment was withheld, the cats went into a state of severe adreno-cortical insufficiency, with almost complete collapse. On recommencing treatment the cats revived.

Thus, in 1930 both groups of workers were able to make potent extracts of the adrenal cortex. Swingle and Pfiffner (1929) used ethanol and Hartman (1930b) used ether to extract the hormone. Britton and Silvette (1931a) confirmed these findings, and thereby afforded final proof of the endocrine nature of the adrenal cortex.

An/

An interesting feature of this assay was that the daily dose to each cat of 30gm. gland extract, represented 100 times the amount of cortex present in the whole cat! Britton (1931b) suggested that this was due to low yields in the extraction procedures. Later, Vogt (1944) demonstrated that the adrenal cortex stored very little hormone.

3. Rats.

Further work on the adreno-cortical hormone demanded that the methods of biological assay be placed on a quantitative basis. Therefore, it became necessary to employ a larger number of test animals per assay. Not only were rats available in large numbers, but it was also easy to control such factors as uniformity of breed, age and weight. Therefore, efforts were made to measure survival and growth in adrenalectomised rats, and to relate this quantitatively to the dose of adreno-cortical hormone administered.

The effects of adrenalectomy in the rat have given rise to more inconsistent results than in any other species. The explanation of the mortality figures which ranged from 10-100% was probably due to variations in the completeness of adrenalectomy and in the/

the use of strains with accessory adrenal tissue. Careful and complete adrenalectomy, together with the removal of periadrenal connective tissue, part of the renal capsule and the adrenal pedicle rarely led to survival of the animal, (Pencharz et al., 1930; Freed et al., 1931; Schultzer, 1935; Cartland and Kuizenga, 1936; Firor and Grollman, 1933).

Kutz (1931) measured survival in adrenalectomised immature rats. They were found to be less variable and more sensitive than adults.

Schultzer (1936) found a 100% mortality in thirty carefully adrenalectomised rats with an average survival period of six days. Treatment with saline increased this period by a further three days. Such animals lived for twenty-one days when injected for this period with a hog adreno-cortical extract. They died within six days of withdrawing the extract. The percentage survivals was proportional to the dose of cortical hormone.

Cartland and Kuizenga (1939) and Kuizenga et al., (1940) using immature rats showed that their untreated adrenalectomised animals died seven-fourteen days after operation. Rats given daily injections of adreno-cortical extract lived for over twenty days, and when treatment was withdrawn, they died seven days later.

later A rat unit was defined as the minimum daily dose which, when administered daily for twenty days to 50-60gm. rats, protected 80% of the animals, and permitted an average growth of 20gm. per rat.

By means of this procedure Cartland and Kuizenga showed that the rat assay gave parallel estimations to that given by using adrenalectomised dogs and measuring their blood urea. On a kg. basis, the rat required 300 times the quantity of adreno-cortical extract as the dog.

Cortical hormone given orally was as effective as when given parenterally.

11-dehydrocorticosterone and corticosterone were shown to be biologically active steroids.

4. Rabbits.

Baumann and Kurland (1927) found an extremely variable survival time in adrenalectomised rabbits ranging from twelve to one-hundred and five days. They also found macroscopic accessory adrenals in 90% of their rabbits. This has made rabbits unsuitable for use in biological assays.

5. Guinea-pigs.

Although these animals show a uniform response after adrenalectomy and have low cortical hormone requirements (Simmons and Whitehead, 1936; Schachter/

Schachter and Bebee, 1939) they have not been used extensively because adrenalectomy is difficult. The adrenalectomised animals survived a maximum of seven days and adreno-cortical extract prolonged their survival.

6. Birds.

Pigeons, chickens and ducks have been successfully adrenalectomised. Parkes and Selye (1936) found that adrenalectomy in ducks and fowls was rapidly and regularly fatal, and that large amounts of adreno-cortical extract were necessary to maintain them. Herrick and Torstveit (1938) found that chickens died 6-15 hours after adrenalectomy and required large quantities of adreno-cortical extract for maintenance.

Bülbring (1937) developed a method for assaying cortical hormone by measuring the survival times in adrenalectomised drakes. Untreated birds lived 5-15 hours and those given injections of cortical hormone lived longer.

She demonstrated a linear log-dose-response relationship with a steep slope. Doubling the dose was adequate to cause significant differences between the mean survival times in groups of ten drakes. The method is not only accurate but quick, an assay being completed/

completed in twenty-four hours. But, drakes are expensive, and ten birds are required per dose. Adrenalectomy requires considerable skill and the dose of cortical hormone required per bird is large. Although Bulbring has suggested the use of this method for the standardizing of commercial adreno-cortical extracts, the technical and financial disadvantages have prevented its adoption.

7. Frogs.

They are unsuitable animals on account of the difficulty in removing adrenal glands without severely damaging the kidneys. Clark, Brackney and Miliner (1944) however, have described a practical operative technique, but they consider that only 30% of their animals were successfully adrenalectomised.

B. Test on Normal Animals or Tissues.

1. Augmented Potassium excretion. Harrop and Thorn (1937) showed that the administration of cortical hormone to normal dogs was accompanied by the retention of sodium and chloride and an increased renal excretion of Potassium. West (1942) investigated these responses in normal rats. They were maintained on a constant diet. Varying doses of cortical/

cortical hormone or desoxycorticosterone-acetate (DCA) were administered, their urine collected for twenty-four hours, and its content of sodium, chloride and potassium estimated. Under the conditions of the experiment, West was unable to obtain consistent results.

2. Sodium retention.

The importance of the adrenal cortex in the control of body electrolytes, developed from observations on the beneficial effects of salt solutions in adrenal insufficiency. Baumann and Kurland (1927) observed a lowered plasma sodium and chloride and an increased K in adrenalectomised cats. Loeb (1932) observed a similar picture in cases of Addison's disease. In a series of balance experiments. the plasma electrolyte picture was correlated with an increased sodium and chloride and diminished K excretion in the urine. It was shown that this picture could be reversed by adreno-cortical extract, not only in adrenalectomised animals but also in intact animals and in normal human subjects (Thorn et al., 1936; Harrop et al., 1933).

Harrop and Thorn (1937) proposed that the effect of adreno-cortical extract on sodium retention in normal dogs be used for bioassay. Dogs whose diet, water and salt intake were controlled, had their

urine/

urine collected by catheter over a period of twenty-four hours. Control animals showed that the maximum variation of excreted sodium was less than 5 m. eq. daily. The minimum amount of extract necessary to produce 15-20% sodium retention in twenty-four hours was used as a standard. Repeated doses of extract were found to be more effective than the same amount given as a single injection.

Hartman and Spoor (1940) adopted the procedure described above, but found it unnecessary to collect urine for more than six hours to obtain accurate values. Later, desoxycorticosterone (DOC) was used as a standard, Hartman et al., (1941). The dose was adjusted so that it caused not less than 35% or more than 65% retention of urinary sodium over the animal's control value. The dose of the unknown substance was adjusted until an equivalent response was obtained.

From comparative assays DOC and DCA were found to be the most active of the chemically pure substances influencing sodium retention. 11-dehydrocorticosterone and corticosterone were about half as active. Adreno-cortical extract was also very active. Hartman and Spoor (1940) described methods for fractionating adreno-cortical extract into a "Na factor" and a factor for life maintenance, "cortin". The sodium retention was directly related to/

to the dose used in treatment. Hartman et al., (1940) also showed that the "Na factor" and "cortin" could be separately demonstrated in adrenalectomised animals.

Thatcher and Hartman (1946) using sodium retention and glycogen deposition methods claimed to have separated a salt active substance from glycogenic substances in a beef adrenal extract. The active substances were adsorbed on an alumina column. The salt active hormone was eluted with methanol and the glycogenic substances with acetone or ethylene dichloride. The identification of the fractions was by means of bioassay. The behaviour of DCA on the column was different from the natural sodium retaining substances.

3. Semi-contraction of melanophores of scales of carp.

Santa and Veil (1939) attempted to assay small quantities of adreno-cortical hormone by observing its effect on the melanophores in the scales of the Cyprinus carpio. They described the melanophores as being well expanded at the beginning of the experiment. On giving an adreno-cortical extract, the melanophores started to contract slowly and in fifteen minutes reached a state of semi-contraction, and remained in that state. All other substances caused either/

either no contraction at all, or a complete contraction. The authors likened this to the "all or none" phenomenon. Adrenaline caused a rapid and complete contraction. Testosterone, progesterone, oestradiol and cholesterol caused no contraction. They claimed that extracts of liver, spleen, kidney, muscle, testis, ovary, pancreas and hypophysis had no activity. But in a later paper, Giroud et al., (1941) using the melanophore test claimed to have detected adreno-cortical extract in minute quantities in the very organs whose extracts were originally used as controls. The weight of tissue used was not stated. They also claimed to have detected cortical hormone in normal blood, and that patients with Addison's disease had one tenth this quantity. Giroud et al., (1940) claimed that adreno-cortical hormone was found only in the zona fasciculata and zona reticulosa of the adrenal cortex. There has been no independent confirmation of the method or results.

4. Growth of the ovipositor of the bitterling.

The bitterling is a small fish found in Western Europe. During the mating season the ovipositor of the female bitterling grows to a length of 3-4cm. by means of which it deposits its eggs. After this function is over, the ovipositor shrinks back/

back to a stump of 1-2 mm. Duyvene de Wit (1941 and 1942) demonstrated that the ovipositor could be made to grow by treating the bitterling with steroid hormones. The fish was placed in a 750 cc. tank at a temperature of 22°C. The addition of progesterone, oestrogens, androgens or adrenal steroids caused the ovipositor to lengthen, its length being proportional to the dose of steroid. He found that the maximum length was reached in 56 hours with oestrogens, in over 6 hours with androgens and in 4 hours with progesterone and adrenal steroids. He compared the effects of DOC, DCA, corticosterone and progesterone, and found the following relative potencies:-

DOC	- 200	times as active as corticosterone
DCA	- 80	" " " " "
Progesterone	- 60	" " " " "
DOC	- 1.3	" " " " progesterone
DCA	- 2.5	" " " " DOC

The dose-response lines for DOC and DCA were steeper than that for corticosterone.

The non-specific nature of the test reduced its value.

C. 1. Growth.

Grollman and Firor (1932a and 1933) assayed adreno-cortical extract by measuring the growth of immature, adrenalectomised rats. When month-old rats were adrenalectomised, they lost weight and died in 1-12 days. If they were treated with an adequate dose of adreno-cortical extract, they continued to grow normally. That is, they showed a daily progressive weight increase in the manner/

manner of unoperated controls. After the 7th day, the treatment was withdrawn and the rats died of insufficiency. By using a series of four to six rats and by varying the dose, Grollman and Firor claimed to have arrived at a "fairly accurate assay of the activity of a given solution".

Grollman and Firor (1932b) showed cortin-like activity in benzene extracts of urine.

Grollman (1939) used DCA as a standard. He found corticosterone less active than DCA, and 11-dehydrocorticosterone to be inactive. He claimed that the average weight gain was linearly related to the log dose of adreno-cortical extract (Grollman, 1941). He used this method in fractionation studies and claimed to have isolated a crystalline substance with a hundred times the potency of DCA. It was also demonstrated that per unit weight of gland, the hog adrenal yielded three times the quantity of cortical hormone as the beef adrenal.

Later work has shown that the 11-oxysteroids retarded or actually suppressed the growth of young adrenalectomised rats, (Wells and Kendall, 1940). Whereas the amorphous fraction caused almost a normal rate of growth, DCA often caused a gain in weight greater than in a normal control. Ingle (1940c) compared the effects of 11-oxysteroids and 11-desoxysteroids on growth in rats and on his muscle test, and/

and demonstrated qualitative differences in these steroid groups.

2. Non-Protein Nitrogen in Dog's Blood.

Rogoff and Stewart (1926) observed that a significant rise in blood urea occurred some time before death in adrenalectomised dogs. Harrop et al. (1930) investigated the changes in blood and urine of normal and adrenalectomised dogs treated with adreno-cortical extract. They found that blood non-protein nitrogen (NPN) and urea started to increase shortly after treatment was stopped. The change usually preceded the appearance of other symptoms of insufficiency by a few hours. The administration at this stage of adequate quantities of cortical hormone restored the blood NPN and urea to normal levels. This effect was used to develop a method for the biological assay of adreno-cortical hormone (Harrop et al., 1932).

In the conduct of the test, the adrenalectomised dog was given injections of an amount of adreno-cortical hormone expected to be more than adequate. The dose was reduced after a period of 7 days until one was reached which allowed the urea to rise by not more than 15 mg. per cent from its normal level. This dose constituted a dog unit. The dogs were free from infections and intestinal parasites, and kept in a constant environment and on a constant dietary regimen. Pfiffner et al., (1934a) modified this procedure/

procedure by taking the end-point of the assay to be a rise in blood urea by 100%. They observed that the hormone requirement of the dog was independent of the time interval which elapsed between adrenalectomy and assay, and varied by only 25% in the same dog for periods of even 16-24 months. The number of insufficiency periods which the adrenalectomised animal had experienced did not affect the assay results. Extracts were compared on the same dog and usually two dogs were used for each dose level.

This method showed that orally administered hormone was about 8% as effective as when subcutaneously administered.

When a massive dose of 2340 dog units of cortical hormone was injected subcutaneously into a normal dog, less than 200 units could be detected in an extract of urine collected for 53 hours. Cortical hormone added to urine could be quantitatively extracted by their methods. It was concluded that the major portion of the hormone was either metabolized or excreted by a route other than the kidneys.

This method of assay has been used by Wintersteiner and his co-workers in their attempts to isolate active crytalline substances from adrenocortical extracts, (Pfiffner et al., 1935; Wintersteiner and Pfiffner, 1935; Wintersteiner and Pfiffner, 1936). They isolated several crystalline compounds, which proved biologically inactive by this/

this method in the doses given by them. Later, these same compounds such as hydrocortisone and cortisone were found to be active in larger doses, and indeed highly active by methods of assay which are specific for 11-oxysteroids. Kendall and his co-workers used this method of assay during fractionation of adreno-cortical extracts and isolation of physiologically active crystalline compounds (Kendall, 1934 and 1940). Mason et al. (1937) isolated corticosterone and showed that it was biologically active on the dog urea test.

3. Performance of Muscular Work.

Muscular fatigue was known to be one of the outstanding symptoms in Addison's disease. The adrenalectomised animal suffered muscular fatigue more rapidly than control animals. This observation has been adapted for assay purposes in a number of ways.

a. In 1932, Everse and de Fremery developed a method for the assay of cortical hormone. An anaesthetised rat was fixed on a board, and its foot was connected to a recording lever. The tetanic contractions of the gastrocnemius were recorded during brief periods of stimulation. An initial faradic stimulation for a period of three minutes was followed by three one minute stimulations at intervals of half a minute. After adrenalectomy, the height of the first contraction remained unchanged, while the excursions produced by the three following/

following stimuli became considerably smaller. Treatment with cortical hormone restored these contractions to the height obtained in intact rats.

The most useful application of this test was in the demonstration of high biological activity for corticosterone, which was one of the numerous crystalline compounds isolated from adreno-cortical extracts by Reichstein (de Fremery, et al., 1937).

Reichstein (1938) found that DCA was ten times as effective as corticosterone in this test. Cortisone was found to have very little activity.

b. Gaarenstroom et al., (1937) found that cortical hormone restored the diminished swimming time of adrenalectomised rats to normal. Waterman et al., (1939) using this test together with the Everse - de Fremery test confirmed that corticosterone and DCA were biologically active compounds.

c. The muscle work performance test of Ingle has yielded in the hands of its originator a wealth of information on adrenal cortical physiology.

Hales, Haslerud and Ingle (1935) found that the gastrocnemius of an anaesthetised rat, stimulated 3/sec. continued to work for over 5 days and that adrenalectomy reduced this period to 36 hours or less. Ingle et al., (1936a; Ingle 1936b) found that the rapid fatigue after adrenalectomy, could not be duplicated by removal of any of the other endocrine glands, nephrectomy or evisceration, and that/

that the adrenal medulla was dispensable for work under such conditions.

The total work performance of adrenalectomised rats was found to be directly related to the amount of cortical hormone injected, until an optimum amount of hormone was used.

The procedure as described by Ingle (1944), was to adrenalectomise and nephrectomise a rat anaesthetised with a barbiturate. The nephrectomy imposed an additional stress, thereby shortening the time required for the test. Such an animal was unable to work for more than 10 hours without treatment. Immediately following the operation the animal was fixed to a board and the gastrocnemius muscle was weighted with 100 gm. and stimulated to contract 3 times per second. The contractions were transmitted to the pulley of an automatic device which recorded the distance the muscle lifted the weight at each contraction. The records of work were read from the dial of a Veeder counter and expressed as the number of recorder revolutions per unit time. This was used as an index of the efficacy of treatment. The animals were enclosed in a constant temperature cabinet. By using 15 animals for each dose it was possible to obtain significant differences in work performances with doses varying from each other by 25%. The log.dose response relationship was linear, and/

and the index of precision $\lambda = 0.140$ (Dorfman, 1951).

Cortisone was used as a standard, and since the sensitivity of the test was found to be constant at different times of the year, it was possible to use a standard curve. The test was found to be sensitive to 120µg. cortisone and 60µg. hydrocortisone.

The procedure was adopted for the assay of extracts and compounds prepared in the laboratory of E.C. Kendall at the Mayo Clinic, and now has a history of usefulness in guiding the chemist during fractionation of adreno-cortical extracts. It was by this method that biological activity was demonstrated for the first time in a pure adrenal steroid viz. - cortisone (Mason et al., 1936). Ingle (1950) says that the choice of the muscle work test in this instance was fortunate, but fortuitous. Its use was rationalized on the grounds of economy of time and test materials. The fact that the work test is highly specific for the 11-oxysteroids was not appreciated by him until 1938.

After that, a large number of fractions of adrenal cortical extracts, adrenal steroids and chemically related compounds have been examined for the presence or absence of the biological activity characteristic of the 11-oxysteroids without any known error. Just now, it is used extensively by the Upjohn Company for screening adrenal steroid-like substances/

substances for biological activity (Ingle, 1952b). Kendall (1935), using the Ingle test and growth-survival tests, was able to demonstrate two qualitatively different fractions in his adreno-cortical extracts. One of them, effective in the work test only, and the other, effective in maintaining adrenalectomised animals normal, as measured by their growth. Subsequent work has shown that these fractions contained the 11-oxysteroids and 11-desoxysteroids.

Ingle (1940c) compared cortisone and DOC on life maintenance and on muscle work in adrenalectomised rats. The two compounds were found to be qualitatively different. Whereas DOC was effective in maintaining life and increasing weight, and ineffective in the muscle test, cortisone had the reverse effects.

These experiments illustrate how the use of biological assay provided information of the possibility that the adrenal cortex contains two or more qualitatively different hormones.

By this test, Ingle (1940b) and Ingle and Kuizenga (1945) compared the relative activity of four different adrenal steroids. If cortisone is considered the standard (100), hydrocortisone has a relative potency of 160, 11-dehydrocorticosterone a value of 32 and corticosterone 46. The same relative potencies are observed in the ability of this compound

to/

to cause glycogen deposition in the liver of the adrenalectomised rat. The effects of the adrenal steroids on work performance in the Everse - de Fremery test differ from these obtained in the Ingle test (1950). In the Everse - de Fremery test, DOC was the most active compound and cortisone was ineffective in the doses tested. This apparent discrepancy has led to some confusion in interpreting the significance of "fatigue" tests in general. Actually, the two tests are different in principle. The Ingle test requires continuous stimulation over many hours, whereas the Everse - de Fremery test determines the extent of recovery from a brief period of stimulation and does not measure the capacity of the animal to resist severe and prolonged stress.

Ingle et al., (1951 and 1952a) have shown that if adrenalectomised-nephrectomised rats were treated with increasing doses of the pure adrenal steroids, cortisone or hydrocortisone given intermittently or by continuous intravenous drip, the work performance remained significantly below the average values for work in nephrectomised controls and adrenalectomised-nephrectomised rats treated with a continuous intravenous infusion of adreno-cortical extract. This suggests that the adrenal cortex secretes substances other than the known adrenal steroids.

4./

4. Water diuresis.

Petranyi (1941) observed that the diuretic response to a given load of water was decreased in an adrenalectomised rat when compared to the intact animal. Cortical hormone restored the diuresis to normal levels.

Rats were adrenalectomised. A load of water, equal to 5% of its body weight, was given by stomach tube, and the urine collected. In untreated animals the volume of urine did not exceed 25% of the water given by stomach tube. The amount of cortical hormone which raised the volume of urine to 50% of the water load was taken as one "rat diuresis unit".

DCA was found effective, and 2 mg. was equal to a unit.

5. Resistance to Various Agents.

Several investigations had revealed that adrenalectomised animals were hypersensitive to noxious agents, such as drugs, poisons, bacterial toxins and foreign proteins. Cortical hormone raised the resistance of adrenalectomised animals to normal. The protection afforded by cortical hormone has been used to develop methods for assaying it. A few examples of this procedure are described.

a. Resistance to water intoxication.

Despite the inital diuresis which follows adrenalectomy, the kidney loses its ability to excrete water administered to the animal. As a result,/

result, if water is given in repeated doses, to an adrenalectomised animal there is intracellular hydration with symptoms of water intoxication, and death. Swingle et al., (1937) showed that adrenalectomised dogs were highly sensitive to water intoxication and that intravenous cortical extract restored them to normal.

Eversole, Gaunt and Kendall (1942) studied the effectiveness of adreno-cortical derivatives in protecting adrenalectomised rats against water intoxication. The rate of excretion of administered water and the occurrence of prostration, convulsions or death were the criteria of response. Normal rats tolerated a given quantity of water and eliminated over 40% of it. They found that quantitative studies were impossible because of the wide individual variations in their animals. However, when the average responses in about 20 rats was considered, DCA was found to be less effective than whole adreno-cortical extract; cortisone was at least three times as efficient as DCA and the amorphous fraction was only weakly effective.

b. Resistance to histamine poisoning.

Scott (1927) observed that adrenalectomised rats were killed by an injection of 1/8th the dose of histamine tolerated by normal rats. This was confirmed by Marmorston-Gottesman and Gottesman (1928). Perla et al., (1931) showed that the/

the injection of adreno-cortical extract protected adrenalectomised rats against four or five times the lethal dose of histamine, and suggested that it be used as a method for detecting cortical hormone. Male and female sex hormones and adrenaline were found to be without effect. A unit was defined as the amount of adreno-cortical extract necessary to protect adrenalectomised rats against 200 mg. histamine acid phosphate.

Perla and Gottesman (1931) proceeded to use this method for assaying cortical hormone activity in normal human urine. A lipoid extract of urine was made and the equivalent of 300 ml. of urine was injected daily into adrenalectomised rats given varying doses of histamine. Since the extract of urine afforded protection they claimed to have demonstrated cortical hormone activity in the urine for the first time.

c. Resistance to potassium poisoning.

Zwemer and Truszkowski (1937) showed that adrenalectomised animals could not deal adequately with certain definite amounts of potassium given in food or by injection, although much greater amounts were tolerated by normal animals. Cortical hormone protected adrenalectomised animals against poisoning by potassium.

Truszkowski and Duszynska(1940) used adrenalectomised mice and showed a linear relationship between/

between the percentage survival following the injection of potassium chloride and the log.dose of cortical hormone. A similar protection was afforded by DCA. Feil and Dorfman (1945) used this method to investigate the cortical hormone content of urine. Male rats were used for the assay. They were adrenalectomised, and an injection of potassium chloride was followed by the administration of hormone; the percentage survival was taken as a measure of cortical hormone activity. Adreno-cortical hormone and DCA protected against potassium poisoning. Under the same conditions, chloroform extracts of urine afforded protection, indicating the presence of cortical hormone in urine.

d. Resistance to shock.

Adrenalectomised rats, tied down to a wire grid by all four legs for one hour exhibit symptoms of shock and die within four hours. The administration of DCA, adreno-cortical extract, and certain 11-oxysteroids prolonged the survival (Elmadjien and Pincus (1944).

e. Resistance to typhoid vaccine.

Typhoid vaccine was found to be poorly tolerated by adrenalectomised animals (Jaffe, 1926). Adreno-cortical extract effectively restored the resistance to normal (Hartman and Scott, 1931). Perla and Marmorston-Gottesman (1931) proposed a method for the/

the biological assay of cortical hormone by measuring its ability to protect adrenalectomised rats against typhoid vaccine.

Lewis and Page (1946) reported the use of this principle for biological assay of cortical hormone. They defined a toxic protection unit, which they found was equivalent to 0.2 mg. of cortisone; 5 mg. DCA exhibited less than one toxic unit. The toxic protection activity of cortical hormone was found to parallel "carbohydrate activity".

6. Glycogen Deposition in Liver.

That the adrenal glands exerted an effect on carbohydrate metabolism was noted in 1908 when Bierry and Malloizel reported that adrenalectomy in dogs was followed by a fall in blood sugar to one-half or one-fifth of its original level. The observation was extended to show that adrenalectomy resulted in a general depletion of carbohydrate levels in blood, liver and muscle. Britton and Silvette (1932) showed that extracts of the adrenal cortex restored the blood sugar, and liver and muscle glycogen of adrenalectomised animals to normal. In their paper they suggested that "the increments in hepatic glycogen produced by the adrenal cortex extract form the basis of a relatively simple and economical means of testing the potency of such material". But it was only 10 years later that Reinecke and Kendall (1942) developed a method based on this effect. It has proved to be of great value and precision.

On/

On the fourth day after removing their adrenals, and after a 24-hour fast, rats were given injections of hormone subcutaneously. They were killed 1-2 hours after the last injection, the liver rapidly removed and its glycogen content estimated. There was a linear relationship between the logarithm of the dose and the amount of liver glycogen. Increasing the dose by a factor of two produced a difference in the liver glycogen deposited of approximately four times the standard error. This represents a high degree of accuracy for the biological assay of cortical hormone. The test was found to be highly specific for the 11-oxygenated adrenal steroids.

Olson et al., (1944a and 1944b), slightly modified the Reinecke and Kendall method. High protein diets were given to the rats. Four 11-oxysteroids were studied in detail. Cortisone was used as a standard. On this basis corticosterone was found to be 75% as active as cortisone, and 11-dehydrocorticosterone had a relative activity of 88%. The fourth steroid, hydrocortisone, was found to have a slope significantly greater than the standard, and therefore, the relative potency was not calculated. The index of precision, λ , varied from 0.077 to 0.141.

Pabst et al., (1947) used the same procedure as Olson et al., No departure from parallelism of slope was found for the four steroids. Corticosterone/

Corticosterone and 11-dehydrocorticosterone were 54% and 48% as active as the standard, whereas hydrocortisone had a relative potency of 155%.

Pabst et al., (1947) also compared this test with the muscle performance test of Ingle. The relative potencies of the adrenocortical extracts and crystalline adrenal steroids were found to be the same by both types of tests. DCA was found to be about 100 times less potent than the 11-oxysteroids.

The use of rats was soon abandoned because their sensitivity was inadequate for the determination of the small amounts of active substances normally present in urine. Long et al., (1940) had observed that adreno-cortical extracts caused a very high deposition of liver glycogen in mice. On the basis of this finding, three groups of workers have studied the liver glycogen response of the adrenalectomised mouse to adrenal steroids. The methods are essentially of two types.

The first, such as that of Venning et al., (1946) depends upon the deposition of glycogen, in the glycogen depleted liver of a fasting adrenalectomised mouse. The second method, employed by Eggleston et al., (1946) and Dorfman et al. (1946b) starts with a normal level of liver glycogen in an adrenalectomised mouse. The test depends on the ability of adrenal steroids to prevent the fall in liver glycogen. According to Venning et al., (1946), male mice are adrenalectomised/

adrenalectomised, and their food is removed on the third post-operative day. Twenty-four hours later, the hormone is injected in divided doses. One hour after the last injection, the liver is rapidly removed, its glycogen content estimated. The relationship is linear when the response is plotted against the logarithm of the dose. The method is sensitive to 10µg. of cortisone and 40µg. of 11-dehydrocorticosterone. The indices of precision for the assay for these two compounds was found to be 0.137 and 0.222 respectively.

Eggleston et al., (1946) start with high levels of liver glycogen, either by injecting adrenocortical extracts before the start of the assay, or by not removing food until immediately before the start of the first injection. What is measured is the ability of hormones to maintain the liver glycogen level. The method is sensitive to 2µg. of cortisone.

When the total fermentable sugar was estimated instead of the glycogen, the sensitivity was reduced but the accuracy was increased about 10 times.

Dorfman et al., (1946b and 1946c) using mice, obtained similar results.

The use of the adrenalectomised mouse compared favourably/

favourably with the use of the rat in that the sensitivity was increased about 25 times, although there was a slight loss of accuracy.

Since no departure from parallelism was found for the 11-oxysteroids and urine extracts, these methods have been usefully applied to estimating the urinary content of these steroids. It has been found that the normal human subject excretes 25 to 85µg. of cortisone equivalent per 24 hours in the urine. It is increased by about three times during stress, (e.g. exercise, infections, operations and burns) and hyperfunction of the adrenal cortex (e.g. Cushing's Disease and after ACTH). There is a lowered output in adreno-cortical hypofunction, (e.g. Addison's disease and Simmond's disease),(Sayers, 1950). Chemical assays give slightly higher levels, probably because such methods may include non-active metabolites.

Paschkis et al., (1950) used the method of Venning et al. (1946) to assay the cortical hormone content of adrenal venous and peripheral blood of dogs. Adrenal vein blood contained 14-50µg. equivalent of 11-dehydrocorticosterone acetate per ml. of plasma. They claimed to have detected very small quantities of cortical hormone in the peripheral blood of dogs; the amount increased after injections of ACTH or adrenaline/

adrenaline or when the animal was subjected to stress.

7. Survival on Exposure to Low Temperatures.

Hartman, Brownell and Crosby (1931) demonstrated a significant difference in the sensitivity of normal and adrenalectomised rats when exposed to low environmental temperatures, and showed that the resistance of adrenalectomised rats could be increased by the administration of adreno-cortical extract.

This phenomenon was adopted for the assay of adrenal cortical steroids by Selye and Schenker (1938). Young adrenalectomised rats were submitted to stress from a cold environment on the day after operation. The rats were divided into groups and given different amounts of adrenal cortical extract during the first hours of exposure. The survival time increased with the quantity of extract administered.

The method has proved to be both rapid and sensitive. Three groups of workers have used this test on a quantitative basis.

The test as designed and used extensively by Vogt (1943), is described in some detail because it has been used in parallel with the mouse eosinophil method in the work presented in this thesis.

Wistar rats, 19-23 days old and weighing 38-45gm. were divided into groups of ten. They were as nearly as possible of the same age and weight.

Litter/

Litter mates were evenly distributed among the groups, and the mean weight of each group was always made equal. The same ratio of males to females was used throughout the groups.

Adrenalectomy was carried out by the lumbar route. Pentobarbitone combined with ether were used as anaesthetics. The rats were given a few drops of 25% glucose solution soon after the operation. They took food overnight, usually gained in weight, and were lively and fit the next morning when the experiment was about to start.

For the test, the rats were distributed into individual numbered cages, given their first injection and simultaneously placed in a large refrigerator at 4°C. At intervals of an hour and a half further injections were given, their total being four or five. From the 5th or 6th hour after the beginning of exposure, a rapid inspection of the rats was carried out every half hour, any deaths recorded, and these observations continued till all the rats had died.

Calculation of the mean survival time was made for each group.

Since the rats in each group were selected so as to have in every group representatives of the same litter, ages, weights, and sexes, the test for "significance" of the result was based on the standard/

standard error of the differences in survival times between corresponding pairs of rats in any two groups and not on the standard error of the survival times themselves. The ratio mean difference/standard error gave the usual measure for "significance".

Vogt has described the following characteristics for the dose-response curve.

1. The response is proportional to the logarithm of the dose.
2. It is necessary to have doses differing by a factor of 2.5 to obtain significant differences in mean survival times.
3. The test is highly sensitive, the smallest dose of an adreno-cortical extract (Eucortone) being 0.06 ml. per rat.
4. Since the mean survival time for a given dose varies from day to day, it is necessary to establish the mean survival time for a "control" group of rats injected with saline only.
5. Since the slope also varies in addition to the sensitivity, it is necessary to have two further groups treated with two doses of standard. This enables a quantitative assay of unknown samples.
6. Two crytalline steroids were compared with adreno-cortical extract. Cortisone gave a linear dose-response curve, which not only differed from day to day for this compound, but also differed from the slope for adreno-cortical extract. DCA and DOC phosphate were ineffective when administered on the day of the test. It was effective if given one day before exposure to cold, but its degree was independent of the dose given. DOC glycoside afforded protection without the latent period, but it had to be given in extremely large doses of 400 to 600µg.

Dorfman et al.,(1944a and 1946a) used the cold protection test in much the same manner as Vogt. The hormone/

hormone was sometimes administered by stomach tube and not by injection. They also concluded that it was necessary to run the standard, unknown and "control" groups simultaneously. The relative potencies of the various adrenal steroids were ascertained. When the potency of cortisone was set at 100, 11-dehydrocorticosterone had a relative potency of 33, corticosterone 9 and DCA 8.

The mean value for λ for 11-dehydrocorticosterone= 0.299.

Vogt (1952) found that young mice gave responses similar to those given by young rats. Since the latter are easier to handle, they were preferred to mice.

Venning et al., (1944) used immature adrenalectomised rats for the cold test. The hormone was administered by stomach tube. A "cold unit" was defined, even though these workers were aware of the necessity of running standard and control groups at each test. The unit was found to be equivalent to 0.05ml. of an adreno-cortical extract, 10µg. of corticosterone or 2mg. of DCA.

The cold test has been extensively used.

Vogt has applied the test for assaying the content of cortical hormone in adrenal venous blood of various mammals, and in particular, the dog. Some of/

of her conclusions are presented:-

A striking discrepancy was found between the amount of chemically extractable hormone from the gland and the quantity present in the venous blood drained from the gland (Vogt, 1943). The quantities found showed that if the gland ceased to synthesize hormone, while secretion was allowed to proceed at a normal speed, it would lose its total hormone content in 6-12 seconds. The adrenal cortex therefore, contains practically no stores of the biologically active hormone, but elaborates the hormone rapidly and in large quantities. Changes in blood pressure and blood flow were without effect on the rate of secretion. Stimulation of the splanchnic nerves or an intravenous infusion of adrenaline caused a rapid and large increase in the rate of secretion (Vogt, 1944). The effect of adrenaline was mediated by release of ACTH by the pituitary (Pickford and Vogt, 1951).

The isolated dog adrenal, perfused by means of a Dale-Schuster pump showed that the gland secreted cortical hormone at a steady rate for a period of 1½ hours, irrespective of whether the perfusing blood came from a normal or hypophysectomised donor. The rate of secretion of the isolated gland was comparable to the gland in situ of a dog, in which the release of/

of adrenaline into the circulation had been avoided. ACTH added to the perfusing blood increased the rate of secretion. A decrease in the Na/K ratio of the plasma caused an accelerated secretion. Adenosine-triphosphate and creatinephosphate stimulated the activity of the cortex, probably because they were ready sources of energy. Glucose, lactate, amino-acids, sodium ascorbate, adrenaline, nicotine, colchicine and morphine were without any effect when added to the perfusate. Histamine in large doses caused a transient increase (Vogt, 1951).

Weil and Browne (1940) using the cold test demonstrated cortical hormone-like activity in extracts of normal urine. Increased quantities were found in Cushing's disease, and in patients with acute infections.

Dorfman et al., (1944a and 1944b) used the cold test to show that the cortical hormone-like activity in urine extracts was abolished in adrenalectomised monkeys and in cases of Addison's disease. Administration of cortical hormone caused significant activity to appear in the urine. These experiments proved that the active substances in extracts of urine originated in the adrenal cortex.

Venning et al., (1944) used the "cold test" to determine the activity of fractions of urine extracts, during/

during various stages of purification.

9. Sodium retention.

Dorfman, Potts and Feil (1947) were the first to develop a sensitive method for adreno-cortical hormone assay by measuring its sodium retaining effects in adrenalectomised rats.

Male rats were adrenalectomised. The following day the animals received the test dose of material. One hour later, radio-sodium as sodium chloride was administered. Urine was collected quantitatively for a period of 6 hours. It was evaporated to dryness, and the radio-sodium contained in the dried residue was counted with a suitable instrument, and the amount excreted was expressed as a percentage of the administered material.

The method was found to be highly sensitive. As little as 1µg. DOC and 10µg. DCA produced significant sodium retention. 25µg. hydrocortisone, 2000µg. of testosterone and the same quantity of α-estradiol caused no significant sodium retention (Dorfman, 1951). However, no dose-response curve has been published, and the test remains a qualitative one.

Spencer (1950) designed an elaborate test to measure sodium retention in adrenalectomised mice. Mice were adrenalectomised and used for testing from the/

the 4th to the 7th post-operative day. Twelve animals were used for the test. After emptying the bladder, each mouse was injected subcutaneously with 2 ml. of a load solution containing Na.K and Ca. They were divided into three groups; the first received a control solution of 5% glucose intra-peritoneally, the second received a standard dose of DCA ($\frac{1}{2}$-4µg.) and the third the substance to be tested for mineral hormone activity. The mice were left 6 hours, during which time their urine was collected, and the Na content estimated by indirect-flame photometry. On each subsequent day, the groups of mice were changed and the assay repeated until all the mice had in turn, acted as control, standard and unknown test animals. The "cross over" design minimized errors due to individual response variation, and any influence of one test phase on the next.

The means of the differences of the percentage Na. retention were taken as a measure of DCA activity. A linear log dose-response relationship was obtained with a steep slope. It was possible to detect 0.5µg. of DCA. Spencer claimed to have detected sodium retaining activity equivalent to 4µg. DCA per ml. of serum in the adrenal venous blood of a dog. No such activity was detectable in carotid arterial blood of the dog or in human blood obtained by catheter from the/

the I.V.C. There has been, so far, no confirmation of this method or of these findings.

Simpson and Tait (1952) developed a method for assaying mineralo-active steroids by their effect on Na and K excretion in the urine of rats. The method is sensitive, rapid, and quantitative. The bioassay is based on the effect of adrenal steroids on the urinary Na^{24}/K^{42} ratio in adrenalectomised rats, after the injection of these isotopes. Male rats were adrenalectomised and the assay was carried out on the 4th post-operative day. The rats were injected with a solution of the hormone in ethanol or with ethanol only in the case of the controls. This was followed one hour later by a subcutaneous injection of a solution of sodium and potassium chloride labelled with Na^{24} and K^{42}. The urine was collected for 2 hours, and evaporated to dryness in Petri dishes. They were placed under an end window Geiger counter and the counts per minute due to Na^{24} divided by the counts per minute due to K^{42} was determined for each sample and for an aliquot of the injected solution. It was found that the lowering of the Na^{24}/K^{42} ratio was linearly dependent on the logarithm of the dose of DCA for a range 0.5 to 5µg. The slopes for adreno-cortical extract and active adrenal steroids were found to be parallel with that of DCA. Simultaneous use of standard and unknown was essential for quantitative/

quantiative assays. The index of precision for the assays was about 0.30. The time required to carry out an assay was about 6 hours.

If the activity of DCA was taken as 100, the relative potencies of other steroids was found to be as follows:

DOC 135,
corticosterone 14.3,
17-hydroxy-desoxycorticosterone 7.99,
hydrocortisone 7.58,
11-dehydrocorticosterone 6.74,
cortisone 5.91 and 1 ml. of a beef adrenocortical extract 150.5.

Progesterone, oestradiol and testosterone were less than 3.

Tait, Simpson and Grundy (1952) have applied this method in an interesting manner. A beef adrenal extract was run on paper against appropriate standards e.g., hydrocortisone, cortisone, corticosterone, 11-dehydrocorticosterone, 17-hydroxy-desoxycorticosterone and DOC. The steroids were identified, eluted and bioassayed for mineral activity. 87% of the mineral activity of the extract lay with the cortisone area. This activity was found to be six times greater than the mineral activity of an equivalent amount of pure cortisone. It was inferred that this/

this mineral activity was due to some compound other than the known crystalline adrenal steroids, and that this compound and cortisone had similar running properties. Subsequent work showed that the "mineral" activity could be completely separated from cortisone by running the fractions for longer distances along the chromatogram. The mineral active compound does not appear to be any of the known biologically active adrenal steroids. For instance, it does not possess the α-β unsaturated ketone grouping (Grundy and Simpson, 1952).

The salt active substance has been demonstrated in the adrenal venous blood of the monkey and the dog (Simpson, Tait and Bush, 1952).

Lockett (1951) introduced a method whereby the activity of cortical hormone was measured by its ability to stabilise the plasma chloride concentration (measured as mg. NaCl%) in adrenalectomised dogs in a state of mild deficiency. Environmental temperature and diet were rigorously controlled. The diet was rich in potassium and low in sodium content, and it was necessary to adopt hand feeding to ensure uniform food intake as the dogs developed anorexia. Standard and unknown doses of adreno-cortical extracts were given alternately by daily injection, the effect of each/

each dose being observed for 48 hours or longer. By this means, the dose of the unknown was adjusted to give responses in plasma chloride levels equal to that given by the standard.

Large quantities of extract were necessary and the assay of one unknown extract on two dogs took 14 days to complete. But Lockett claims a very small error of ± 6%. The method is used for standardising commercial preparations of adreno-cortical extracts.

10. Effect on lymphocytes.

Dougherty and White (1944)showed that the injection of ACTH in mice, rats and rabbits produced a temporary fall in the number of blood lymphocytes. Adrenalectomy abolished this effect. Injection of adreno-cortical extracts caused a lymphopenia in the absence of adrenals. The lymphopenic action of ACTH was therefore mediated by the adrenal cortex. Schrek (1949) found that corticosterone and hydrocortisone and extracts of the adrenal cortex had a direct cytocidal action on rabbit lymphocytes in vitro. He proposed that the lymphocytotoxic action be used for the bioassay of the adrenal cortex hormones.

The method of assay (Schrek, 1951) consisted of incubating cellular suspensions of lymphocytes derived from/

from the rabbit thymus with adreno-cortical extracts and adrenal steroids at 37°C. for 24 hours. The number of viable cells in treated and untreated suspensions was determined by adding a solution of safranine, and noting the number of unstained cells. The pyknotic nucleus of a dead cell took the safranine stain. A cytocidal unit was defined as that amount of steroid or extract which killed 50% of the cells as compared to a control. The cytocidal unit was found to be 0.025µg. for hydrocortisone and 0.1µg. for corticosterone and cortisone. 50µg. of DOC had no effect. The test seems specific for the 11-oxysteroids.

Feldman (1950a) found adrenal cortex extracts and 11-dehydro-corticosterone (200µg./ml.) to be lympholytic. At this level of sensitivity he could not detect adrenal cortex activity in dog adrenal venous blood (1950b).

METHOD OF ASSAY AND ITS APPLICATION TO A COMPARATIVE STUDY OF ADRENAL STEROIDS.

Introduction.

Although the eosinophil cell was described over one hundred years ago, its relationship to the adrenal cortex was first suggested by Dalton and Selye (1939). The application of acute damaging stimuli to mammals elicited a group of symptoms and signs, which Selye called the "Alarm Reaction". The syndrome included, amongst other findings, an adrenal cortical hypertrophy and a consistent fall in the number of circulating eosinophils. The control exercised by cortical hormones on eosinophils was conclusively demonstrated in man by Hills et al., (1948). Whereas the injection of ACTH caused a fall in the circulating eosinophils in normal subjects, it failed to do so in patients with Addison's disease. When these patients were injected with hydrocortisone, there was a considerable fall in eosinophils. They concluded that a decrease in the number of circulating eosinophils was associated with an increased adreno-cortical activity.

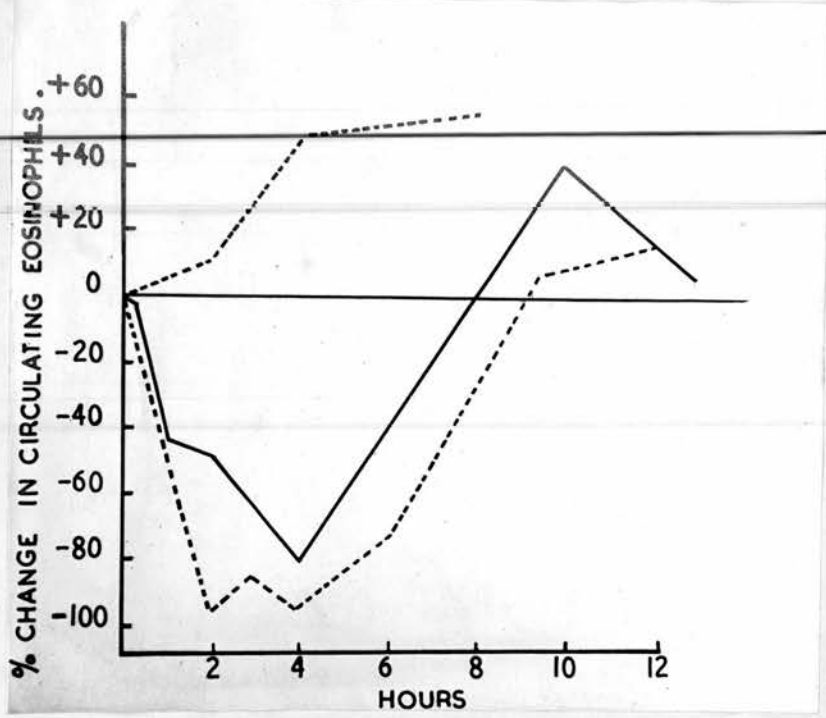

Fig. 1

Effect of stress and cortical hormone on circulating eosinophils in mice.

continuous line- normal mice subjected to stress.
upper interrupted line- adrenalectomised mice subjected to stress.
lower interrupted line- adrenalectomised mice injected with hormone.

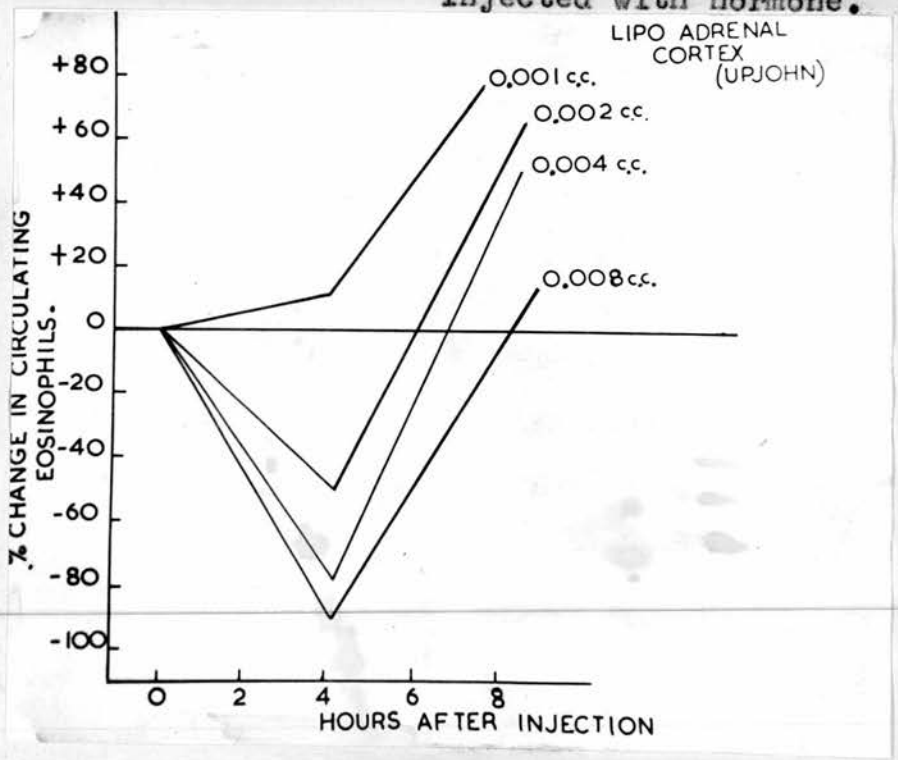

Fig. 2

Effect of graded doses of cortical hormone on the circulating eosinophils in adrenalectomised mice.

Speirs and Meyer (1949) demonstrated the effects of stress and cortical hormones on mouse eosinophils. Undisturbed, normal mice were subjected to the stress of having their tails cut in order to obtain blood for an eosinophil count. Subsequent eosinophil estimations were made at hourly intervals, and the change was expressed as a percentage of the initial count. There was almost a complete disappearance of eosinophils from the circulation in four hours. When the adrenals were removed, the application of stress did not cause a disappearance of eosinophils. The count at the fourth hour showed either no change or a slight rise from the first count. When adrenalectomised mice were treated with cortical hormone, they responded with a fall in eosinophils, which reached its maximum in two to four hours after the injection. These results are illustrated in Fig.1. It was further shown that the percentage fall in eosinophils is related to the dose of an adreno-cortical extract administered (Fig.2). Oestrone (30µg.), testosterone (150µg.) and DCA (25µg.) were without effect. Cortisone (3µg.) and 11-dehydrocorticosterone (25µg.) were strongly eosinopenic compounds.

These findings formed the basis of the assay procedure/

procedure described below.

METHODS.

Male white mice from a closely inbred strain and weighing from 25 to 30gm. were used throughout. They were kept in a thermostatically heated room at a temperature of 23°-25°C. for some time before, and always after adrenalectomy. The assays were carried out in the same room, and the mice were subjected to a minimum of disturbance.

Adrenalectomy was performed under ether anaesthesia. The adrenals were approached by the lumbar route. Care was taken to remove the gland intact, together with the periadrenal fatty tissue and the anterior third of the renal capsule. A pellet of DCA weighing 5mg. was inserted subcutaneously and the incision closed. Recovery from the anaesthetic was rapid, and thereafter the mice remained lively. They were given tap water for drinking.

The mice were used for the first assay a week after adrenalectomy, and used again at weekly intervals for four or five weeks. At the end of this period, the DCA implant was nearly completely absorbed, and the mice were destroyed.

On the day of the assay each mouse received 5µg. adrenaline/

adrenaline subcutaneously. Adrenaline pre-treatment was adopted for two reasons. It caused a higher initial count, and saline injected controls showed a lower percentage fall when so pre-treated. Three hours after the injection of adrenaline, tail blood was sampled for its eosinophil count (for counting procedure see below), and the test substance in a volume of 0.2 ml. was injected subcutaneously. Four hours later, a second eosinophil count was made, and the percentage fall from the first count calculated. The mean percentage fall in ten mice represented a response for any given dose.

Each mouse was kept in a separate cage and subjected to the minimum of disturbance throughout the assay. The usual procedure was to compare two or three solutions by giving each of them to five mice on one day and to five other mice on the next day. Therefore two samples required ten mice on each successive day, and three samples required fifteen mice each day.

A difference in mean responses expressed as a multiple of the standard error of this difference gave the usual value for "t". The probability that such/

such a difference was due to chance was determined by means of Fisher's table (1941).

Procedure for obtaining a blood sample in the mouse.

The procedure used to obtain blood from mice is important. A highly standardized procedure ensures a minimum of variation in eosinophil estimations.

A mouse is heated under a 100 watt lamp. This is done in a small metal case containing about half an inch of sawdust at the bottom. After a period of ten minutes, or as soon as the animal becomes agitated and there is a dilatation of the tail vessels, it is introduced into a perforated metal cylinder, one end of which is closed. Its exit at the other end is blocked by means of a U-shaped wire, which permits the tail to protrude freely from between the arms of the U. A sharp razor blade is used to make a transverse nick over a tail blood vessel. Large drops of blood form swiftly over the cut, and a sample is drawn into a white cell pipette until it reaches the 1 mark. The tail wound is compressed to prevent bleeding and the eosinophil diluting fluid is quickly drawn into the pipette to the 11 mark, diluting/

Procedure for obtaining blood from mouse tail.

Figure 3

Cutting the tail.

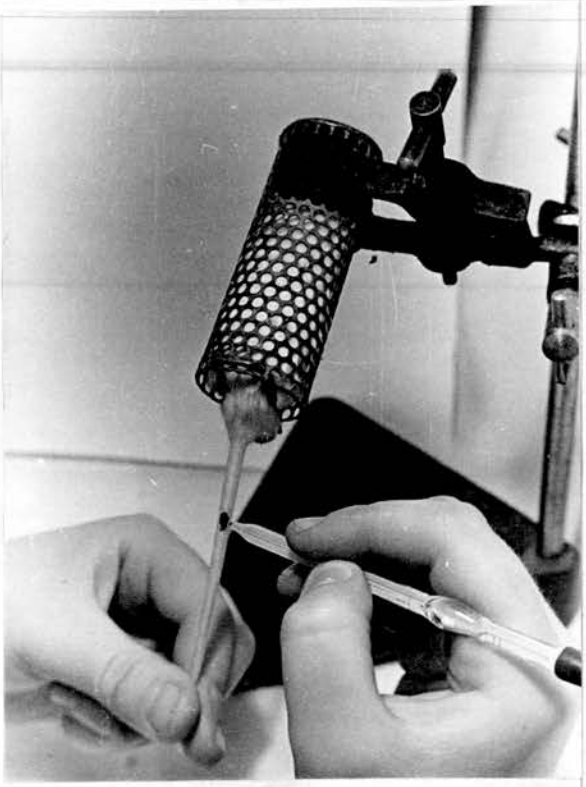

Figure 4

Taking blood sample.

Figure 5

Tail compressed to prevent bleeding.

diluting the blood ten times. The pipette is gently shaken fifty times by hand, and the four sides of two Fuchs-Rosenthal counting chambers are filled by capillarity after discarding the first four drops. A chamber is placed on the stage of a microscope (magnification 8 x 15), and allowed to stand for five minutes before the cells are counted. During this five minute interval, the pipette is washed and the test substance is injected subcutaneously.

It is necessary to carry out these manipulations with speed to prevent clotting of blood and to minimise blood loss from the tail.

The procedure is illustrated in Figures 3, 4 and 5.

In this manner, eosinophil estimations may be carried out at fifteen minute intervals. While one count is being carried out, another mouse is being heated for the next count. Ten to fifteen estimations can be completed before it is time again to do an eosinophil count on the first mouse, i.e., four hours after its first count.

Eosinophil Counts./

Eosinophil Counts.

Eosinophil cells are found in relatively small numbers in normal blood. Their estimation by means of blood films is not only time consuming, but is also an inaccurate procedure, in that it totals the errors inherent in the two procedures of total count and differential count. The introduction of the absolute count method for estimating eosinophils (Dunger, 1910), was therefore a distinct advance on the previous method. Dunger reported a diluent for blood which stained the eosinophils and destroyed the other blood cells. It was based upon the known fact that the eosinophil is the most resistant cell of the blood. Several such diluents have been introduced from time to time in clinical work. (Randolph, 1944; Rud, 1947; Manners, 1951).

Speirs and Meyer (1949) introduced such a diluent suitable for use in rats and mice. This was slightly modified for use in the work presented in this thesis. It was freshly made up on each day of an assay, and consisted of:

5 ml. 0.1% Phloxine in water
15 ml. distilled water
5 drops of 0.5% Byprox (detergent)
2.5 ml. Acetone.

Phloxine is a good (eosinophil) stain for

bringing/

bringing out the eosinophil granules in the acetone solution. The small amount of detergent helps to obtain a better distribution of cells. The eosinophils are recognized as follows: the granules within the cell stand out as red bodies, which may be in a mass in the centre or scattered around the periphery. Usually, the cell is seen only faintly and the nucleus is colourless.

Errors.

Apart from the biological variation in the responses of individual mice to treatment, there are sources of variation in the purely technical procedure of blood counting. In any routine analysis of this kind, each step in the process may introduce some degree of error.

Berkson (1939) has classified these sources of error into three categories:

(1) Pipette error - due to differences in calibration and differences involved in the manipulations of filling, diluting and mixing.

(2) Chamber error - due to differences in calibration and differences involved in the procedures of filling the chamber. These two sources of variation/

variation are negligible, and may be said to cancel out because the same diluting pipette and same counting chamber are used for every determination, and great care is taken to duplicate manipulations exactly.

(3) "Field" error - that which is due to the settling of cells by chance on the ruled field of the counting chamber. It is a chance error which is unavoidable with any technique dependent upon sampling, and may be considered to be the minimum error to which the count is subject. The error can be decreased by increasing the number of cells counted, but cannot be eliminated.

"Student" (1907), has shown that the distribution of cells which have settled out of a liquid on the ruled field of a haemocytometer follows the Poisson Law. According to this Law, the variance of a count is equal to the number of cells counted, i.e., the standard deviation (S.D.) is equal to the square root of the number of cells counted.

To illustrate this principle, Plum (1936), made several cell counts from the same pipette and demonstrated a marked agreement between the observed error and/

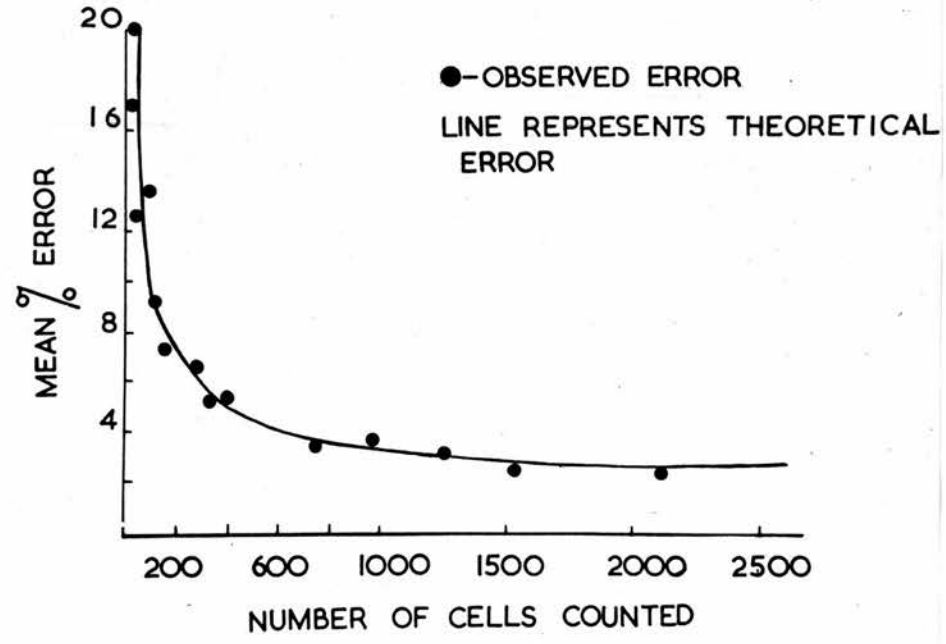

Figure 6

Relation between observed and theoretical (expected) error in absolute cell counts (Plum, 1936).

and the error calculated from the Poisson Law. Fig.6 summarises Plum's findings.

To test this distribution in the experiments reported in this thesis two duplicate counts were made from each of sixty pipettings covering a wide range of eosinophil concentrations. The value of χ^2 was 46.06, which for sixty degrees of freedom corresponds to a probability of 0.8 indicating satisfactory agreement of the data with the Poisson Law. To give some practical applications:-

If 100 cells are counted, the S.D. would be 10% of this count;

if 400 cells are counted, the S.D. could be only 5%.

To quote another example:-

A difference of 22% between the two counts 36 and 28 would not be significant, whereas, a similar percentage difference between 360 and 280 becomes highly significant.

Since then the error of a count is diminished as the number of cells counted is increased, at least 100 cells were counted at each estimation. In order to/

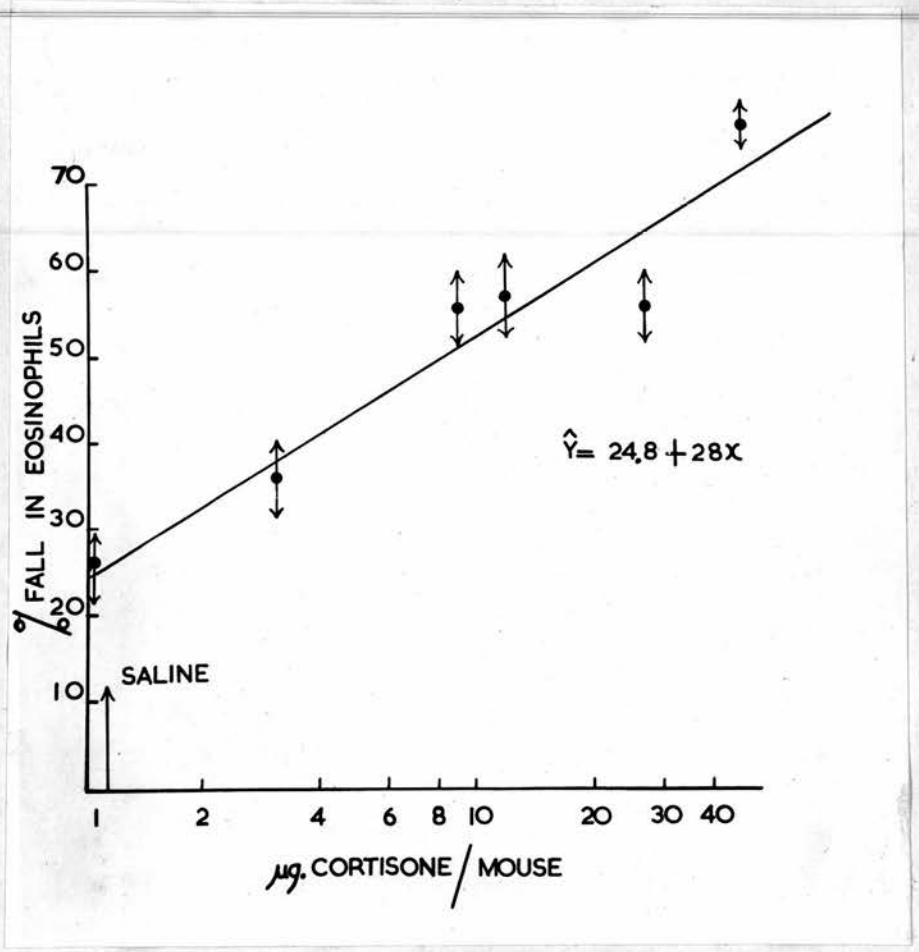

Figure 7

Dose response curve for cortisone.

to ensure this, blood was diluted 1:10 instead of the customary 1:20. Four chambers were filled at the first count for each mouse and all four counted if a two chamber count did not add up to a hundred cells. It can be calculated that a difference of 25% is statistically significant if the two counts are 108 and 81 cells. A 25% fall usually indicated 1µg. of cortisone.

DOSE-RESPONSE CURVES.

1. CORTISONE (Free Alcohol).

A standard solution of cortisone was made in acetone. Subsequent dilutions were made with saline so that the required dose was injected in a volume of 0.2 ml. The acetone was evaporated before injection.

Fig.7 represents the dose-response curve for cortisone, where the percentage fall in circulating eosinophils is plotted against the log. dose of cortisone in micrograms. The line drawn was that calculated from the regression equation. Each point on the graph represents the mean response of ten mice, and the arrows indicate the standard error.

Tables 1, 2 and 3 summarise the results and their statistical treatment.

TABLE 1

TABLE 1

DOSE	0.2 ml. Saline	µg. Cortisone/mouse					
		1	3	9	12	27	48
Mean % fall in eosinophils	11	26	36	55	57	56	77
Standard error	3.5	4.9	4.1	4.1	4.8	4	2.1

TABLE 2

CALCULATION OF REGRESSION EQUATION

Notation

x = log. dose

n = number of doses

y = mean response at each dose

Σ = sum

$$\bar{x} = \frac{\Sigma x}{n}$$

$$\bar{y} = \frac{\Sigma y}{n}$$

$\bar{x}$	$\bar{y}$	$\Sigma(x-\bar{x})^2$	$\Sigma(x-\bar{x})(y-\bar{y})$
0.94	51	1.9	53.27

$$Y = \bar{y} + \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})^2}(x-\bar{x})$$

$$Y = 51 + \frac{53.27}{1.9}(x - 0.94)$$

$$Y = 24.8 + 28x$$

TABLE 3

ANALYSIS OF VARIANCE

Variation	df.	Sum of squares	Mean square	Variance ratio
Regression	1	1494	1494	
Deviation from regression	4	218.81	54.7	0.29
Between doses	5	1712.81	342.6	
Within doses	49	9367.19	191.17	
Total	54	11,080	205	

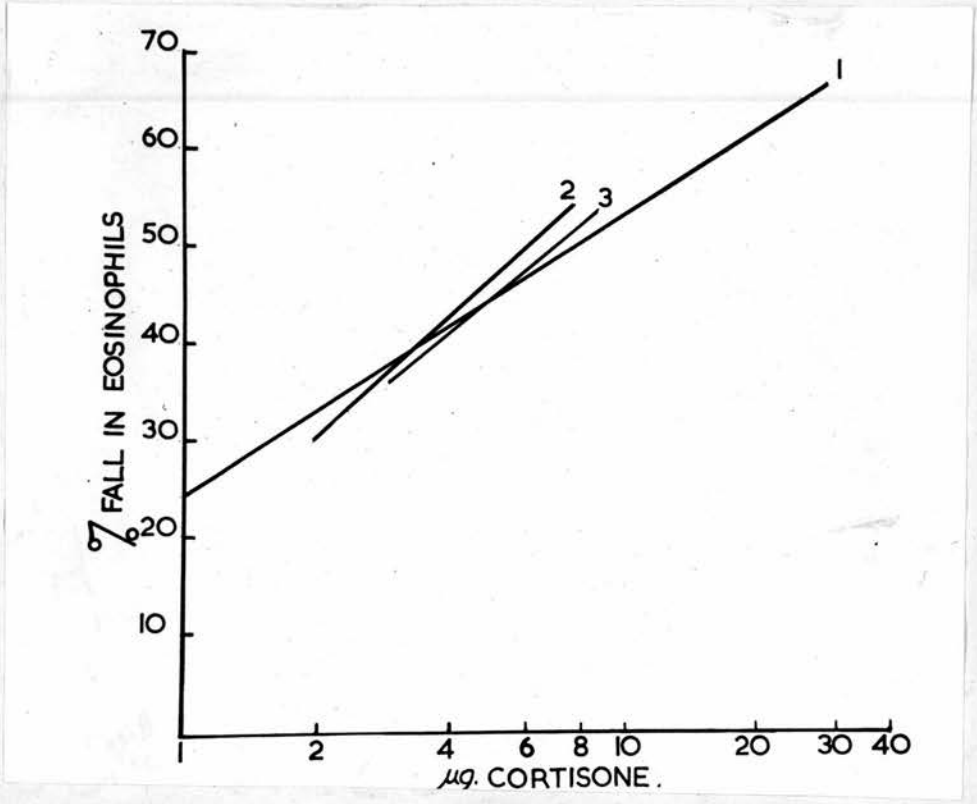

Figure 8

Three dose-response curves for cortisone.

1. **Feb. 1951**
2. **June 1951**
3. **Sept. 1951**

The dose-response curve for cortisone has the following characteristics:

1. The response to one microgram is significantly different from saline. The response indicated for saline is the highest mean effect obtained in a series of four control groups.

2. The curve is very flat. The dose must be increased by a factor of 4 for mean responses to differ significantly.

The index of precision λ - 0.56. This measure of inaccuracy reduces the value of the test. It is the most inaccurate of procedures for biological assay of 11-oxysteroids (see table 7 for comparative λ).

3. The ratio between the deviations from regression and the deviations within doses is equal to 0.29, which does not approach the 0.05 probability level of 2.5. Hence there is no significant departure from linearity.

4. There is a stable variance throughout the varying doses.

5. Three dose-response curves constructed in 1951 at intervals of four months showed no significant departure in slope or potency from one another.

Fig. 8 illustrates these curves.

Table 4 gives the values for their slopes and standard errors.

Table 4/

TABLE 4

Date	Slope	S.E. Slope
Feb. 1951	28	4
June 1951	40	10
Sept. 1951	30	13

The curve for cortisone was used as a standard curve when this method was used for estimating the hormone content of plasma extracts.

Speirs and Meyer (1951) published a dose-response curve for cortisone. The mice were pre-treated with adrenaline on the day of the assay. Their data were used for calculating the slope and λ. Between 1 and 6µg, their slope is twice as steep (regression coefficient = 70) and λ = 0.25. However, only six mice have been used at each dose level and dose-response curve was constructed only once.

2. OTHER ADRENAL STEROIDS.

Five other biologically active steroids (free alcohols) and two of their acetates were examined for/

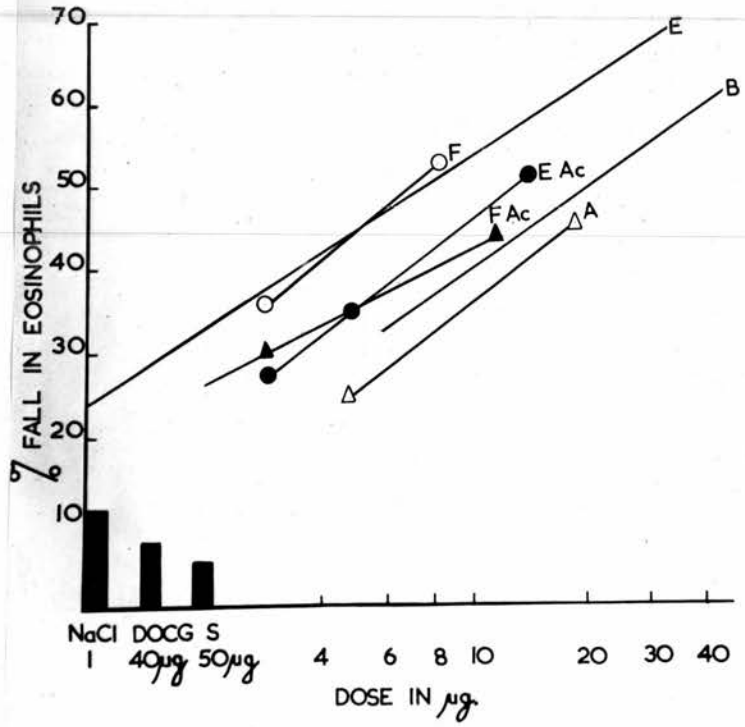

Figure 9

Comparative assay of steroids.

F = hydrocortisone

E = cortisone

E Ac = cortisone acetate

F Ac = hydrocortisone acetate

B = corticosterone

A = 11-dehydrocorticosterone

DOCG = desoxycorticosterone glucoside

S = 17-hydroxydesoxycorticosterone

for eosinopenic activity in adrenalectomised mice. The steroids were initially dissolved in acetone. Subsequent dilutions were made in saline and the acetone evaporated by heating. With 17-hydroxy-desoxycorticosterone, the saline contained 5% acetone in order to keep the steroid in solution.

Fig. 9 illustrates the dose-response curves for the following steroids:-

1. Cortisone
2. Hydrocortisone
3. Cortisone Acetate
4. Hydrocortisone Acetate
5. Corticosterone
6. 11-dehydro-corticosterone.

The effects given by desoxycorticosterone glucoside and 17-hydroxydesoxycorticosterone are indicated by the black columns. They are placed beside the saline effect for purposes of comparison.

Table 5 summarises these results and gives the calculated values for slopes and their standard errors.

Table 5/

TABLE 5

STEROID	Dose in µg/ mouse	Mean % fall in eosinophils	S.E.	Slope	S.E. Slope
Hydro-cortisone	3	36	6.7	42	16
	9	56	3.8		
Hydro-cortisone acetate	3	30	4.4	23	11
	12	44	5.3		
Cortisone acetate	3	27	5.0	33	11
	5	39	3.6		
	15	51	8.1		
Cortico-sterone	6	33	5.1	30	7
	15	44	5.0		
	24	55	4.7		
	45	58	3.0		
11-dehydro-cortico-sterone	5	24	4.7	35	10
	20	45	3.9		
Desoxy-cortico-sterone Glucoside	40	6	3.2	-	-
17-hydroxy-desoxy-cortico-sterone.	50	4	1.9	-	-

The relative potencies between the steroids or groups of steroids were calculated. Since there was no significant departure from parallelism between the slopes for individual steroids, a common slope was first calculated for the two steroids whose relative potency was being determined. The common slope was then substituted in the original regression formulae, so that the regression lines became exactly parallel. The vertical distance between these two lines expressed as a ratio of the slope gave an estimate M, which is the horizontal distance between the lines. This estimate is the log. difference between equi-active doses of the two steroids compared. The value for M expressed as a ratio of its standard error, gave the usual value for t. The probability that the value for M was due to chance was determined from Fisher's table (1941).

The antilog. of M gave an estimate of the relative potency.

Table 6 represents the results of these calculations.

Table 6/

TABLE 6

STEROIDS	M	s.e.(M)	t	p	Relative Potency (antilog.M)
Cortisone Hydrocortisone	0.104	0.119	0.873	0.50	1.3
Cortisone and Hydrocortisone Cortisone and Hydrocortisone Acetate	0.312	0.111	2.810	< 0.01	2.0
Cortisone Corticosterone	0.440	0.106	4.150	< 0.001	2.7
Cortisone 11-dehydrocorticosterone	0.643	0.098	6.670	< 0.001	4.4
Corticosterone 11-dehydrocorticosterone	0.157	0.108	1.454	0.10	1.4

Comments.

The test is specific for the 11-oxygenated adrenal steroids, 40µg. of desoxycorticosterone glucoside or 50µg. of 17-hydroxydesoxycorticosterone were without effect, whereas the four 11-oxysteroids were eosinopenic. Speirs and Meyer (1949) reported that 25µg. of desoxycorticosterone and 50µg. desoxycorticosterone acetate were ineffective.

Since there is no significant departure from parallelism between the slopes for the four 11-oxysteroids, their relative activities may be compared. If cortisone is considered the standard with a value 100, hydrocortisone has a value of 78, corticosterone 37 and 11-dehydrocorticosterone 24. The difference between cortisone and hydrocortisone is not statistically significant, whereas the difference between cortisone and the other two steroids is significant.

The order of relative potencies for the 11-oxysteroids as determined by the mouse eosinophil test closely parallels that obtained in two other tests specific for the 11-oxysteroids, viz. the Ingle muscle test and the glycogen deposition test. In the latter tests, hydrocortisone was more potent than cortisone/

cortisone, but in the eosinophil test, no significant difference was detected between these steroids. Speirs and Meyer (1951) published results in agreement with this conclusion for cortisone and hydrocortisone.

Table 7 compares the relative potencies of the 11-oxysteroids as determined by these three tests.

Table 7/

TABLE 7

STEROID	Mouse Eosin-ophil Test	Muscle Test Ingle (1940b)	Rat Glycogen Deposition.Test	
			Olson (1944b)	Pabst(1947)
Cortisone	100	100	100	100
Hydrocortisone	78	160	-	155
Corticosterone	37	46	75	54
11-Dehydro-corticosterone	24	32	88	48
λ	0.56	0.141	0.149	0.141

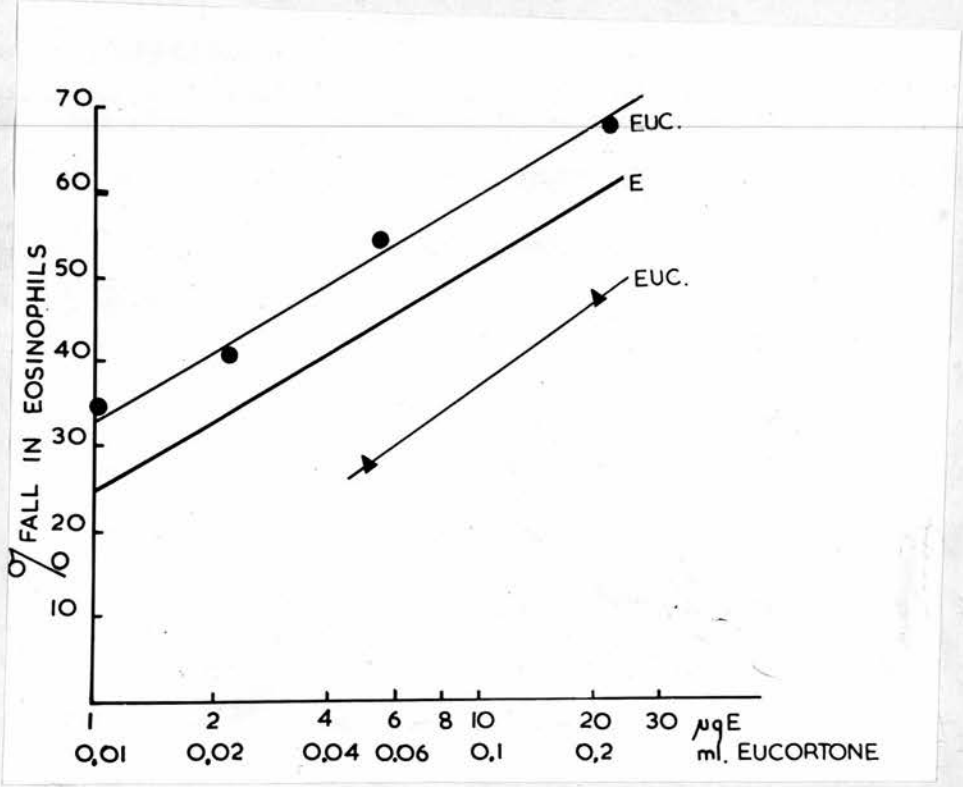

Figure 10

Comparison of dose-response curves obtained with cortisone and cortical extract.

The relative potency between the free alcohols and the acetates of cortisone and hydrocortisone is = 2 (p = 0.01). The difference is greater than can be accounted for by the difference in molecular weights. It may be due to differences in the rate of absorption or utilisation. Speirs and Meyer (1951) found no significant difference in the relative activities of cortisone and its acetate.

3. ADRENAL CORTICAL EXTRACT

The cortical extract used was "eucortone" (Allen and Hanburys). Dilutions were made with normal saline, so that the required dose was injected in a volume of 0.2 ml. per mouse. Two different batches of eucortone were tested, one in February, 1951 and the other in February, 1952. The latter had been stored in the refrigerator for over one year.

Table 8 gives the results and Fig.10 represents their regression lines together with the regression for cortisone.

Table 8/

TABLE 8

Date of Assay	Ml. encortone per mouse.	Mean % fall in eosinophils.	s.e.(effect)	Slope
February, 1951	0.01	35	5.0	29
	0.05	53	3.9	
	0.25	74	4.0	
February, 1952	0.05	28	4.6	33
	0.20	48	6.4	

There is no significant difference in the slopes for eucortone and cortisone. The cortisone contents of the two batches of extract are 219µg./ml. and 30µg./ml. respectively.

THE ESTIMATION OF 11-OXYSTEROIDS IN DOG PLASMA

1. Recovery Experiments.

Cortisone or hydrocortisone (free alcohol) was added to 15 ml. of dog arterial plasma. This mixture, together with 15 ml. of arterial plasma to which nothing was added, were extracted with organic solvents.

Method of extraction.

The organic solvent consisted of a mixture containing two parts of ethyl acetate and one part of freshly distilled ether. The plasma was extracted four times with its own volume of solvent. The combined extracts were evaporated in vacuo to near dryness in a 100 ml. round bottomed Quickfit and Quartz flask, (bath temperature 40°C.). The residue was taken up in 15 ml. petroleum ether (b.p. 40-60) and transferred to a separating funnel containing 25 ml. of petroleum ether. The flask was washed four times with 2 ml. of 70% ethanol. The washings were used to extract the petroleum ether solution twice. The ethanol extracts were combined and concentrated in vacuo (bath temperature 30°C.) to less than 1 ml. in a Quickfit and Quartz tube, by which time the ethanol had been driven off.

The/

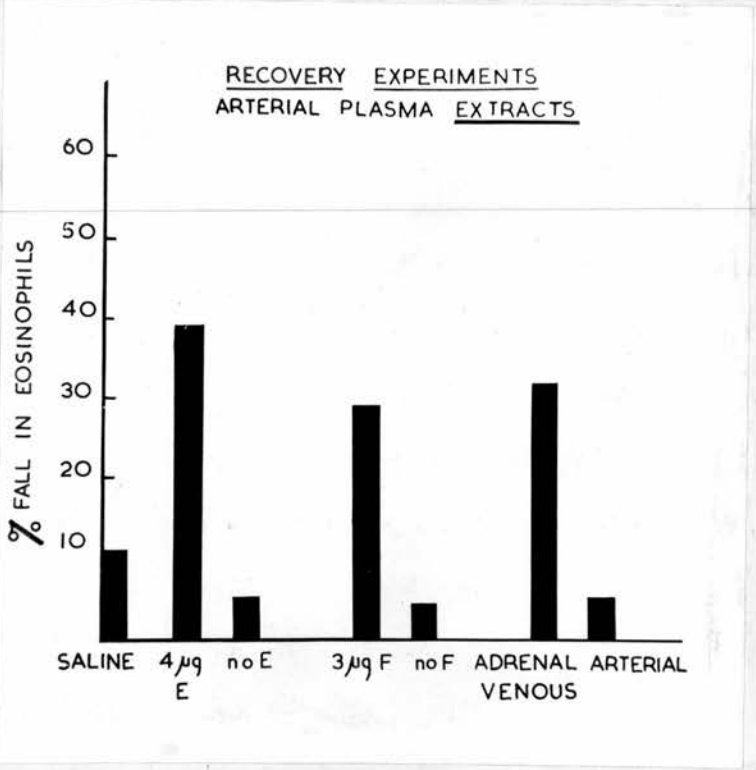

Figure 11

Recovery experiments.

E - cortisone

F - hydrocortisone

Each column represents the mean response in 10 mice.

The concentrate was transferred to a graduated centrifuge tube, the Quickfit and Quartz tube washed thrice with 0.5 ml. portions of distilled water and the washings added to the concentrate, which was made up to a volume of 2.5 ml. with more water, if necessary. Each mouse was injected with 0.2 ml. of this solution (≡ 1.2 ml. plasma).

Table 9 gives the results of recovery experiments with cortisone and hydrocortisone. Figure 11 illustrates two of these results and also the results with adrenal venous plasma extracts. The fiducial limits of recoveries show that there is no significant difference between the observed and expected responses.

The method of extraction described above was communicated by Bush (1951), and it is a modification of methods originally used by Pfiffner et al. (1934b) and Pfiffner, Wintersteiner and Vars (1935).

TABLE 9

Steroid	µg. added	µg. found (fid. limits in brackets)	% recovery
Cortisone	4	3.2 (1.2 - 8.4)	80
	nil	< 1	
Hydro-cortisone	3	2.0 (1 - 3.2)	67
	nil	< 1	
Hydro-cortisone	3	4.5 (2.3 - 10)	150
	nil	< 1	
Hydro-cortisone	3	2.1 (1.2 - 3.5)	70
	nil	< 1	

Eosinopenic effect of dog plasma proteins in adrenalectomised mice.

These experiments resulted from an attempt at assaying cortical hormone activity in dog plasma by injecting the mice with the untreated plasma. It was found that both arterial and adrenal venous plasma caused significant and about equal falls in eosinophils. In eighteen out of twenty-one instances, 0.1 ml. of dog arterial plasma caused a significant fall. Ethyl acetate extracts of arterial plasma did not give this effect unless cortisone was previously added. Arterial plasma obtained from an adrenalectomised dog caused a significant fall in eosinophils (see last column in Fig.14). The eosinopenic effect of untreated arterial plasma was therefore not due to any cortical hormone content. At this stage the plasma proteins were suspected of causing the eosinopenia. Hence, they were isolated in several ways and tested on adrenalectomised mice.

1. Fig.12 illustrates the results obtained with different preparations from the same arterial sample. Each column represents the mean effect on ten adrenalectomised mice. The first column shows the/

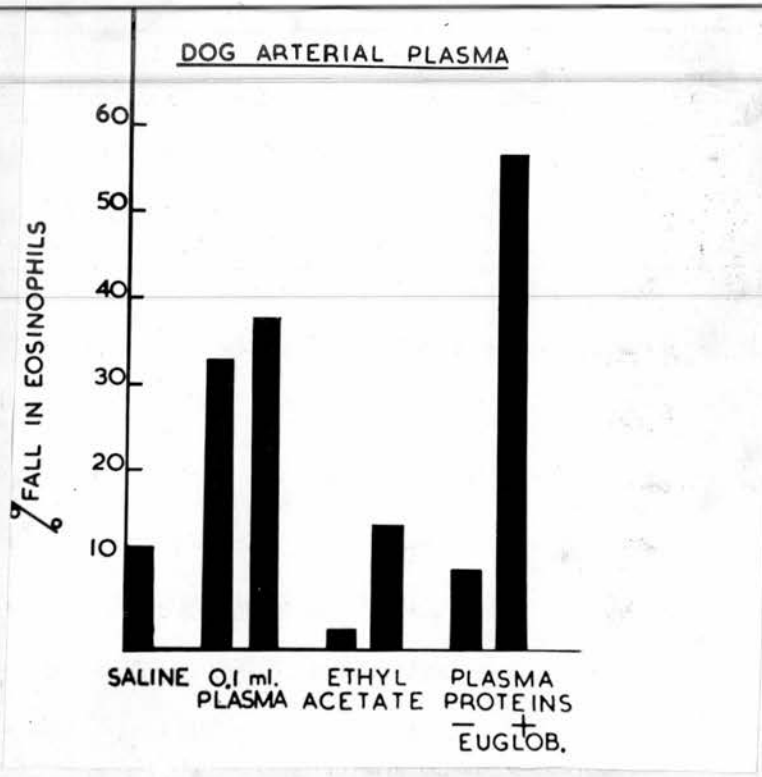

Figure 12

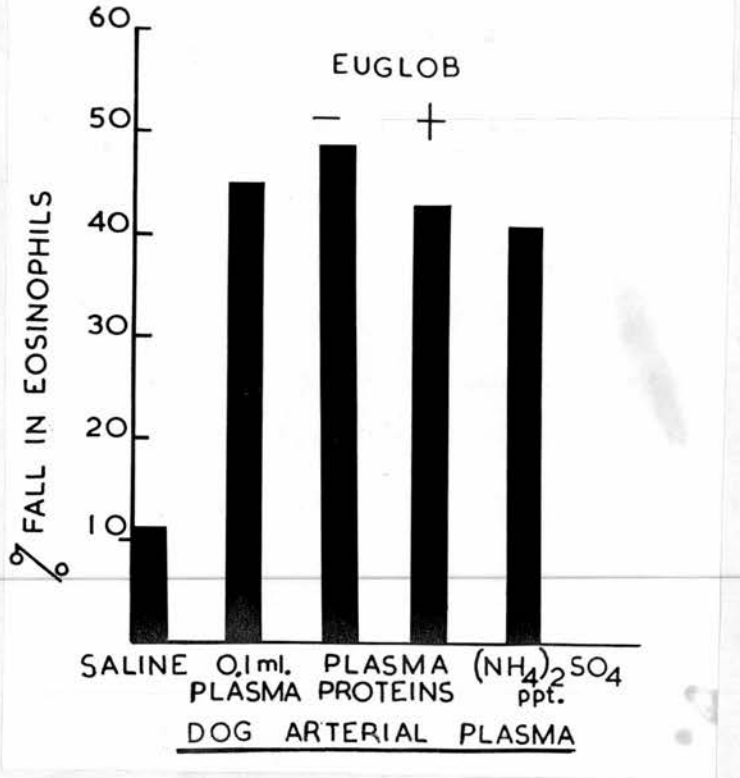

Figure 13

the effect with saline, and the second and third columns show the effects with the untreated plasma injected on two different days. They are significantly different from the saline effect. The fourth and fifth columns represent the mean effect with ethyl acetate extracts. These are not significantly different from the saline effect. Another portion of the arterial sample was fully saturated with ammonium sulphate. The protein precipitate was introduced into a "sausage" cellophane bag and the salt dialysed off for twenty-four hours. In the absence of salt, the euglobulins remained as a fine precipitate in the bag. This precipitate was separated by centrifuging and was dissolved in normal saline. This fraction and the euglobulin-free fraction were tested separately. Columns 6 and 7 show that the euglobulin fraction was markedly eosinopenic, whereas the protein fraction without euglobulins had no effect.

2. However, such a difference between the two fractions was not obtained in another arterial sample. Fig.13 shows that both fractions were as eosinopenic as the untreated plasma or the total protein precipitate

3./

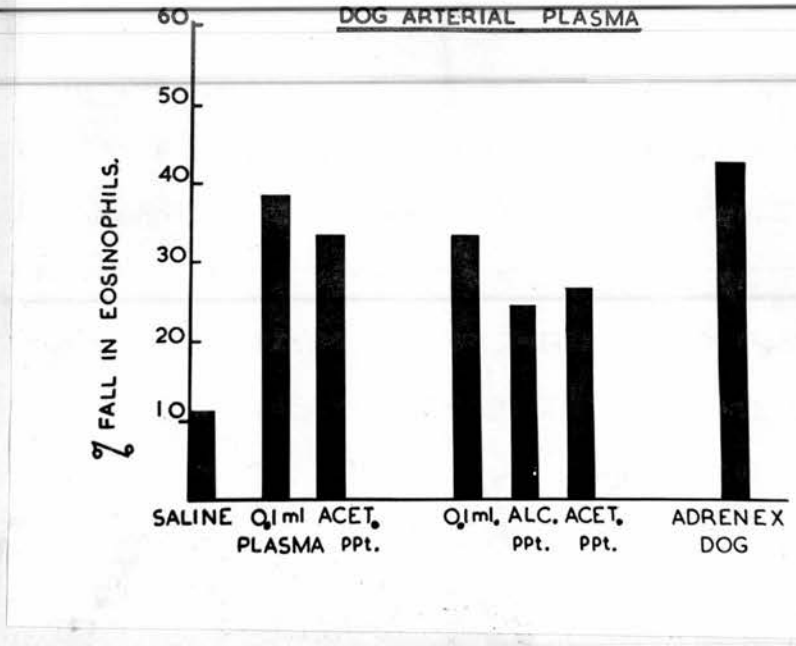

Figure 14

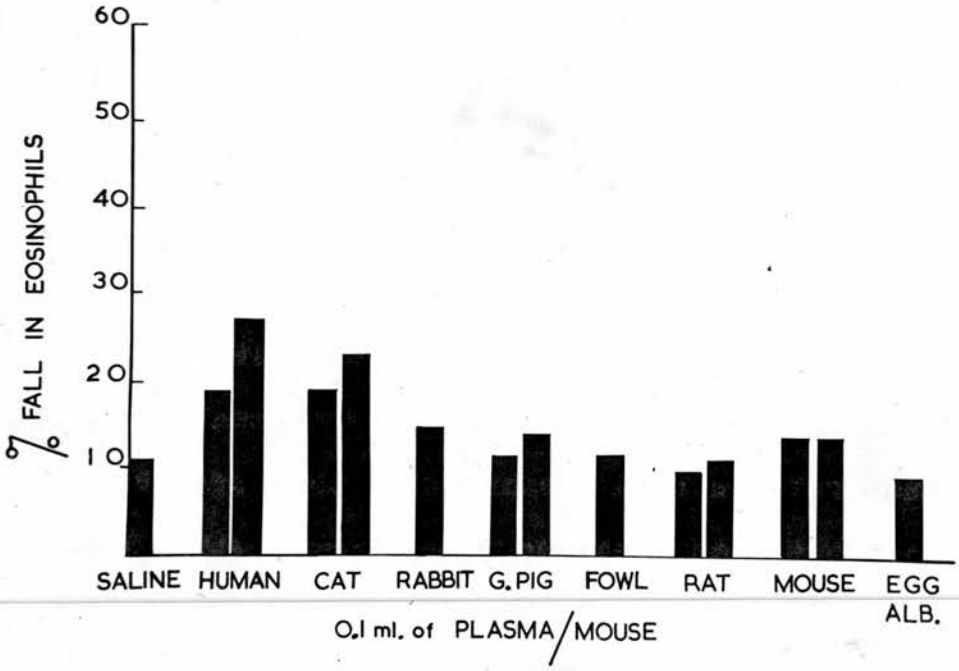

Figure 15

3. In a third arterial sample, the proteins were precipitated with alcohol or with acetone at -4°C. At this temperature the proteins are not denatured. Consequently, they remain soluble. The protein precipitate was redissolved in saline and tested. Fig.14 shows that the protein fractions gave effects which did not differ significantly from that given by the untreated plasma, and all of these were significantly different from saline.

4. Arterial plasma was obtained from several other species. These samples together with two human venous samples and a sample of egg albumen, were tested for eosinopenic effects. The results are seen in fig.15. Except for one human sample, none of the others gave effects which differed significantly from saline.

Comment.

These results have an important bearing on the mouse eosinophil method of assay. It is evident that the eosinopenic response in adrenalectomised mice is not specific for adrenal steroids. Plasma proteins from the dog give this effect. It is possible that proteins from sources other than plasma/

plasma will have a similar effect. If, therefore, the mouse eosinophil response is used for assaying cortical hormone in biological fluids, it is necessary first to extract the hormone with organic solvents.

Effect of horse serum on the eosinophils of sensitised adrenalectomised mice.

It is difficult to explain the eosinopenic effects of dog plasma protein. It may be a hypersensitivity phenomenon. It is possible that mice are congenitally hyper sensitive to dog plasma protein just as rats are congenitally hypersensitive to egg albumen (Selye, 1949). An experiment was designed to see whether sensitised adrenalectomised mice given a challenging dose of the homologous antigen would respond with an eosinopenia.

The method of sensitising mice has been described by Dougherty (1950). Ten mice were sensitised with horse serum (1.2 ml./mouse) given intraperitoneally in two divided doses, 19 and 21 days before the day on which the challenging dose was injected.1 ml. of the same serum was freeze-dried and preserved for use as the challenging dose. Twenty-four/

four hours after removing the adrenals of the sensitised mice as well as of ten non-sensitised controls, eosinophil counts were done on their tail blood. They were then given 0.0002 ml. of the horse serum in 0.05 ml. saline intravenously, and four hours later a second eosinophil count was carried out.

The sensitised mice responded with a 4% fall and the non-sensitised controls showed a 5% fall. When the challenging dose was doubled, three out of five sensitised mice died within an hour of injection, and the other two showed no significant eosinopenia.

Comment.

There is no significant eosinopenia in either group; nor is there a significant difference between the responses in the sensitised and non-sensitised groups. However, this negative result was obtained where a hypersensitive state was induced. The experiment throws no light on the manner in which dog plasma protein produced its eosinopenic effect in adrenalectomised mice.

Effect/

Effect of hydrocortisone on eosinophils in vitro.

Godlowski (1952) reported that cortisone acetate had a direct cytolytic action on eosinophils in vitro. The steroid was added to heparinised blood, in a concentration ranging from 0.05 to 5mg./ml. Muehrcke et al., (1952) also reported a similar effect with cortisone acetate when it was used in a concentration of 1mg./3ml. blood. The concentrations of steroid used in these experiments far exceed the concentrations found in blood under physiological conditions. Therefore, lower concentrations of steroid were used to determine whether they had a direct cytolytic action on eosinophils in vitro.

Procedure.

8 ml. of rat carotid arterial blood was obtained by means of a cannula after an intra-arterial injection of 1 mg. heparin. The blood was immediately distributed into six small tubes, each tube receiving 1 ml. Each tube was sampled for its eosinophil count after which a given quantity of steroid or saline was added in a constant volume. It was placed on a mechanical rocker inside an incubator at 37°C, agitated for four hours, and a second eosinophil count/

count was made. Two tubes received 0.2 ml. saline each and served as controls; two tubes received 2µg. hydrocortisone each in 0.2 ml. saline and the other two tubes received 5µg. hydrocortisone each in 0.2 ml. saline.

Another experiment was carried out with mouse blood. Three mice were used for withdrawing blood from the carotid artery by means of a cannula. Each mouse received 0.2 mg. heparin intravenously. 0.5 ml. of blood was introduced into each of six tubes and the procedure repeated as described for rat blood. The doses of hydrocortisone used were 1µg. and 2.5µg. in 0.05 ml. saline, and the two control tubes received 0.05 ml. saline.

Results.

Table 10 shows the mean percentage fall (2 tubes) for the control and steroid added tubes. The differences between them are not significant.

Therefore, under the conditions of this experiment, hydrocortisone did not have a cytolytic action on rat or mouse eosinophils in vitro. The removal of blood for the first eosinophil count and the addition of a certain volume of fluid (saline or steroid) would by itself, have accounted for a 17% fall in the rat blood experiment and a 10% fall with mouse blood. But in each/

each tube the observed fall exceeded this amount. Since this observation was common to the control and test samples, it was inferred that the agitation by itself was responsible for a certain measure of eosinophil cell destruction.

TABLE 10

Species	Dose Hydrocortisone	Mean % fall
Rat blood	Saline	34
	2 µg.	36
	5 µg.	41
Mouse blood	Saline	45
	1 µg.	38
	2.5 µg.	45

Distribution/

Distribution of adrenal steroid between cells and plasma.

There has been no independent investigation into the distribution of adrenal steroids between the cells of the blood and its plasma. Vogt (1943), has used plasma for assaying the hormone content of adrenal venous blood. She separated the cells and plasma within a few minutes of collecting the samples. Paschkis et al., (1950), have also used plasma. Nelson (1952), used whole blood at first when estimating 17-hydroxysteroids in peripheral blood. Later, they found double the quantity of steroid in plasma as in whole blood, when equal volumes were used. This observation indicated that adrenal steroids were transported in the plasma, and that they did not enter the cells in detectable quantities. An investigation was undertaken to determine this distribution.

Procedure.

Dog adrenal venous blood was used in these experiments. Anaesthesia was induced with ether and completed with chloralose. The abdomen was opened, the viscera retracted and the left adrenal was exposed. A segment of the left adreno-lumbar vein, lateral to the/

the gland was mobilised, its tributaries ligated and the vein cannulated. At this stage heparin was given intravenously. When the entrance of the adreno-lumbar vein to the vena-cava was tied, adrenal blood flowed into the cannula and was collected into ice-cooled centrifuge tubes. In two experiments, half this blood was centrifuged almost immediately. The other half was left standing at 25°C. for four hours with occasional mixing after which it was centrifuged. An equal volume of water was added to the packed cells. The cells and plasma were separately extracted in the manner described previously. In a third experiment, the blood was left standing for four hours after collection before the cells and plasma were separated and extracted. The extracts were tested on adrenalectomised mice.

TABLE 11

Hours after collection.	µg. Cortisone/ml.	
	Cells	Plasma
1	< 1	-
4	< 1	8.6
1	< 1	1.3
4	< 1	2.5
4	< 1	9.5

Results/

Results and Comments.

Table 11 shows the results of estimating the hormone content in cells and plasma.

In five sets of estimates, no hormone was detected in the cells, whereas appreciable quantities were found in the plasma. This is true for a period of four hours after the hormone has entered the blood from the gland. These observations, coupled with that of Nelson (1952), seem to indicate that the hormone does not enter the cells, at least in quantities detectable by the methods used for assay.

The rate of secretion of the dog adrenal gland in situ in terms of cortisone.

The output of the dog adrenal in situ has been determined by several workers. Vogt (1943), estimated the output in terms of an adrenal extract (eucortone) and expressed it as an equivalent of grams of glandular tissue. According to her estimates, the amount of hormone released from one adrenal per minute ranged between 2.6 and 20 'g. gland', with a mean value of 6.0 'g. gland'. If a 'gm. gland' contained the equivalent of 3 µg. cortisone (Olson et al., 1944a), Vogt's/

Vogt's estimate works out approximately to be 18 µg. cortisone per minute, as the output of one adrenal.

Nelson et al., (1951) found between 11 and 15 µg. of hydrocortisone being secreted per minute by the dog adrenal. A chemical method was used for this estimation.

Bush (1952) used paper chromatography to isolate steroids from the adrenal venous blood of dogs and he estimated an output of 2.7 to 6 µg. hydrocortisone per minute.

Paschkis et al., (1950) used the liver glycogen deposition test to assay the hormone content of dog adrenal venous blood. The average output per ml. expressed as µg. 11-dehydrocorticosterone was found to be 26. In terms of cortisone or hydrocortisone, this would amount to over 100µg. per ml.

Method.

The procedure for obtaining adrenal venous blood from the dog and extracting the plasma has been previously described. The time taken to collect the blood and the weight of the gland were noted. The splanchnics were left intact.

Results/

Results.

The results of six estimates are found in Table 12.

TABLE 12

Dog	µg. cortisone/g/min.
1	9.7
2	10.3
3	12.0
4	13.5
5	2.7
6	19.4
Average =	11.3

These estimates are in good agreement with the kind of output found by Vogt, Nelson et al., and Bush but not Paschkis et al. In the mouse eosinophil assay, there is no significant difference between the potencies and slopes for cortisone and hydrocortisone. And/

And since the dog adrenal secretes mainly hydrocortisone (Nelson et al., 1950; Bush, 1952), the figures in Table 12 may be even expressed as µg. hydrocortisone.

These results confirm the original findings of Vogt that the adrenal cortex has a high hormone output.

Observations on comparative biological assays of cortical steroids.

Comparative biological assays have often been used for the comparison of pure adrenal steroids either with each other or with cortical extracts. In the experiments reported below, comparative assays have been used to obtain information on the nature of hormone secreted by the dog adrenal cortex. Adrenal plasma obtained from perfused adrenals and from glands in situ was extracted, (as described previously) and the extracts were assayed in parallel by means of the 'cold test' and the mouse eosinophil test.

The cold tests were performed by Dr. M. Vogt. Eucortone (Allen and Hanbury's) was used as a standard in most of the cold tests. One ml. of this extract was equivalent to 30 µg. of cortisone in the mouse eosinophil test.

The/

The comparisons were made with two objects in view: first, to get figures for the secretion rate in terms of cortisone and, secondly to try and see whether changes in the rate of secretion would be reflected in the same way in both tests.

The effect of potassium on the output of the perfused adrenal.

One of the problems of the adrenal cortex is whether its natural secretion contains compounds which fall into two groups, one of them highly active in survival and sodium retention tests, and the other group predominantly active in tests using muscle work performance, (Ingle's method), glycogen deposition or a fall in circulating lymphocytes or eosinophils. The belief, held for a long time, that DOC regulated mineral metabolism has become increasingly unlikely when chromatographic analysis of adrenal blood did not reveal this compound. The 17-OH derivative of DOC (Reichstein's compound S) has very low biological activity (Masson et al, 1950). It has been suggested that hydrocortisone or corticosterone would account for the mineral activity of cortical secretion. On the other hand, experiments by Ingle, Nezamis and Morley (1952) have shown that these steroids are

inferior/

inferior to whole cortical extract in restoring functional deficiency produced by adrenalectomy. Spencer (1950), reported high salt retaining properties of serum from adrenal blood of a dog. Tait, Simpson and Grundy (1952) and Simpson, Tait and Bush (1952) have demonstrated an unknown compound with high activity on mineral metabolism in adrenal extracts and in the adrenal venous blood of a dog and monkey.

Vogt (1951) has shown that potassium increases the cortical secretion in the perfused gland, as measured by the cold test. It is possible that this increase is due to the secretion of a hormone acting on mineral metabolism. The cold test, unlike the mouse eosinophil test, responds more indiscriminately to adrenal corticoids. There are two reasons for suspecting that survival at low temperature is not prolonged exclusively by compounds carrying an oxygen in the C_{11} position. One is the lack of parallelism of dose-response curves obtained with adrenal extracts and with cortisone, and the other is the efficacy of DOCA in the cold test. The latter argument has to be used with caution as DOCA has to be given on the day preceding the exposure in order to be effective, and may/

may therefore be oxidised in the body. The water soluble DOC glucoside is effective on the day of exposure if its dose is sixty times that needed of cortisone.

In spite of some uncertainty as to what action of corticoids is measured in the cold test, it was used together with the mouse eosinophil test to assay samples of adrenal plasma extracts obtained from perfused adrenals.

Methods.

The adrenal gland of dogs was isolated and perfused in the manner described by Vogt (1951).

The dogs were anaesthetised with ether and 300-500 ml. blood, required for filling the perfusion pump, collected from the carotid artery into a flask containing heparin (100 units heparin/10 ml. blood). Chloralose (70mg./kg.) solution was injected intravenously and the ether was withdrawn. Both splanchnics were divided. The viscera, kidneys and right adrenal were removed, and the left adrenal together with an adjoining piece of aorta, vena cava and left renal artery were isolated. Perfusion was carried out through the superior mesenteric artery, and the effluent collected by means of a cannula tied into/

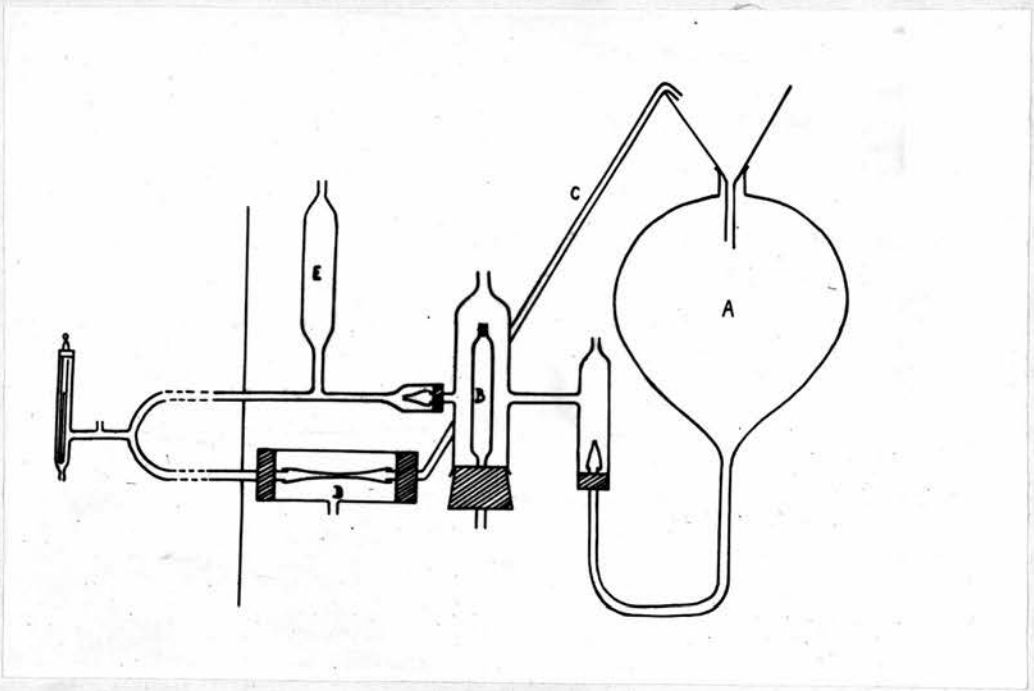

Figure 16

Circuit of Dale-Schuster pump.

into the adreno-lumbar vein.

The circuit of the Dale-Schuster pump (see fig.16) consisted of a pulsating fingerstall B pumping blood from the reservoir A along two channels, one leading to the arterial cannula and the other through the shunt C back to the reservoir. The shunt containing an adjustable resistance D determined the perfusion pressure. The air cushion E provided an "elastic recoil" to the system. The formation of vasoconstrictor substances through stagnation was prevented by filling the pump before proceeding with the dissection of the adrenal gland and by keeping the blood circulating through both channels from the moment the pump was filled till the onset of perfusion.

The effluent leaving the adrenal was collected in ice cooled centrifuge tubes. Since none of the blood was returned to the reservoir, it did not require reoxygenation. Throughout the perfusion, the temperature of the blood was kept at 38°-39°C. by means of a water bath surrounding the pump and by an adjustable lamp placed near the arterial cannula. The rate of flow was kept within certain limits by altering/

altering the perfusion pressure.

Potassium chloride (12 to 18 m. eq./litre of plasma) was added to the reservoir by means of a syringe and long needle.

The adrenal effluent was centrifuged and the plasma extracted in the manner described previously. Samples were always collected for equal periods of time. Each sample was divided into two portions for the two tests. The doses given to rats and mice were the same in that they represented equal collection times.

Results and Comments.

Table 13 shows the results of a series of comparative estimations of adreno-cortical secretion from the perfused gland before and during KCl. The arrows indicate the point at which KCl was added to the reservoir. The estimates of hormone output are expressed in terms of cortisone per gram gland per minute. In some of the cold tests standards were not run simultaneously. Consequently, estimates in terms of cortisone are absent from the data, but the manner in which KCl affects the secretion is seen in the mean survival times.

If the output of the perfused gland before the addition of KCl. is considered, it is seen that the isolated/

isolated gland yields a smaller amount of hormone than the gland in situ (data in Table 13) especially with intact splanchnic nerves. The output in terms of cortisone does not vary a great deal, the mean obtained, appears a little smaller by the mouse assay than by the rat assay, but the difference is non-significant.

In the attempts made to increase the cortical secretion by means of KCl, the limitations of the assays made themselves apparent. The increases produced by KCl. were often hardly large enough to be demonstrated when the assays were done by the cold test, which is generally able to detect a significant increase when it is $2\frac{1}{2}$-fold. But the mouse eosinophil test suffers from even poorer discrimination between doses, a fourfold increase being necessary to obtain differences which would be significant. There are two experiments (No.4 and 6) in which small increases in hormone yield shown by the cold test were reflected by similar, but non-significant increases when the assay was done by the eosinophil test. When the secretion was diminished after KCl (No.5 and 8) both tests reflected it. These falls were unexpected and may have been due to perfusion with blood/

blood of dogs which had suffered from distemper and had an accelerated sedimentation rate. Under these circumstances the blood flow through the gland diminished in the course of the experiment and this may adversely affect the secretory performance of the tissue. The impression created therefore, is that the activity measured is always due to secretion of compounds with an oxygen at C_{11}. Whether this be due to the limitations of the methods or to the fact that KCl. really increases the secretion of compounds with an oxygen at C_{11}, these assays have not produced any evidence for the dual nature of cortical secretion.

TABLE 13/

TABLE 13

Exp. No.	Sample	Mouse Assay		Cold Test	
		% fall	µg.E/gm/mt	Mean survival hours	µg.E/gm/mt
1	1 → 2	47 31	6.9 1.9	10.3 9.4	⩾3.3 >3.3
2	1 → 2	17 15	< 1 < 1	9.3 10.8	-
3	1 2 → 3	36 38 38	1.9 2.2 2.2	9.7 10.2 9.9	>3 >3 >3
4	1 → 2 3	35 36 41	5.5 6.0 9.1	8.5 9.4 9.6	7.5 10.8 11.6
5	1 → 2	34 24	4.5 2.1	8.9 8.5	3.9 2.9
6	1 → 2 3	23 26 26	< 1 2.1 2.1	8.3 9.0 8.5	-
7	1 → 2	20 24	< 1 1.5	6.7 7.4	
8	1 → 2	29 22	3 < 1	9.1 8.8	5.8 2.4
9	1 → 2 3	23 28 21	< 1 2.3 <1	8.8 9.2 8.7	-
	Average output-isolated gland with splanchnics cut	**4.4**			**4.7**
	Average output of gland in situ with splanchnics intact	11.3			

The effect of ACTH on the output of the perfused adrenal.

Vogt (1951), using the cold test showed that adding ACTH to blood perfusing the isolated adrenal increased its hormone output by 2-4 times. Nelson et al., (1951), also obtained a 2-4 times increase in hydrocortisone output of the dog adrenal in situ when ACTH was injected intravenously. Bush (1950) found that ACTH caused a quantitative increase in steroid output without qualitative changes in the ratios of the known steroids isolated by his chromatographic techniques.

The present investigation was undertaken to see whether the cold test and mouse eosinophil test would reflect any increased output in the same way.

Method

The method of perfusing the isolated adrenal has been described previously. The ACTH (LAIA), dissolved in saline was infused straight into the arterial cannula instead of adding it to the reservoir because there it might have been inactivated by the blood. For the infusions, a short T-piece was inserted into the rubber tubing leading/

leading to the arterial cannula. The blood flowed through the horizontal part of the T, while its vertical part was occluded by a rubber bung through which a syringe needle had been pushed. A piece of thin plastic tubing was fitted over the tip of the needle. The tubing ended near the nozzle at the inside of the cannula. A short length of fine rubber tubing clamped by a small clip was slipped over the butt of the needle. A syringe was connected to the rubber tubing, and the solution of ACTH was slowly infused from the syringe, the rate of infusion being controlled by a screw attached to the plunger.

Results and Comments.

Table 14 summarises the results of four experiments.

The arrows indicate the point at which the ACTH infusion was started. The infusion lasted during the collection of the next sample after which it was stopped and another sample collected except in the fourth experiment.

The start of the ACTH infusion was sometimes followed by a vasodilatation and an increased flow. This was controlled by reducing the perfusion pressure, in/

in order to keep the flow constant within limits.

Three out of four mouse eosinophil assays and two out of four cold tests showed a significant increase in hormone output after ACTH. (Unfortunately, the cold test failed in experiment 1 because litter mates were not obtainable for all groups). The increase caused by ACTH was 3-4 times the output measured before the infusion. Experiment 1 shows that the increase was maintained for an hour after the infusion was stopped. In experiment 2, the mouse assay showed a fall in hormone output after ACTH, but this was not significant ($t = 0.450$). In the same experiment, the cold test indicated an increase, but this too did not reach significant values ($t = 1.365$). Statistically, therefore, all samples in this experiment must be considered to have come from the same "population".

TABLE 14/

TABLE 14

Exp. No.	µg.ACTH infused	Mouse Assay		Cold Test	
		% fall	µg.E/gm/mt	Mean survival hours	µg.E/gm/mt
1	8.8	18	1.7	8.0	
		34	3.6	8.7	4.3
		33	3.5	8.7	4.3
		33	3.5	8.8	4.3
2	16.5	43	4.7	8.0	2.6
		40	3.6	9.2	6.0
		28	1.4	8.5	4.5
3	18.5	24	1.4	9.2	approx. 3
		24	1.4	8.7	" 3
		36	3.5	10.1	> 3
		32	3.5	9.7	> 3
4	19	26	0.7	6.9	
		21	< 0.7	4.8	
		43	3.1	8.2	
				7.9	

The hormone output in terms of cortisone show fairly good agreement in both tests considering the errors inherent in the two methods of biological assay used. The impression therefore, is that the increased activity measured is due to secretion of compounds with an oxygen at C_{11}.

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