

CYTOLOGICAL CHANGES IN THE ENDOCRINE
GLANDS OF ALLOXANIZED HYPERTENSIVE
DIABETIC RATS

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Abstract.

Rats made diabetic with alloxan and fed high protein and salt diets showed hyperactivity of the pituitary-adrenal systems, as indicated by adrenal hypertrophy, depletion of adrenal cholesterol and increased pituitary acidophils. Typical signs of hypertension such as arteriosclerosis, nephrosclerosis, fatty degeneration of the liver and hemorrhages were common. Insulin therapy and omission of protein from the diet did not lessen the extent of the pathological conditions. It was concluded that the animals were responding to alloxan as an agent of stress in conformity with the adaptation syndrome and that the high salt content of the diet aggravated and sustained the resulting hypertension.

The pathology of alloxan diabetes, which differs from that of diabetes mellitus, is changed to that of the latter by the salt diet. Because of the salt craving of diabetics, it is suggested that the hypertension often associated with diabetes is largely due to the salt intake.

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I. Introduction To The Problem:

An experiment on alloxan diabetes in rats given high salt and protein diets, conducted by Dr. Chute in Toronto, provided the source of experimental animals used in this problem. Dr. Chute's interest involved the high dietary salt requirements of diabetic human patients. During the course of the experiment, it soon became apparent, however, that the problem, though an exceedingly fruitful and interesting one, was a duplication of one of Selye's many projects concerning stress and the adaptation syndrome. Preliminary work consisting of histological brain studies was done at this university by the author and Mr. W.A. Rivers. The findings of this investigation will be referred to in connection with the results of this thesis. It will be shown by cytochemical methods that the animals were in the throes of the adaptation syndrome due to the action of alloxan as a direct or indirect agent of stress and that the diet had a direct bearing on the severity of the accompanying pathological conditions. Because of this, the hypertensive aspects of the case will receive more emphasis than the diabetic, since the alloxan served directly as a vehicle for the former.

II. Historical Background.

A. Alloxan Diabetes.

1. History.

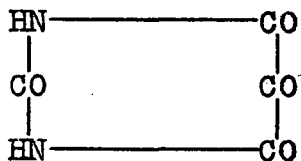
Alloxan is the ureide of mesoxalic acid. When injected into many lab animals it produces a selective necrosis of the beta cells in the Islets of Langerhans of the pancreas. This is followed in 24-48 hours by diabetes mellitus. It has been found that most animals are susceptible, with the exception of the guinea pig, duck, owl, chicken, frog and toad. (3) Symptoms of diabetes mellitus are polydipsia, polyurea, hyperglycemia and glucosuria.

A short summarizing history of alloxan is as follows:

- 1838 - synthesized by Woehler and Leibig.
- 1862 - discovered in intestinal mucus of humans by Liebig.
- 1866 - found in human urine by Lang.
- 1931 - excretory route studied by Cerecedo (14).
- 1937 - action on sugars noted by Jacobs (42).
- 1943 - islet necrosis discovered by Dunn et al (27).
- 1943 - alloxan diabetes in rabbits by Bailey (4).
- 1943 - alloxan diabetes in rats by Dunn (60).
- 1943 - alloxan diabetes in dogs by Goldner (36).

2. Chemistry of Alloxan.

Alloxan, a pyrimidine, is white in colour, water and alcohol soluble and is acid in solution. It cannot be neutralized without inactivating it. (Human blood plasma inactivates it.) Its formula is:



It may act either as an oxidizer or a reducing agent. As an oxidizer it is itself reduced to dialuric acid whereas acting as a reducing agent it is oxidized to parabanic acid. In vitro, alloxan inhibits the conversion of glucose - 1 - PO₄ to glucose - 6 - PO₄. This is the second step in the accepted metabolism of glycogen. This inhibition is believed to be due to the action of alloxan as an enzyme destroyer. (48)

Alloxan can be detected in the blood and in the pancreas immediately after its intravenous injection but it has all been destroyed or removed from the blood within five minutes of its injection. (47) The fate of alloxan in the body was thought by Cerecedo (14) to be a chemical combination with sulfuric acid with a subsequent excretion in the bile as ethereal sulfate. A small amount of alloxan is converted to alloxantin and excreted in the urine as murexide.

3. Alloxan and Sulfhydryl Compounds.

Labès and Friesburger were the first to suggest that alloxan might react with sulfhydryl compounds. (45) Leech and Bailey (47) discovered that non-glucose reducing substances in the blood as well as glucose were markedly decreased after the injection of alloxan into rabbits. Since glutathione, a sulfhydryl compound, comprises most of the reducing substance of the blood, an interaction is indicated. Glutathione remained below normal levels for 18-24 hours, at which time diabetes

began to appear.

The exact way in which alloxan produces islet necrosis and diabetes is not known. It has been suggested that islet cell destruction is a result of an action by the alloxan on cellular enzymes, possibly sulphhydryl enzymes, which are known to react with alloxan. (46) Bruckmann and Wertheimer (11) suggest that alloxan may act in one of three ways:

- (i) By a selective accumulation of alloxan in toxic amounts in the beta cells.
- (ii) By competing with a structurally similar compound for an enzyme with resulting disorganization of the cell metabolism and eventual destruction of the cell.
- (iii) By a specific reaction of alloxan on the islet cell, with or without an accumulation. Such a reaction would probably consist in the inactivation of an enzyme system intimately connected with insulin synthesis.

That the islet cell necrosis is due to the direct action of the alloxan was shown neatly by Gomori and Goldner (37), who temporarily ligated the arteries to one half of the dogs' pancreas, injected alloxan and then released the ligature five minutes later. This half remained normal. Hypophysectomy does not affect the destructive action, (5) (6) (31). A diet rich in lard increases a rat's sensitivity to alloxan, while protein decreases it.

4. Pathology.

The course of alloxan diabetes differs somewhat from naturally occurring diabetes in the following ways. (26)

- (i) There is a selective destruction of the B cells in the Islets of Langerhans.
- (ii) There is no effect on alpha or non-granular cells of the pancreas.
- (iii) Fibrosis or hyalinization is missing when alloxan is used as contrasted to that seen in naturally occurring diabetes.
- (iv) There is acidosis, coma, cataracts and retinal hemorrhage in the experimental diabetes.
- (v) There is temporary damage to the liver, adrenals, pituitary and kidney.
- (vi) There is no diabetic neuritis or arteriosclerosis.

5. Comparison with other diabetogenic methods:

There are three other commonly used methods for producing experimental diabetes.

- (i) Injection of anterior pituitary extract (A.P.E.)
- (ii) Depancreatization.
- (iii) Injections of glucose.

A.P.E. is effective on dogs and partly depancreatized rats and cats. A previous hyperglycemia is necessary and injections must be repeated over several days or weeks. It also acts on beta cells. Depancreatization is difficult since the gland is often very diffuse. Glucose administration is effective on cats only. Its action is very slow and again involves the beta cells.

6. Effect of alloxan on humans:

It was hoped that hyperinsulinism due to tumours or

other causes could be treated with alloxan. Unfortunately, results were poor, since alloxan is too toxic to humans. Conn (15) had no good results and found after removing the pancreas surgically that no damage had been done to tumour cells but instead the normal cells had been affected.

B. Hypertension As A Disease Of Adaptation To Stress.

1. The adaptation syndrome.

The production of nephrosclerosis and hypertension, in the absence of sexual anomalies, in experimental animals treated with D.C.A. led to the theory that a selective increase in the endogenous mineralo corticoids might be the cause of corresponding syndromes in man. Selye's concept of the diseases of adaptation was largely based upon this observation, since it is known that certain corticoids are produced in excess during adaptation and defence to any type of damaging agent.

As previously mentioned in the introduction, the problem was seen to resemble one of Selye's stress experiments. Under the influence of stress, occasioned by a variety of non-specific damaging agents, the organism responds with the general adaptation syndrome, whose manifestations are essentially independent of the specific nature of the stress employed and depend mainly on the degree and duration of the exposure. The syndrome evolves in three distinct phases, (1) the alarm reaction, (2) the stage of resistance, and (3) the stage of exhaustion. Hypertension is essentially a disease of the stage of resistance.

2. The catabolic impulse.

Certain types of hypertension are caused by excessive exposure to stress. A variety of stresses elicit metabolic changes, the so-called 'catabolic impulse', which are prominent during the shock phase of the alarm reaction. This catabolic impulse apparently influences the anterior pituitary to produce an excessive amount of A.C.T.H. The latter causes increased corticoid production by the adrenal, resulting in involution of the thymus and lymph nodes as well as certain metabolic changes, such as retention of sodium, gluconeogenesis from protein, and production by the liver of alpha 2 globulin (hypertensinogen). Directly or indirectly through their metabolic action, the corticoids also cause a renal type of hypertension with nephrosclerosis. (73)

During stress, other factors such as sympathetic stimulation, liberation of adrenalin, and possibly even a discharge of pituitary vasopressin may contribute to the raised blood pressure.

It has been found that many entirely unrelated non-specific damaging agents produce the catabolic impulse, which is characterized by a breakdown of body proteins and an increase in the concentration of protein catabolites and proteolytic enzymes in the blood. This is accompanied by loss of weight and by histological evidence of a generalized breakdown of cells. There is also characteristic adrenal-cortical enlargement, gastrointestinal ulcers, involution of lymphatic organs, etc., at this time. Prolonged stress leads to hyper-

tension and nephrosclerosis, especially in animals sensitized by unilateral nephrectomy and high sodium, high protein diets.

3. Activation of the pituitary-adrenal system.

Sayers and Sayers (62) believe that stress increases the consumption or utilization of corticoid hormones by tissues and that the resulting decrease in their blood-level causes a discharge of corticotrophin by the pituitary and is thus responsible for the cortical enlargement. There is an excessive production of corticoids at this time however, (73) (81) (82) so that there must be an increased utilization.

C.N.H. Long believes that the pituitary-adrenal system is activated by epinephrine (52) while Sayers and Sayers maintain that there is an inverse relationship between the blood levels of A.C.T.H. and A.C.H. that determines the relative secretory activity of each gland. The latter workers regard the effect of epinephrine as due to its capacity to increase the utilization of cortical hormones, thus reducing their levels in blood traversing the pituitary. In order to further his contentions, Long devised the following series of experiments.

A group of rats were hypophysectomized and three days later homologous transplants of pituitary tissue from rats of the same strain were inserted into the anterior chamber of the right eyes. The eosinophil decline in the peripheral blood was selected as an index of adrenal cortical activity and hence of A.C.T.H. release from the grafts. A subcutaneous injection of epinephrine produced such a response, as did direct injection

of epinephrine into the treated eye chamber. The same minute dose given into the chamber of the intact eye did not cause the reaction. When the animals were subjected to stress, they again responded by eosinopenia. After removal of the eye containing the transplant, epinephrine caused no response. The grafts were assayed after removal and found to contain A.C.T.H. This set of experiments proved conclusively that adrenaline has a direct stimulatory effect on the pituitary.

Either hypophysectomy or adrenalectomy prevents stress from causing hypertension or nephrosclerosis, while extracts of either gland administered in excess will cause hypertension. The hypertension of the shock phase may so decrease the blood supply of the kidney that renin production is increased as it is after the Goldblatt clamp. (33) (34) Selye has shown further that mineralo-corticoids such as D.C.A. cause hyalinization of individual glomeruli, thus diminishing blood supply in the kidney after corticoid overdosage. He believes that such hyalinized glomeruli may produce the pressor substance of hypertension.

There is a wealth of data showing that almost any type of nonspecific damage or stress causes essentially the same alarm reaction symptoms. It is noteworthy, however, that upon prolonged exposure to stress, diseases of adaptation are not always produced. Different types of stress may yield different end results, e.g. exposure to cold tends to produce nephrosclerosis, while forced muscular exercise or subcutaneous

injections of formaldehyde are more apt to cause hypertension. Yet, if the mechanism through which stress produces nephrosclerosis and hypertension is the liberation of mineralo-corticoids, it is difficult to understand why the results of stress are not invariably the same. The excess production of corticoids is a nonspecific effect of stress, but perhaps the catabolic impulse is more dependent upon the specific nature of the damaging agent and intermediate metabolism plays an important role in determining whether the excess mineralo-corticoids produced result in nephrosclerosis and hypertension. It can readily be shown that A.P.E. or D.C.A. are made more damaging after sensitization by diets rich in sodium and protein or by partial nephrectomy. Perhaps the specific metabolic effects of stress may similarly sensitize the organism to excess mineralo-corticoids.

Recent publications indicate that the diseases of adaptation develop in man in essentially the same manner as in experimental animals. Increased corticoid elimination in the urine has been demonstrated by bioassay, (81) (82) increased width and activity of the adrenal cortex has been shown by histological methods (Zamchek) and increased renin content of the renal vein has been proven in hypertensive humans. (39) In relation to the previously mentioned effect of sodium, it has been found that low sodium diets or treatment with ammonium chloride sometimes prove effective in depressing the blood pressure (72) and that diets consisting mainly of rice and fruit juices (43) likewise tend to depress the blood pressure in hypertensive

patients. It is believed that the latter do not act by means of a pressor substance but rather by their low sodium and protein content.

Metabolic Changes Induced by Administrrating A.C.T.H. & A.C.H.

1. Functions of the adrenal cortex.

At present, the functions of the adrenal cortex appear to be the following:

1. Regulation of electrolyte metabolism.
2. Regulation of carbohydrate, protein, and fat metabolism.
3. Regulation of lymphoid tissue and circulating leukocytes.
4. Androgenic function.
5. Regulation of pigmentation.

Electrolyte-regulating adrenal steroids, particularly 11 - desoxycorticosterone, are commonly referred to as sodium-retaining hormones, since this is their most conspicuous effect. The interrelation of carbohydrate, fat and protein metabolism, particularly under fasting conditions, is most effectively controlled by 11, 18 - oxysteroids and to a lesser extent by 11 - oxysteroids. The 11, 17 - oxysteroids are the most potent in promoting gluconeogenesis and in causing atrophy of the thymus, lympholysis and eosinopenia. The androgenic effect appears to be caused by hormones resembling androsterone. The factors responsible for changes in pigmentation have not been identified as yet.

It has always been the custom to discuss the function of the adrenal medulla separately from that of the cortex.

It is well known that the secretion of epinephrine is under the control of the autonomic nervous system whereas the liberation of cortical hormones is dependent upon pituitary A.C.T.H. which in turn is functionally integrated with the autonomic nervous system. Now, by histological methods, it is possible to demonstrate that the medullary hormone, epinephrine, acts as a stimulator of pituitary A.C.T.H. secretion in man.

(58) Proof that the action is a direct one was provided by Long. (See previous discussion in B-3).

D.C.A. exerts its effect by the renal retention of sodium, chloride and water, with excretion of potassium, and upon the concentration of sodium and chloride in perspiration.

(16) (38) Excessive hydremia, edema, hypertension and cardiac enlargement may be observed in both lab. animals and patients treated with excessive doses of D.C.A. (38) (78) (61) (70)

The hypertension associated with D.C.A. is enhanced by sodium chloride administration, but is not dependent solely on the increase in blood volume. (55) Recent studies by Schroeder et al. suggest that D.C.A. injected intravenously may exert a direct pressor effect, particularly in hypertensive patients.

(35) Excessive retention of sodium and depletion of potassium over long periods of time may cause tendon contractions and flaccid paralysis. It is also possible that sudden death occasionally reported in D.C.A. treated human patients may be related to the detrimental effect of a low serum potassium level on cardiac conduction and contraction. (78) Symptoms of potassium deficiency are enhanced by administration of glucose.

2. The pituitary-adrenal system.

In patients with an intact pituitary-adrenal system, stress causes a rapid depression of circulating eosinophils (30) Patients with Addison's disease and panhypopituitarism fail to show this change. (58) Because of these facts, the measurement of circulating eosinophils can be used to indicate the integrity of the pituitary-adrenal relationship, abnormal A.C.T.H. production, ability to meet a stress such as a major operation, etc. (76)---The application of stress has been correlated with epinephrine production in explaining this eosinophilic action. The role of epinephrine and of the sympathetic nervous system in the early response to stress was described some time ago by Cannon. (12) At the beginning of this century, it was noted that repeated injections of epinephrine to rabbits caused adrenal enlargement. (2) Only recently (1945), has the link been established between the increased activity of the adrenal medulla and the stimulation of the adrenal cortex under stress. Vogt (83) noted an adrenal enlargement in normal rats given epinephrine, which was prevented by hypophysectomy. Long et al (50) showed that a crystal of epinephrine placed directly on the pituitary of a rat led to adrenal cortical stimulation, characteristic of that induced by A.C.T.H. Recent studies have shown that epinephrine administered to either dogs or humans results in rapid eosinopenia (79) This depends on the pituitary-adrenal combination being entire. (58)

Epinephrine also causes increased excretion of urinary hormones. Prolonged administration, however, does not give results

in the same amount or rate as direct A.C.T.H. administration. This suggests that an early refractoriness of the pituitary has been attained. It is likely that the action of epinephrine is limited to the liberation of preformed A.C.T.H., without otherwise stimulating A.C.T.H. production.

Although there is ample evidence that epinephrine is capable of inducing increased output of pituitary A.C.T.H., it is by no means established that it is the only activator. Preliminary evidence suggests that a neural mechanism located in the hypothalamus is also activated by epinephrine and in turn stimulates the pituitary. (41) The mechanism whereby A.C.T.H. secretion is sustained for a prolonged period would not appear to be based on the continuous action of epinephrine.

D. Methods Of Studying Adrenal Cortex Activity.

In the study of the function of an endocrine gland it is important to determine the circumstances under which an increased supply of its particular hormone is made available. Both the circumstances that cause an increased secretion and the method of production must be considered. The mechanism of the biological synthesis and release of any hormone is imperfect at the present time. This is particularly true in the case of the adrenal cortex, whose characteristic hormone has only recently been synthesized in small amounts by a laborious technic.

The methods that have been developed for the determination of an increased secretory activity of the adrenal cortex are as follows:

1. Urinary Excretion.

Cortical hormones in urine are assayed either by the ability of the hormone to increase the liver glycogen of rats and mice or by its capacity to protect adrenalectomized animals against the effects of exposure to cold. By using these techniques, it has been clearly demonstrated that there is an increased urinary excretion of cortin in man after trauma, burns and infections. The large amounts of urine required limit its use to man or large animals.

2. 17 - Ketosteroids.

The excretion of 17 - ketosteroids has been suggested by some workers as an indication of the cortical activity in man. (49) (53) However there are two facts that throw discredit on this method. Firstly, the 17 - ketosteroid level in the urine does not parallel the level of cortin. Secondly, the 17 - ketosteroid excretion, at least in the male, is also complicated by the formation of these substances from the testicular hormone. Until methods are devised for more specific determination of the ketosteroids with C 11 substitution, the value of this approach will remain uncertain. However this method, along with the first, has the advantage that it can be performed without the use of operative procedures.

3. Assay of adrenal blood.

Vogt has shown recently that the blood plasma drawn from the adrenal veins of dogs contains sufficient quantities of cortical hormone to be determined by the Selye-Schenker

method of assay. In this manner he has shown a daily secretion in dogs equal to 250 c.c.'s of commercial extract. Even greater outputs were obtained by splanchnic nerve stimulation or by epinephrine.

4. Hormone Content of the Gland.

One of the most widely used methods for the study of variations in endocrine activity is the determination of the actual quantities of hormone present in the gland under different conditions. One general difficulty of this method is to decide whether a decreased content of hormone in the gland indicates a greater secretion or a decreased formation, a matter that is usually settled by actual measurement of hormone released into the blood. A special difficulty in the case of the endocrine glands is the small amount of hormone present at any one time in the gland itself. Even by using biological technics it is not possible to directly measure changes in hormone quantity in the adrenal cortex.

5. Associated Metabolic Changes.

(a) Cholesterol and ascorbic acid.

Another approach to the problem is available however. It may be assumed that increased hormone production, particularly in a gland that only has a small reserve, will be accompanied by characteristic metabolic changes associated with the synthesis and release of the hormone. This is even more evident when a precursor or an essential constituent of the hormone is present. Two instances of this are known. The first is the

relation of iodine metabolism to the thyroid hormone and the second is the presence in the adrenal cortex of two chemical substances, both of which appear to be specifically related to the formation of the hormone and both of which appear to reflect any changes in the rate of secretion. These two substances are cholesterol and ascorbic acid.

The presence of these two organic compounds is the outstanding chemical characteristic of the adrenal cortex. No other organ of the body except the corpus luteum approaches the organ in its high concentrations of both these substances. Another characteristic of the adrenal cholesterol is that approximately 90% of it is present in the gland in ester form. This is to be compared with the 50% of the ester found in the liver and the 10% found in the brain. It is generally understood that a high proportion of esterified cholesterol indicates a high rate of turnover of both the steroid and its associated fatty acids.

There has been considerable speculation concerning the possible relationship between the adrenals and the general cholesterol metabolism of the body, although no definite association has yet been shown. The fact that the amount of cholesterol in the adrenal fluctuated greatly under different circumstances was known. This latter subject has been reviewed by Sayers et al (67) who have pointed out that a lowering of adrenal cholesterol is associated with exposure of the organism to a variety of stresses.

The high concentration of ascorbic acid in the adrenals furnished the first source for its isolation in pure form. Fluctuations are again present under various circumstances and the relation of the vitamin to the natural resistance of the organism has also been suggested. (56) It has been reported that the adrenal contains water-soluble steroids in which the steroid is associated with ascorbic acid. (86) Compounds of this type which possess the biological activity of the cortical hormones have been isolated.

(b) Biogenesis of the steroid hormones.

The introduction of isotope studies has given new information concerning an old belief. The demonstration that cholesterol had a similar chemical structure to many biologically important substances had led to the hypothesis that cholesterol might undergo transformation in the body into such related substances as the steroid hormones and bile acids. Recently it has been shown by use of cholesterol tagged with deuterium that this substance is actually converted into cholic acid (8) and that in women this is followed by the excretion of pregnanediol containing the isotope in the urine. (9) This last experiment is of particular interest in relation to similar transformations in the adrenal cortex, since pregnanediol is the urinary excretion product of progesterone, a substance of very similar composition to the adrenal cortical steroids.

Rittenberg has reported experiments on mice in which a constant deuterium content of 1.5 atom percent was maintained.

After 60 days the cholesterol in these mice showed a relation between deuterium and hydrogen content which was half that found in body fluids. It was concluded that at least 22 hydrogen atoms in the cholesterol molecule are exchangeable with the deuterium of the body fluids in some step of its biogenesis. The fact was interpreted to mean that cholesterol is synthesized in the body from smaller molecules. The possibility of hydrogen-deuterium exchange reactions has been eliminated, since the amount of deuterium present in the cholesterol is greater than could thus be accounted for. All these findings suggest that cholesterol is first built up from one, two or three carbon compounds and subsequently degraded to the steroid hormones.

A comparison of the molecular structure of pregnenolone to that of any of the steroid hormones shows a possible relationship in biogenesis. If the hormones are derived from cholesterol, this compound would appear to be the logical parent substance of all steroid hormones, as it differs from cholesterol only in its side chain. Pharmacologically, pregnenolone also occupies a rather unique position in that it is the only compound known to possess all the independent steroid hormone actions. It appears that the specialization for a certain pharmacological action occurs gradually and always at the expense of other properties of the parent compound.

- (c) Effect of A.C.T.H. on adrenal cholesterol and ascorbic acid.

Studies with A.C.T.H. have proved that this pituitary

hormone caused depletion of both cholesterol and ascorbic acid in the adrenal cortex. (51) In the rat, a single injection of 1-4 mg./100 gr. is followed by a rapid fall in adrenal ascorbic acid and a slower fall in adrenal cholesterol. (63) (64) (65) (66) (67) Some twenty minutes after the injection, the adrenal ascorbic acid has been reduced by 30% and in an hour by 60%. It then begins to rise again and by the ninth hour has returned to normal. The cholesterol, on the other hand, does not reach its maximum fall until the third hour and the return to normal is prolonged until 24 hours after the injection. These effects of A.C.T.H. upon the composition of the gland are accompanied by definite indications of an increased output of the cortical hormones themselves. Both increased liver glycogen (67) and maximum lymphopenia (25) reach their height between the 6th and 9th hours. Both these effects are specific adrenal cortical hormone results.

The guinea pig, like man, is unable to synthesize ascorbic acid. It has been found that administration of A.C.T.H. to this animal when on a non-ascorbic acid diet causes a fall in ascorbic acid and cholesterol as in the previously mentioned rats but that the reformation of ascorbic acid in the gland is retarded. (64) This slow return of ascorbic acid is believed to be due to a withdrawal of the vitamin from the blood and a subsequent storage in the cells of the gland.

It should be emphasized that comparable changes in cholesterol ascorbic acid levels of other tissues do not occur

with A.C.T.H. administration. Since A.C.T.H. has no known physiological effect other than stimulation of the adrenal cortex, we may conclude that the changes in adrenal cholesterol and ascorbic acid following its injection are indicative of an increased rate of formation and release of the hormone from the gland.

(d) Adrenal cholesterol and ascorbic acid as precursors of cortical hormones.

The problem of whether or not ascorbic acid and cholesterol combine to form cortical hormones is still relatively new. The evidence is strengthened by the fact that cholesterol is known to be convertible to progesterone in man. The association of vitamin C with the cortical hormones was first suggested by Zwemer and Lowenstein.(86) These workers have isolated from the adrenal a water soluble steroid in which they believe that the vitamin C is attached to ring D of the steroid nucleus, apparently by carbon-to-carbon linkage. The compound has a high degree of instability in water and breaks down in an acid medium to form a steroid, without cortical activity, plus vitamin C.

This important claim clarifies the alterations observed in the adrenal cortex after A.C.T.H. injections. It would also account for the rapid and early depletion of ascorbic acid in response to stimulation of the gland, since this may represent the discharge of preformed hormone which is followed by a slower change in cholesterol content as new quantities of hormone are formed.

The effects of various stresses such as hemorrhage, burns, cold, muscle trauma and painful stimulation of peripheral nerves have been shown to parallel the effects of A.C.T.H. on the adrenals. Thus the falling and subsequent rising of both ascorbic acid and cholesterol proceeds at a very similar rate in the two types of experiments. (67) (57) (38) It is now generally accepted that one of the effects of stress is an increased demand for cortical hormones and that the initiation of the gratification of this need is an increased A.C.T.H. output. Evidence for this latter fact can be obtained from a comparison of the effects of stress on normal and hypophysectomized animals. (51) (80) These experiments also illustrate the rapidity of the adrenal response, since changes can be easily discerned within half an hour after the stress has been applied.

Since the agents that cause depletion of adrenal ascorbic acid and cholesterol are so diversified, the question of what common denominator is possessed by such stimuli arises. That the alterations in adrenal composition depend on a preliminary activation of the pituitary has been mentioned previously. Three mechanisms may control the secretory activity of the anterior pituitary in the liberation of A.C.T.H. These are (i) excitation of a nervous secretory mechanism (ii) changes in composition of the blood traversing the blood, causing secretion (iii) the level of cortical hormones in the blood. The latter view is held by Sayers and Sayers (68) who found

that a previous administration of cortical hormones to rats exposed to cold for one hour prevented the usual fall in adrenal ascorbic acid.

The question of a direct nervous control of anterior lobe secretion is a debatable one at the moment, but there is definitely one type of nervous activity associated with all of the various stresses. This is an excitation of the sympathetic nervous system and the release of its specific hormone, epinephrine. Long (51) has investigated the results of injections of epinephrine on the adrenal and has found that there is a fall of both ascorbic acid and cholesterol in intact but not in hypophysectomized rats. (Epinephrine still retains its glycogenolytic effect in hypophysectomized animals). These experiments would indicate that the effect of epinephrine on the adrenal constituents is also mediated by the pituitary. (84) If the activation of the adrenal depends on release of epinephrine, then animals with complete adrenal denervation or demedullation should have difficulty in meeting stress. This may be so, as the work of Wyman suggests (85) although such animals are not very sensitive to stress. It is not to be implied that the pituitary is innervated by the sympathetic nervous system but that the epinephrine acts as it does on skeletal muscle, by giving a striking metabolic effect.

E. The Chemical Cytology Of The Pituitary and Adrenals.

The ideal goals of chemical cytology are similar to those of biochemistry - to ascertain qualitatively and quantitatively

atively the events of the various metabolic processes. Cytology possesses the advantage of permitting exact location of various metabolic substances. Even non-specific methods may yield valid results if morphological changes can be related to physiological states.

1. The pituitary gland.

Considerable effort has been made to correlate the cytological appearance of the cell types with different physiological states. (1) (74) Two kinds of granular cells have been described, the acidophils and basophils, and one nongranular type, the chromophobes. In humans, these are in the percent ratio of 37:11:52 respectively. (54) The number may change with age, sex, sexual maturity, etc. The cells are classified by their staining behaviour when stained by one of the triacid procedures. Thus basophils stain with anilin blue, acidophils with acid fuchsin or azocarmine and chromophobes reject these dyes. The cell types were first recognized by Schonemann (71) after staining with either eosin-methylene blue or hematoxylin and eosin. Recently (1945) the basophilic cells of the anterior lobe have been investigated by Desclin (24) and Dempsey and Wislocki (23) who found that digestion of sections with ribonuclease destroyed the cytoplasmic basophilia. Thus the basophils apparently contain a considerable quantity of cytoplasmic ribonucleoprotein. In many other locations such a concentration of ribonucleoprotein occurs in cells engaged in rapid protein synthesis. Consequently it seems likely that the pituitary basophils are engaged in rapid protein formation.

Although the chemical natures of most of the protein hormones of the anterior pituitary are not known in detail, it has been established that several of them contain a high percentage of carbohydrate residues such as glucosamine and therefore may be classified as glycoproteins. Attempts have been made recently to locate the site of hormone production by identifying the carbohydrate residue of these glycoproteins. Catchpole (13) utilized periodic acid to free the carbohydrate which was then stained with Schiff's leucofuchsin reagent. By this procedure some of the cells of the anterior pituitary were differentially stained. Similarly, Dempsey has succeeded in impregnating such carbohydrates with silver after alkaline hydrolysis and oxidation. (21) Such methods have only recently been introduced and hold great promise for future work.

There has been a lack of agreement regarding the specific cell type in the anterior hypophysis which is responsible for the secretion of A.C.T.H. (1) (75) Post mortems from Addison's disease and from terminal stages of total adrenalectomy reveal diminution and degeneration of both acidophils and basophils. (44) Crooke and Russell (17) state that the constant features in pathological adrenal insufficiency are the extreme decline in the number of basophilic cells, a variable reduction in the number of acidophils, the presence of a series of abnormal basophilic cells and large chromophobes. The possibility that such a disease might be primary in the hypophysis or in the adrenals with opposite appearances in the cytology of

the hypophysis must always be considered. Reese et al (59), studying pituitaries of bilaterally adrenalectomized rats found diminution in number and size of acidophils correlated with a progressive loss of granules and regressive changes in the Golgi apparatus. The question may be raised as to whether the changes were a result of a modification in pituitary-adrenal relationship or of abnormal metabolism of the body as a whole. (69)

Conversely, Heinbecker and Rolf (40) in studies on the dog found that following hypophysectomy, progressive atrophy of the adrenal cortex occurred. Studies were also made on "puncture" dogs in which the infundibular stem and the fibers running caudally from the paired paraventricular nuclei in the hypothalamus were severed. Under such conditions the acidophils are maintained, the basophils decrease in number, the adrenal cortex remains normal, while the gonads and thyroids atrophy. This suggests that the acidophils may be the source of A.C.T.H. while the basophils are the source of F.S.H. and thyrotrophin. However Albright and Elrick (1) attribute only F.S.H. to the basophils. Daughaday et al (18) correlate the acidophils with A.C.T.H. production because of increased adrenal function in acromegaly, a condition usually caused by acidophil tumours of the hypophysis.

The response to unilateral adrenalectomy is compensatory hypertrophy of the remaining gland. Since this does not occur in hypophysectomized animals it appears to be dependent

upon an increased A.C.T.H. production. Under the former conditions there is a concomitant rise in acidophils. (1) (29) Hypertrophy is increased by stress, which also increases the acidophils.

2. The adrenals.

A relatively large number of cytochemical components have been detected in the adrenals. On gross examination the gland does not appear to stain heavily with basic dyes but on microscopic examination it can be seen that the lipid droplets of the cells compress the cytoplasm into thin, heavily basophilic strands. This cytoplasmic basophilia is more intense in the peripheral zones of the gland than in the reticularis, where the protoplasmic strands are distinctly eosinophilic. This observation suggests the presence of nucleoproteins and of rapid protein synthesis.

The adrenal cortex contains considerable amounts of alkaline phosphatase. After hypophysectomy, phosphatase rapidly disappears from the parenchyma but increases in the endothelium for a time before gradually decreasing. Elftman (28) has described sex differences in the distribution of phosphatase in the adrenals of mice, and has shown that injections of sex hormones modify its location.

Ascorbic acid has been demonstrated in the adrenal cortex by virtue of its reducing power in acid solutions (32). This is important from a physiological viewpoint in that it is linked metabolically with the production of cortical hormone.

(51)

The lipid content of the adrenal cortex has received intensive study. The sudanophilic and birefringent properties of the lipid droplets have led to the revelation that a high concentration of cholesterol is present in the adrenal cells. More recently, the identification of the adrenal hormones as steroids has stimulated interest in locating the active compounds in the adrenal gland. In 1940, Bennett (7) showed that lipids located in the outer layer of the fasciculata in the cat's adrenal cortex exhibited a number of reactions similar to those demonstrated by active hormones in chemical experiments. These were (i) acetone solubility, (ii) reactivity with semicarbazide and phenylhydrazine, i.e. ketone reactions, (iii) reduction of alkaline silver salts, and (iv) formation of digitonides. The lipids in other regions do not exhibit this array of reactions. Later, (1943), in attempting to locate the steroids of the ovary, Dempsey and Bassett (20) defined the following histochemical characterizations of ketosteroids: (i) acetone solubility, (ii) reactivity with carbonyl reagents, (iii) autofluorescence, (iv) birefringence, and (v) reactivity with concentrated sulfuric acid as in the Liebermann - Burchardt reaction. Each of these reactions depends upon a different physical or chemical property of the steroid molecule. No single one of them is specific for ketosteroids, but no substance other than ketosteroids is known to exhibit all five reactions. The most extensive research of this nature has been made on the rat by Deane and Greep (19). In the rat, both the glomerulosa

and outer fasciculata react positively with the entire series of ketosteroid procedures. After hypophysectomy, the ketosteroids of the fasciculata disappear while those of the glomerulosa remain. Conditions leading to adrenal stimulation (exposure to cold, noxious agents, certain vitamin deficiencies) cause augmented ketosteroid reactions in the fasciculata but do not alter the glomerulosa. Injections of corticosterone cause a disuse atrophy and subsequent disappearance of the ketosteroids of the fasciculata. On the other hand, the glomerulosa becomes atrophic and depleted of ketosteroids after injections of desoxycorticosterone into either intact or hypophysectomized rats. Since after hypophysectomy carbohydrate metabolism is markedly deficient but salt regulation is relatively unimpaired, these experiments point towards a separate site of origin of these two principles. The evidence supports the hypothesis that desoxycorticosterone - like compounds which regulate electrolyte balance are formed independently of the pituitary in the zona glomerulosa, and the 11 - oxygenated steroids which regulate carbohydrate metabolism are formed under the influence of the pituitary gland in the zona fasciculata.

In addition to the steroids, other lipids occur in the adrenal cortex. This material is mostly neutral fat with some fatty acids. The reticularis also contains variable quantities of brownish yellow pigment which is not readily extractable from the tissues. Little is known concerning the nature of these cytochromes.

The methods for identifying specific lipids in tissue sections leave much to be desired. The characteristic solubilities of lipids in non-aqueous solvents are used frequently, but since lipids enter into both loose and firm combinations with proteins their solubilities are not entirely trustworthy indices. They may be characteristically stained with Sudan dyes - compounds possessing no polar groups which stain by virtue of their solution into the lipid of the tissue.

The first of these oil soluble dyes was Sudan III, introduced by Daddi in 1896. Sudan IV, was later introduced by Michaelis in 1901 and has since been the most popular of the oil soluble dyes.

Some lipids possess active groups which aid in their detection and identification. Such compounds as phospholipins and steroids have such distinguishing features. The steroids exhibit a number of characteristic features. (21) Many of the biologically active compounds contain ketone groups and react with carbonyl reagents such as leucofuchsin and phenylhydrazine. The steroids form spherocrystals which are strongly birefringent. When treated with concentrated sulfuric acid together with dehydrating agents such as acetic anhydride, a characteristic play of colours results. This phenomenon depends on the unsaturated nature of their polycyclic ring structures. In Schultz's method for determining cholesterol, the Liebermann - Burchardt sterol reaction has been adapted to cytology. When irradiated with ultraviolet light, some steroids fluoresce, usually a stable

green colour. Under polarized light, the long rhomboidal crystals of cholesterol light up and are extinguished alternately once in each 90° of rotation. Finally, steroids are readily soluble in acetone or alcohol and it can, therefore, be determined if each of the above phenomena is destroyed by dissolving out the lipids. No one test is specific for steroids, but no other substance displays the entire battery of reactions.

(7) (20) (22)

F. Histology Of The Adrenal Gland.

The adrenals were first described by Eustachius in 1856. More than 300 years later, Vulpian discovered that the cells of the medulla differ in their staining ability from those of the cortex. He noted that if a slice across an adrenal is immersed in ferric chloride solution, the medulla takes a greenish tinge, while the cortex does not. Subsequently, in 1865, Henle discovered that certain granules in the medulla gave a rust coloured precipitate with dilute potassium bichromate, the so called chromaffin reaction.

The histological structure of the adrenal medulla is quite different from that of the cortex. Differentiation is not difficult, although cell cords of the cortex may reach deep into the medulla and vice versa. The medullary cell is polygonal and measures 18 - 30 microns, with a vesicular nucleus about 7 microns in diameter. One of the most characteristic features of the medullary cells is their chromaffinity, which is a good index of their adrenaline content.

The cortex consists of three structurally different layers. The external zone, immediately under the connective tissue capsule, is called the zona glomerulosa. It contains small, irregularly arranged cells that contain scanty cytoplasm. The nuclei are small but rich in chromatin. The next layer is the zona fasciculata, which consists of regular rows of large polygonal cells with vesicular nuclei. The cell columns are arranged radially, with sinusoids between them. The walls of these sinusoids are studded with reticulo-endothelial cells. The zona reticularis, the innermost layer of the cortex, consists of irregular strands which form a network of small cells with dark nuclei. Many melanin and iron-pigment containing phagocytes are found in this zone. Although there is considerable variation, the zona fasciculata is always the widest layer.

Because of the numerous lipid granules, the cortical cells, especially in the fasciculata, have a vacuolized cytoplasm and are called spongiocytes. Around the central vein, the cortex is invaginated towards the medulla.

The regeneration of the cortex is believed by some to occur from the outer surface inwards, but this view is not unanimously accepted.

Islets of lymphatic tissue are often found within the adrenals, especially under certain pathological conditions such as Addison's disease.

The adrenal arteries enter the gland at various

points on its surface and the vein emerges at the hilum. All of the blood flows thru the cortex before reaching the medulla, so that the latter is exclusively supplied with venous blood, maximally saturated with the metabolites of the cortex. There are important muscular sphincters in the wall of the central vein. Their periodic contraction and relaxation helps to collect and release the hormone saturated blood in accordance with the requirements of the organism.

III. Methods.

As mentioned previously, the rats were treated initially by Dr. Chute in Toronto. All animals, including the controls, received a diet of known protein and salt content. The rats are identified as follows: (for example PS 3 B 11).

PS = 40% protein, 10% salt diet.

B = subgroup in the experiment.

11 = the number of the experiment.

3 = the number of the rat in the sub-group.

The subgroups are as follows:

A = Alloxanized diabetic, weighed diet only.

B = Alloxanized diabetic, weighed diet plus insulin.

C = Normal control, ad lib diet.

D = Alloxanized diabetic, ad lib diet.

E = Alloxanized diabetic, ad lib diet plus insulin.

F = Alloxanized, not diabetic, ad lib diet.

G = Alloxanized diabetic, weighed protein and salt diet plus ad lib normal diet.

CO = Alloxanized intravenously with kidney pedicles clamped for 10 minutes before and twenty minutes after the injection, with hot saline packs applied to the abdomen.

From the above subgroups it can be seen that various methods were used to ensure that the rats ingested the given salt and protein. The CO group was used to determine whether or not the kidneys were affected directly by the alloxan.

The animals were placed on test at approximately two months age and kept there for 3 - 4 months before being sacrificed or dying spontaneously.

The livers, kidneys and heart were examined in the Toronto laboratory, while the adrenals, pituitaries and brains were studied at the University of British Columbia.

The autopsies performed here followed this procedure: the hypophysis was removed by raising the calvarium, lifting the brain upward and backward to expose the optic tracts and adjacent areas; dura surrounding the gland was carefully removed and the gland was freed from the cranial vault.

Both pituitaries and adrenals were examined grossly for signs of hemorrhage, degeneration, etc. The adrenals were weighed to find signs of hyperplasia, hypoplasia, atrophy, hypertrophy, etc.

The adrenals were examined microscopically using three types of stains.

1. General stains such as hematoxylin and eosin were used to show tissue changes, hemorrhages, and the number of mitosis.
2. Trichrome connective tissue stains were used to show connective tissue infiltration and thickening of walls of blood vessels.
3. The most important stains used were the fat stains, the stain of choice being Sudan IV. It was used to stain the cholesterol of the adrenal cortex to indicate the metabolic state of the hormone-producing

cells. The method, adapted from Lillie, used the saturated isopropanol method for Sudan IV. The adrenals were sectioned on a freezing microtome at 15 microns, stained in Sudan IV and hematoxylin and mounted in Apathe's Gum Syrup.

The pituitaries were stained with hematoxylin and eosin as a general stain. Sections were also stained by the Briseno-Castrejon method for differential cell analysis. (10) Azocarmine staining of acidophil granules is excellent for sharp differentiation. Prestaining of the nuclear membranes by a short immersion in alum hematoxylin brings out the nuclei sharply, making counting much easier. The complete procedure for the rat hypophysis is as follows:

1. Fix in Zenker-formol, 6 - 12 hours.

Potassium bichromate 25 gr.

Mercuric bichloride 50 gr.

Ringer's solution 1000 ml.

Add 1 c.c. neutral formalin 10 ml. solution before using.

2. Wash 6 - 12 hours in running water.

3. Dehydrate in alcohols:

30, 50, 70, 80, 95% - $\frac{1}{2}$ hour each.

100% - 2 changes - 1 hour each.

4. Clear:

(a) absolute alcohol - cedarwood oil (equal parts),
1 hour.

(b) cedarwood oil, 1 hour.

- (c) xylene, 15 min.
5. Infiltrate in hard paraffin (56 - 58° C).
 - (a) 1 change into paraffin (5 min.).
 - (b) 3 changes ($\frac{1}{2}$ hour each).
 - (c) embed.
 6. Section at 4 microns.
 7. Remove paraffin with xylene, 2 changes, 3 min. each.
 8. Two changes absolute alcohol, 3 min. each.
 9. Alcohol, 95%, 3 min.
 10. Distilled water, 3 min.
 11. Lugol's solution (4 I: 6K I: 100 H₂O), 3 min.
 12. Sodium thiosulfate, 0.5% aqueous, 3 min.
 13. Distilled water, 3 min.
 14. Alum hematoxylin, 30 sec.
 15. Tap water wash.
 16. Distilled water, 3 min.
 17. Alcohol, 80% 3 min.
 18. Anilin alcohol, 15 min.

aniline oil	1 ml.
95% alcohol	1000 ml.
 19. Stain in azocarmine G at 60°C, 45 min.

azocarmine G	1 gr.
Distilled water	100 ml.

Warm and allow to cool to room temperature.

Filter with filter paper (all day).

Add glacial acetic acid, 4 ml., to filtrate.

Place in oven 1 hour before using.

20. Rinse in distilled water.
21. Differentiate in anilin alcohol (solution 18)
2 - 3 min.
22. Wash in acid alcohol, $\frac{1}{2}$ - 1 min.

Glacial acetic acid	10 ml.
Alcohol, 95%	1000 ml.
23. Phosphotungstic acid, 5%, 1 hour.
24. Dehydrate in alcohols:

Alcohol 70%	2 min.
Alcohol 95%	2 min., not longer.
Absolute alcohol	2 min. or more.
25. Counterstain in acid green solution, 5 min.

Acid green	0.1 gr.
Orange G	0.5 gr.
Clove Oil	100 ml.

Use fresh stain for each 20 slides.
26. Xylene, 1 min.
27. Two changes xylene, 30 min. each.
28. Mount.

After the above staining, alpha cell granules are purplish red, beta cell granules are light green, nuclear membranes are well defined, mitochondria are orange-red, red blood cells are brilliant orange, Golgi apparatus shows as a negative image, chromophobes show little or no cytoplasm, which is colourless to pale green.

The counting method was modified from Rasmussen,

1922. The rat pituitary is sectioned in a horizontal plane. All the cells are counted in every fifth field in each of three horizontal sections at equidistant levels in the gland. Most variation in cell percentage exists between the central and peripheral regions, which are both sampled adequately by a center section. About 1200-1400 cells can be counted thus, using one central section of the pituitary gland. The use of various colour filters facilitates counting.

The type of fixation of the hypophysis markedly influences the percentage of acidophils, so it is important to consider this factor in comparing the cell counts of different investigators. Fixation by different techniques can be divided into 3 groups: those containing mercuric bichloride, without acetic acid (Zenker - formol); those without mercuric bichloride but with acetic acid (Bouin's); and those which contain osmic acid. Each has its own advantages and disadvantages. The Zenker - formol type gives excellent colour differentiation in subsequent staining. It can be criticized against its use in cell counts in that it preserves mitochondria, which are difficult to distinguish from acidophil granules. Bouin's gives the least cellular distortion but does not provide as sharp tinctorial differentiation. The acetic acid contained therein dissolves out mitochondria from the scene. Osmic acid methods are excellent for cytological detail, especially for Golgi apparatus, but are of little use in differential counts because of variability and tissue damage.

The type of fixation seems to be fundamental in determining the approximate control value as reported by different investigators. Thus Wolfe, using Regaud's found 32-40% alpha cells, as did Pfeiffer, Finerty and Briseno, using zenker - formol; Finerty, Meyer and Marvin, using Bouins, found 24 - 27%.

IV Results:

Rat No.	Adrenal Wt. in mgm.	Gross Observations.
PS 3 A 11	27	
7	41	
9	32	
12	31	
13	34	
14	20	
16	22	
17	29	
18	<u>28</u>	
Average	<u>30</u>	
PS 2 B 11	34	
3	27	
4	--	
5	52	Very fat.
7	54	Pituitary hemorrhage.
8	35	
10	42	
11	25	
12	27	
13	31	
14	<u>56</u>	
Average	<u>38</u>	
PS 1 C 11	34	
2	16	

Rat. No.	Adrenal Wt. in mgm.	Gross Observations.
3	39	
4	20	
5	39	
6	<u>17</u>	
Average	<u>27</u>	
PS 2 D 11	35	
4	45	Small hemorrhage.
5	--	
6	30	
7	52	
8	43	Dark discolouration.
10	38	Spotty discolouration.
11	40	
12	<u>24</u>	
Average	<u>38</u>	
PS 1 F 11	31	
3	33	
4	40	
6	50	
7	44	
8	37	
9	<u>31</u>	
Average	<u>38</u>	
PS 1 G 11	35	
2	33	
3	38	

Rat. No.	Adrenal Wt. in mgm.	Gross Observations.
5	36	
6	<u>49</u>	
Average	<u>38</u>	
S 1 D 10	20	
3	25	Dark discolouration.
5	24	Hemorrhage.
9	44	
12	30	
13	<u>30</u>	
Average	<u>28</u>	
S 2 E 7	35	
5	<u>45</u>	
Average	<u>40</u>	
C O 10	50	
12	30	
15	40	
23	36	Hemorrhage.
29	--	
41	32	Dark discolouration.
46	<u>42</u>	
Average	<u>39</u>	

2. Small hemorrhages were frequent both in the pituitaries and adrenals of all experimental subgroups except the controls.

(Fig. 4) A more common occurrence was the engorgement of the

blood sinuses of these glands to about twice their normal width. (Fig. 5).

3. Several classical examples of severe arteriosclerosis were seen. Two examples of endarteritis obliterans are shown, one in the adrenal cortex (Fig. 1) and the other in an unrelated part of the body, the salivary gland (Fig. 2). Proliferation of the media with complete disappearance of the elastica interna was common in the brain. (Fig. 3)
4. Mitotic figures were surprisingly rare. Chromatin granules, which some authors refer to as prochromosomes, were abundant in both glands. (Fig. 8).
5. All experimental animals except the controls and groups F & A showed severe depletion of cholesterol in the adrenals. Fig. 6, 7 and 8) Groups F & A had only a relatively small drop in cholesterol. It is important to note that there was no apparent difference in the extent of this decrease when comparing alloxanized animals with those that had been alloxanized and given insulin therapy. The CO group also showed the same drop in cholesterol content. Cells in which the cholesterol was in the process of being utilized more quickly than in the normals were easily discernable due to the fact that as the cholesterol was removed from its spherical globule there was left behind a characteristic crescent shape (Fig. 7 and 8) before the cholesterol disappeared completely.
6. The following table shows the percent ratio of the three

cell types in the pituitary as shown by the Briseno-Castrejon method. (See Fig. 9)

Rat. No.	% Ratio.		chrom.
	alpha	beta	
PS 3 A 11	47	6	47
9	46	7	47
PS 3 B 11	52	7	41
7	67	6	27
8	54	7	39
PS 1 C 11	41	7	52
3	40	7	53
4	40	7	53
PS 2 D 11	50	7	43
4	54	6	40
PS 1 F 11	43	6	51
3	45	6	49
PS 2 G 11	55	6	39
6	53	7	40
S 1 D 10	56	6	38
3	52	7	41
6	56	7	37
13	55	6	39
S 5 E 7	57	6	37
CO 10	46	7	47
15	70	6	24
23	83	4	13

7. A comparison of the percent increase in adrenal weight with the percent increase in pituitary acidophils is given in the following table:

Group	% Increase in Adrenal Wt.	% Increase in Pit. acidophils
PS - A	11.1	15
PS - B	40.7	45
PS - D	40.7	30
PS - F	40.7	10
PS - G	40.7	35
S - D	3.7	35
S - E	48.1	43
CO	44.4	65

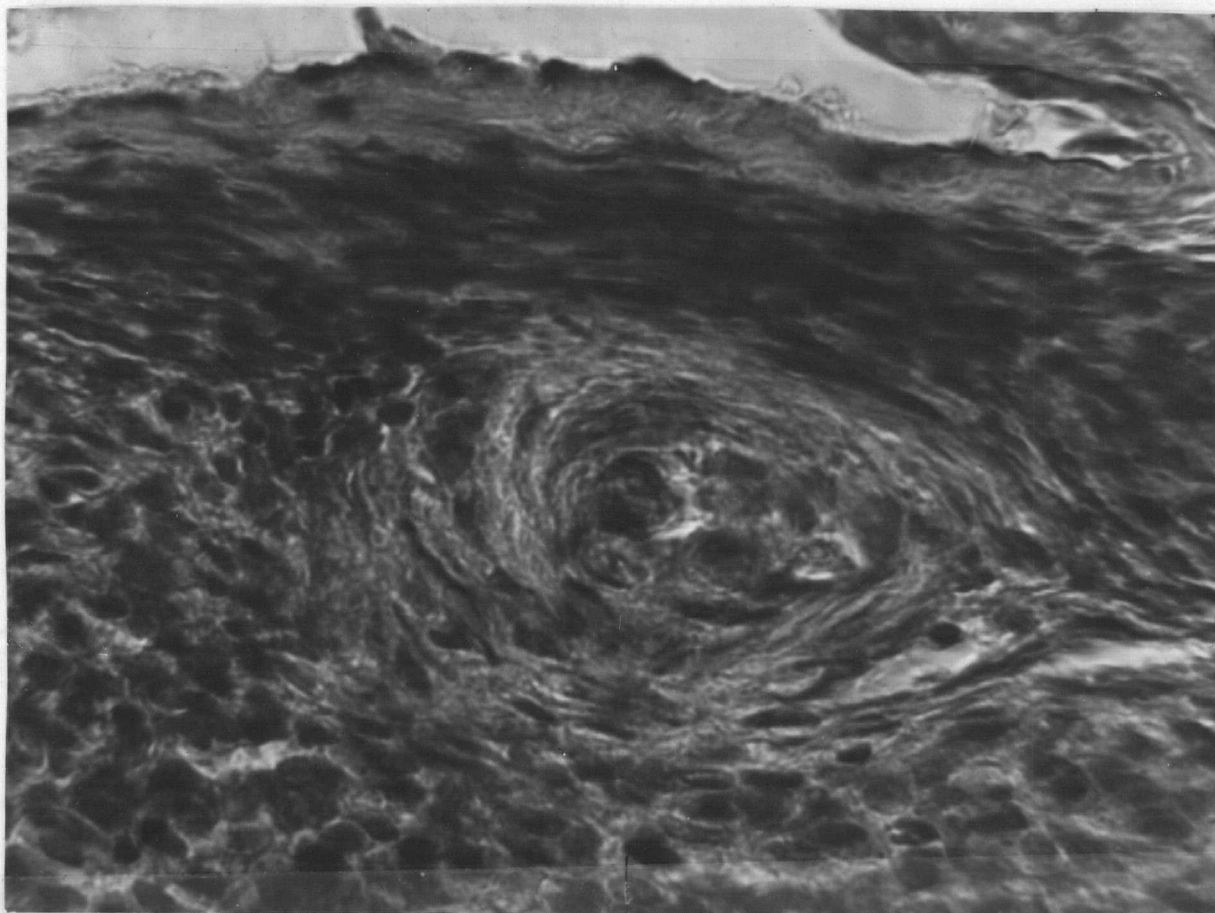


Fig. 1.

Endarteritis obliterans in an artery of the adrenal cortex of rat PS 12 A 11. Note the size of the lumen, which is almost non-existent. x 1000.

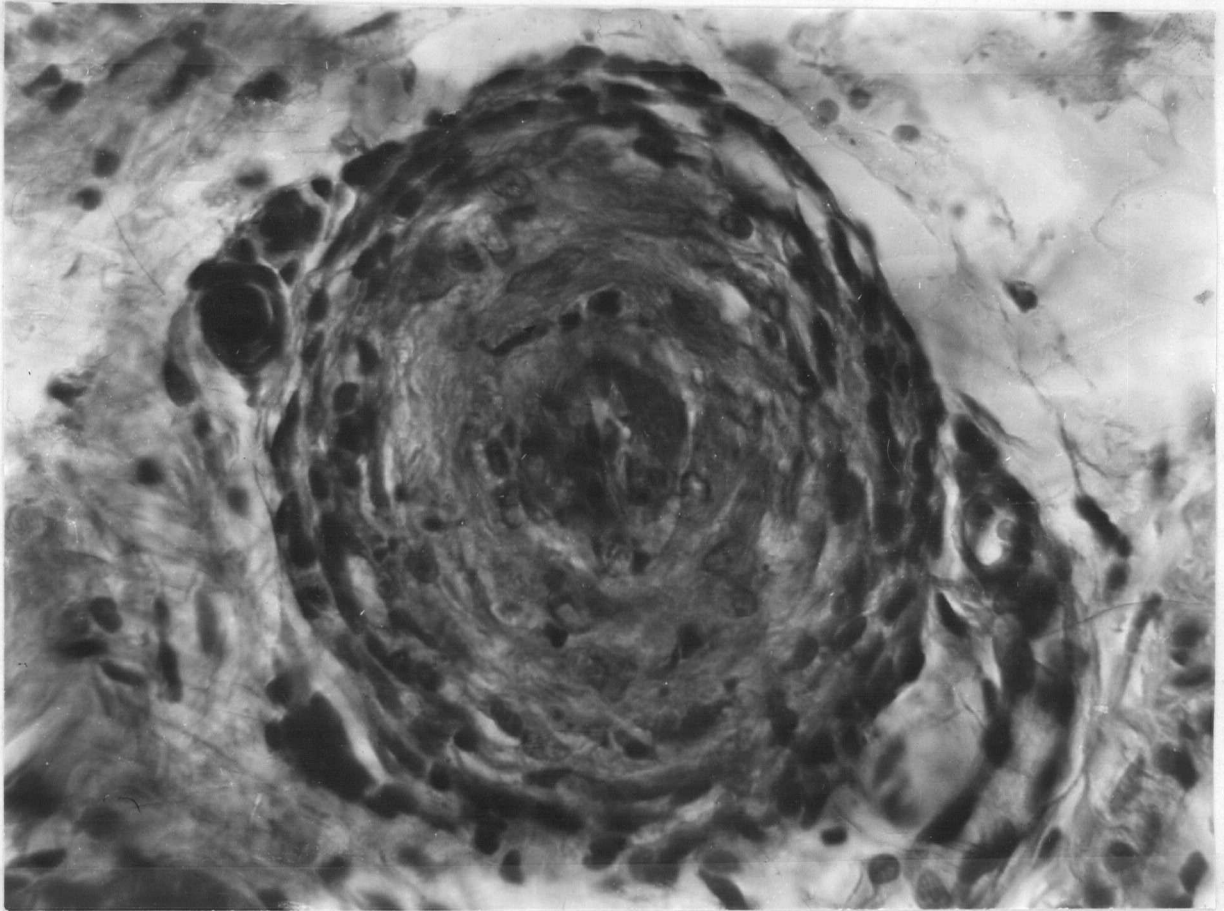


Fig. 2.

Endarteritis obliterans in the salivary gland of rat S 9 D 10, showing the laminated nature of the occluding tissue. x 1000.

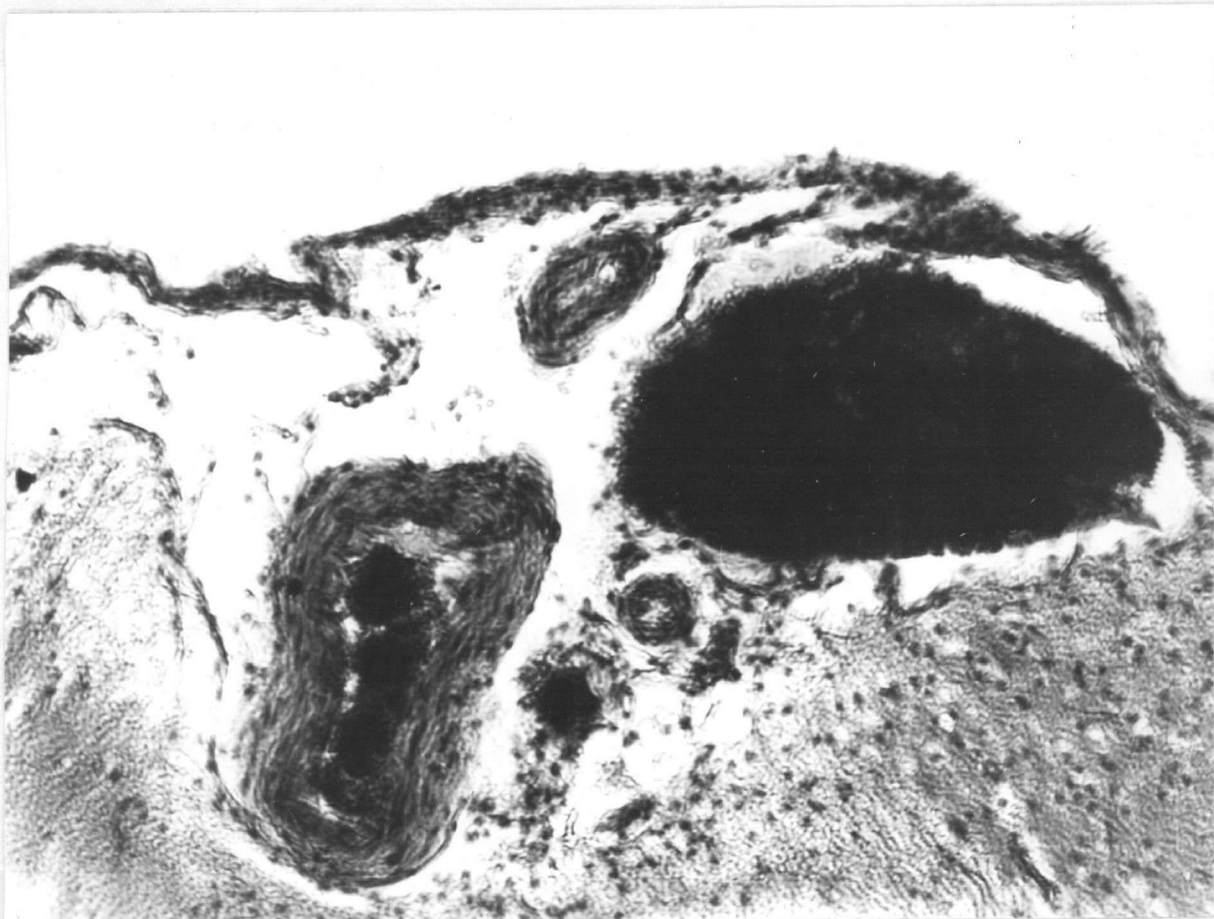


Fig. 3.

Proliferation of the media in three arteries of the cerebral cortex of rat S 3 D 10. Note the disappearance of the elastica interna. x 440.

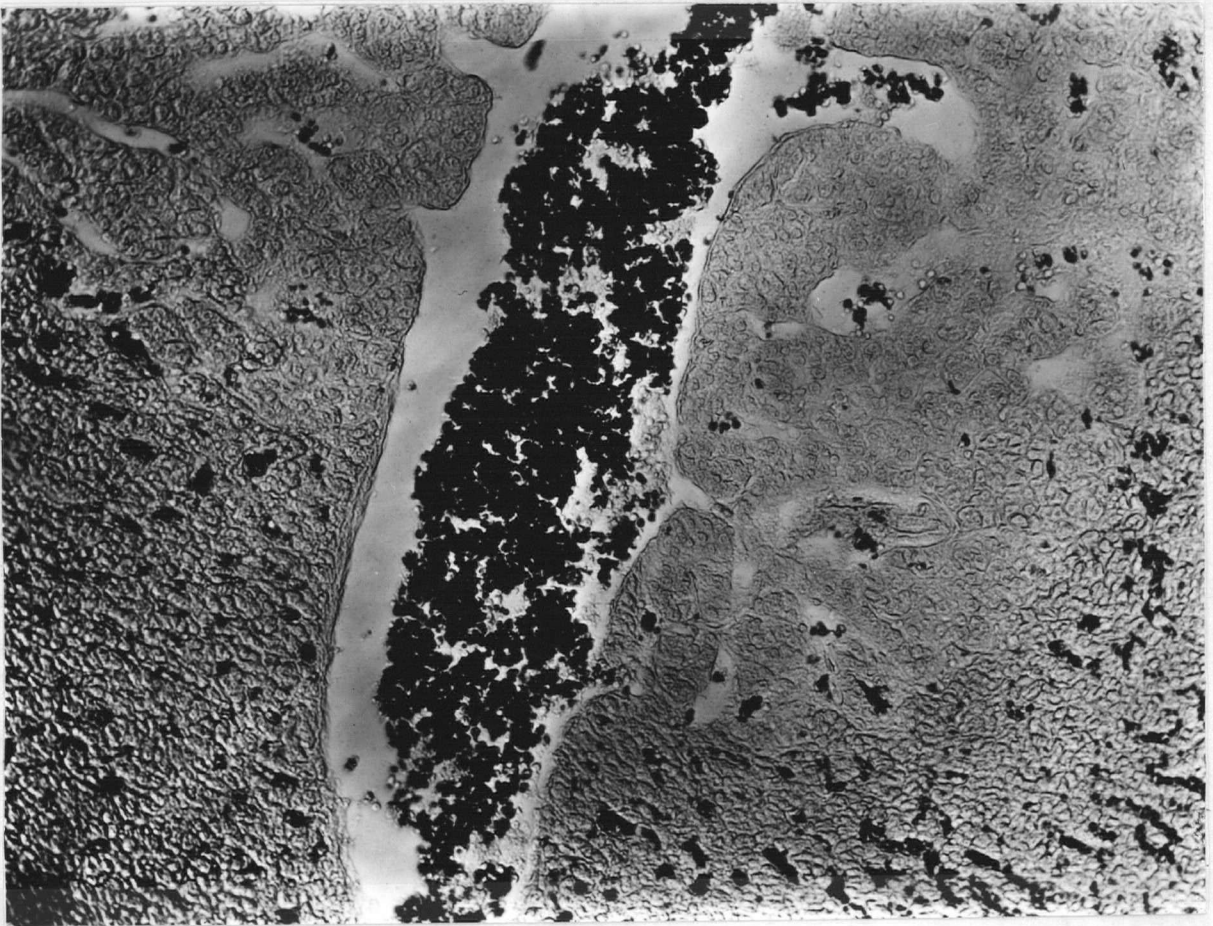


Fig. 4.

Large internal hemorrhage in the adrenal cortex of rat PS 12 A 11. The hemorrhage was situated across the junction of the zona fasciculata and the zona reticularis.

x 800.

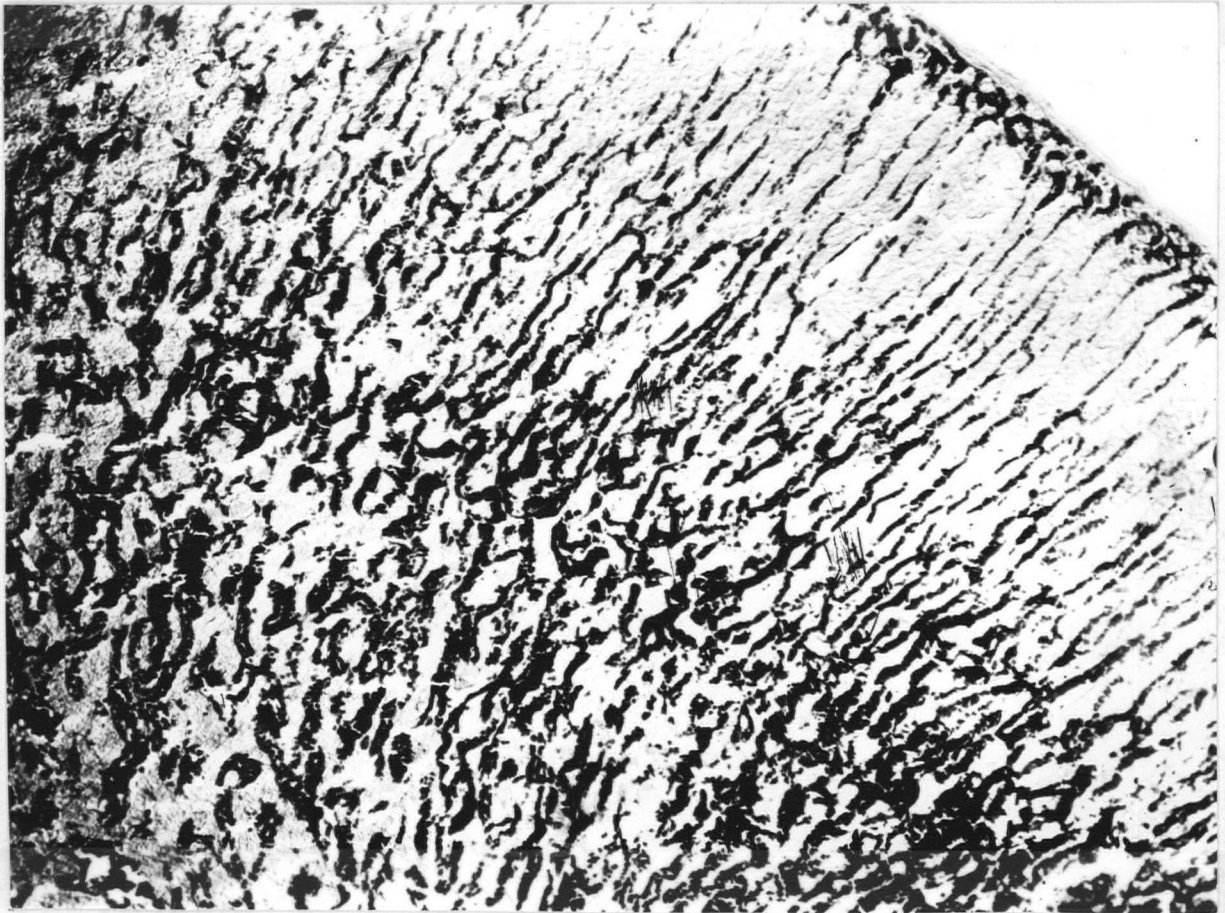


Fig. 5.

A low power view of the adrenal cortex of rat PS 5 D 11 showing the greatly expanded blood sinuses. Note the relative distribution of blood to the three layers. (Mallory-Heidenhain rapid connective tissue stain). $\times 180$.

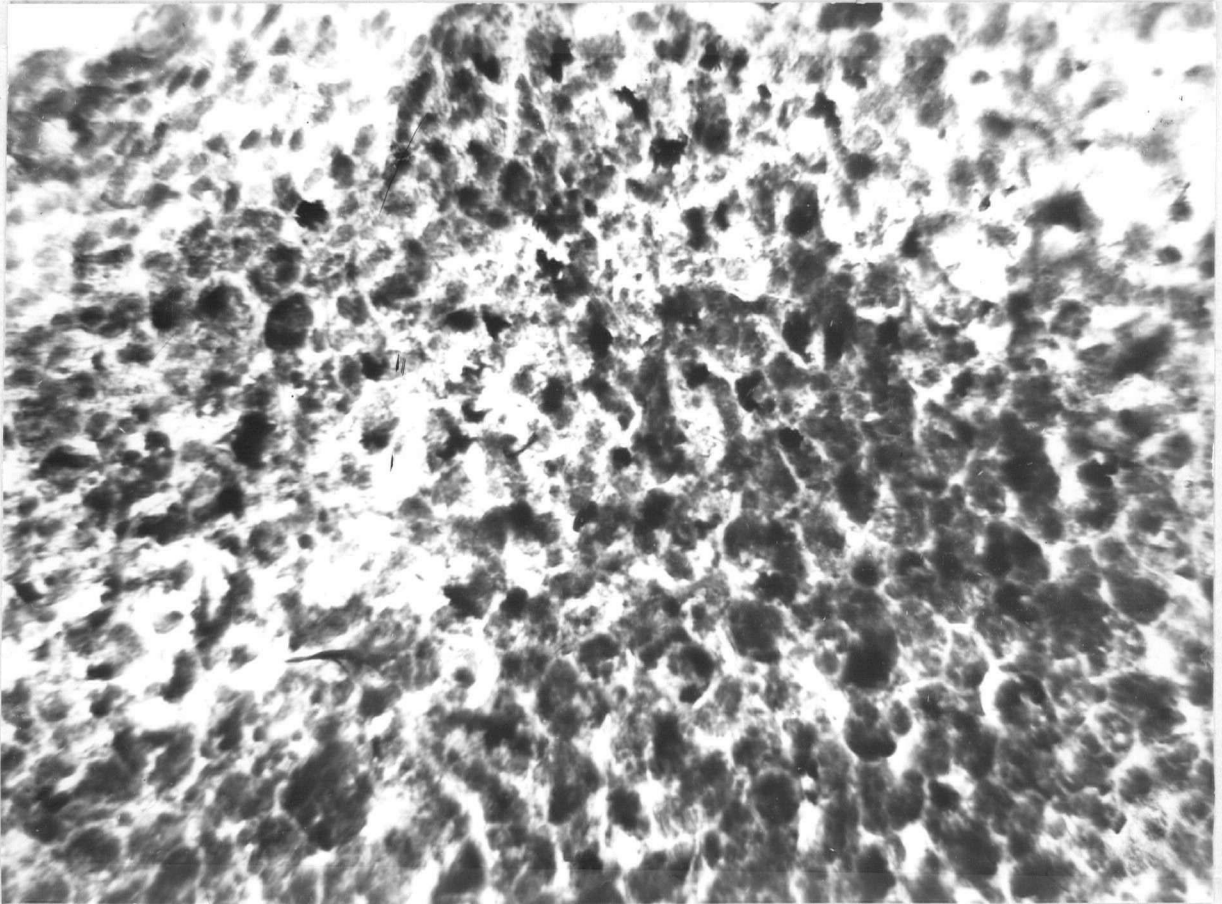


Fig. 6.

Low power view of the adrenal cortex of rat PS 4 C 11 stained with Sudan IV in isopropanol to show the normal distribution of lipid material. x 400.

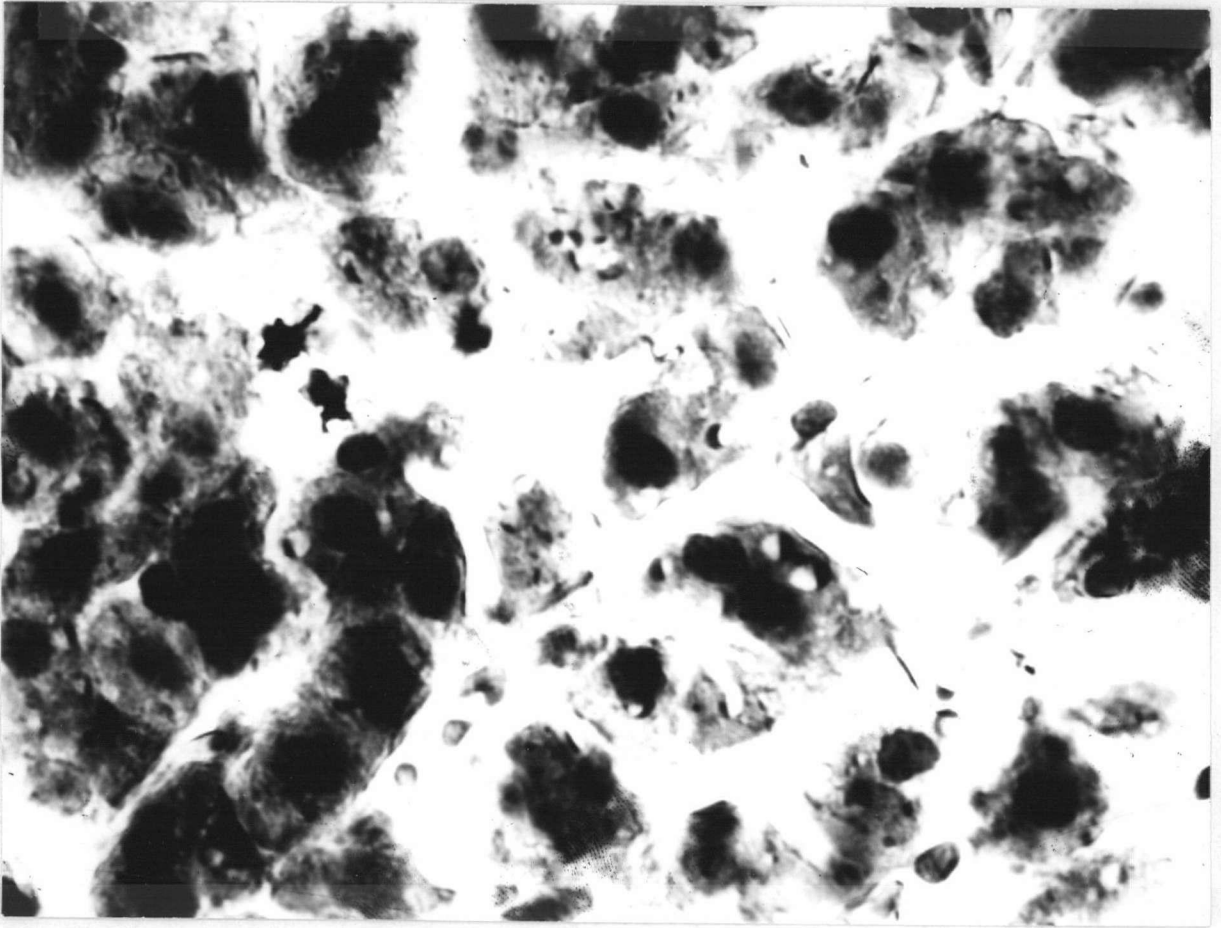


Fig. 7.

Greatly magnified view of nearly exhausted adrenal cortex of rat PS 5 G 11 stained with Sudan IV. Note the typical crescent shaped structure formed as cholesterol is utilized from the globule in which it is stored. x 3500.

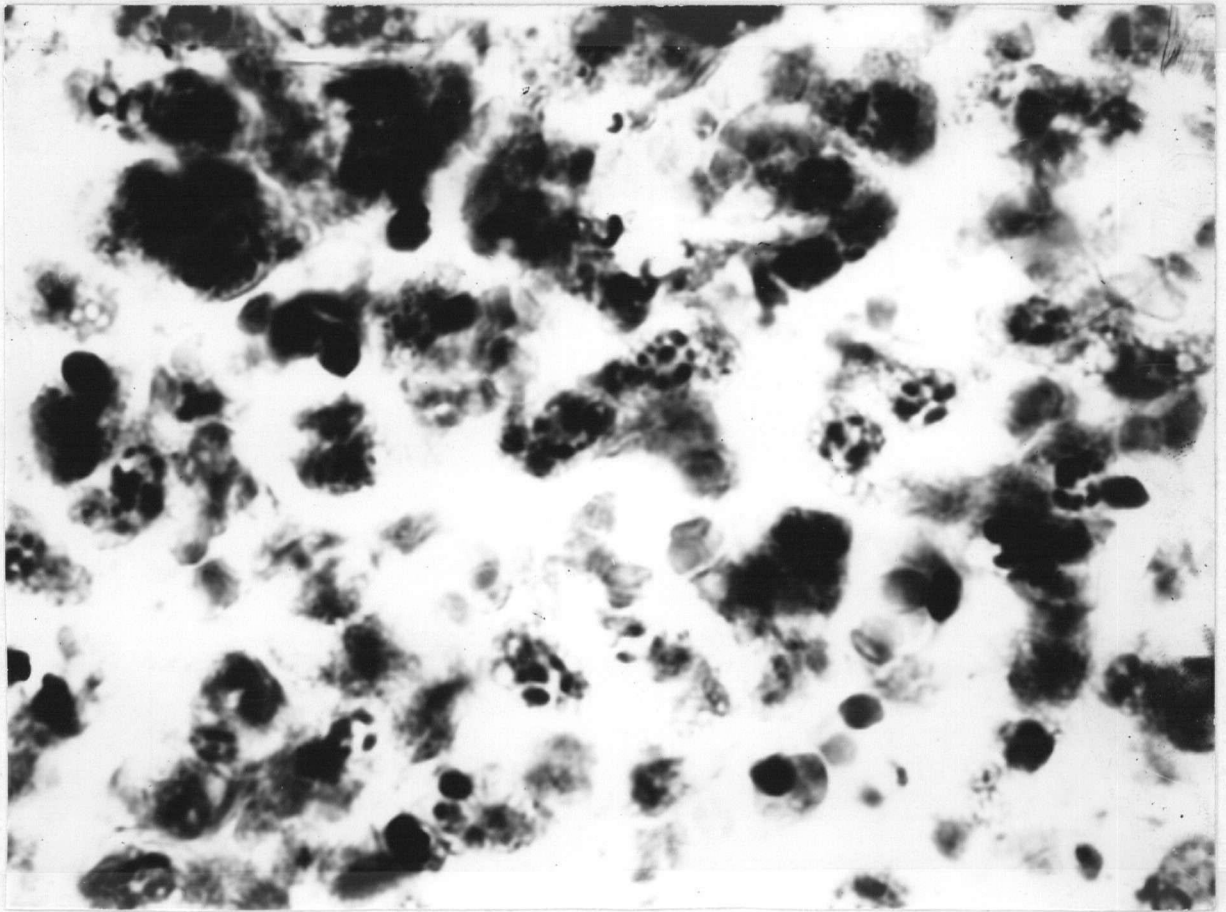


Fig. 8.

A similar condition to that seen in Fig. 7, but slightly more severe. Note the typical crescent in the upper centre of the photograph. Chromatin material of the nuclei is also clearly visible in many of the cells. $\times 2500$. Rat CO 10.

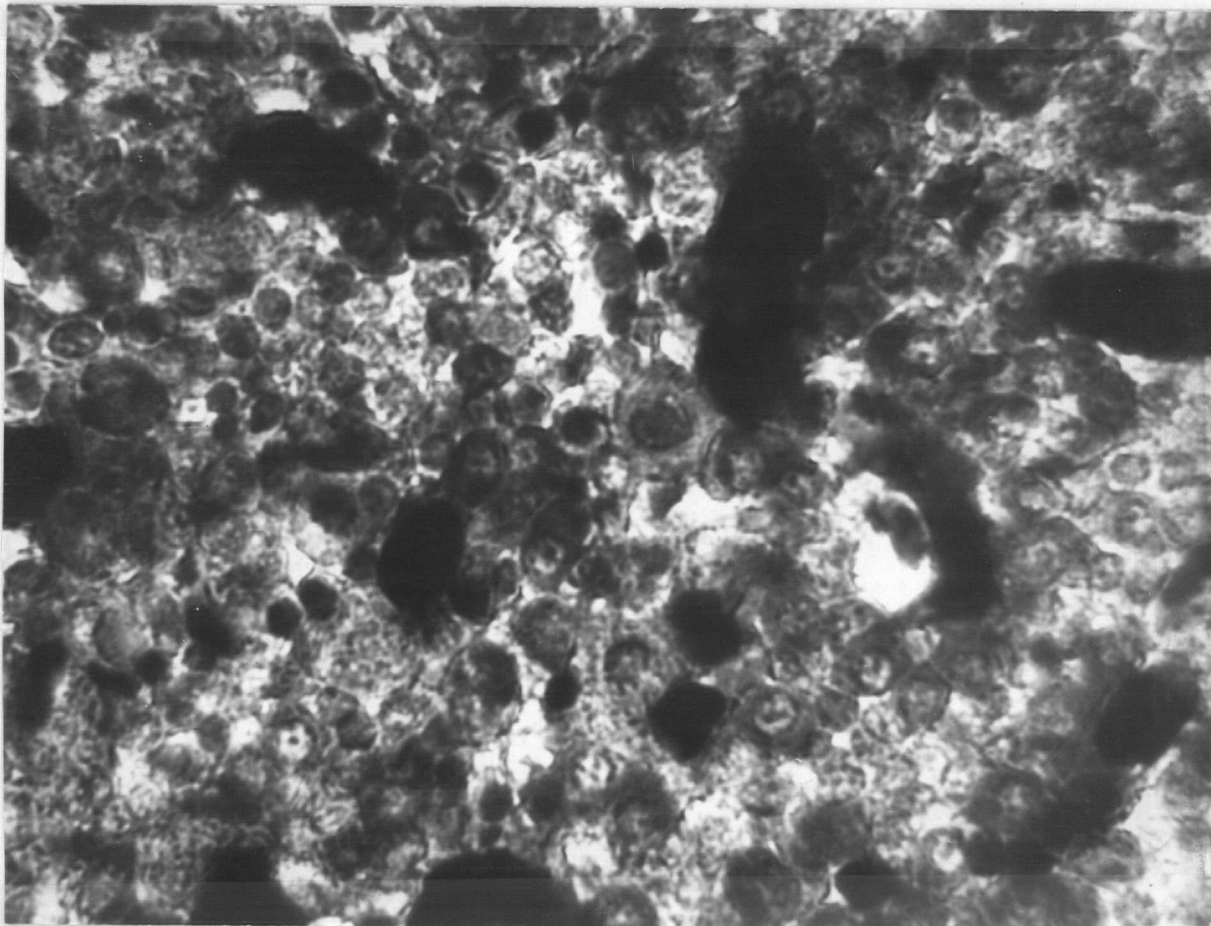


Fig. 9.

Briseno-Castrejon pituitary stain in rat PS 2 G 11.

A typical basophil cell with plentiful lightly stained cytoplasm is seen near the centre. The numerous acidophils have darker stained cytoplasm while the chromophobes have little or none. The acidophil count is high, the % ratio of alphas: betas: chromophobes = 55: 6: 39. x 1700.

Discussion and Conclusions:

It is apparent from the results obtained that the pituitary-adrenal systems of the experimental animals were in a state of hyperactivity. In many types of experiments, as in unilateral adrenalectomy, the pituitary returns to normal when the remaining adrenal regains the necessary level of cortical function thru hypertrophy. In the case with which we are concerned, however, there is a sustained depletion of adrenal cholesterol and a constant increased pituitary acidophil count. Therefore, the demand for cortical hormones must be continuously high. At this point, three facts should be noted, (i) that all animals, including the controls, received a high protein, high salt diet, (ii) that alloxanized animals received only one dose of alloxan and that such a dose is completely destroyed in the body five minutes after its injection, and (iii) that there was no difference in the results obtained for alloxanized animals when insulin was given to remedy the diabetes. It can be seen then that the diabetes is not a factor in the pathological conditions with which we are concerned. Similarly, the diet alone is not a sufficient stimulus. It therefore seems logical that the alloxan is the initial agent of stress, subsequently aided and abetted by the dietary protein and salt. That such a small factor as a single dose of alloxan acting over such a short period of time could have such a great effect is made plausible by two facts. One is that during that short time, the alloxan is capable of destroying the insulin-producing beta cells of the islets of Langerhans. The other is that

hypertension, comparable to what is produced in these animals, has been produced by a former student at this university using only a single small dose of desoxycorticosterone acetate. (70) The nature of the action of alloxan in the adaptation syndrome is probably the same as that of the host of other non-specific agents of stress. But it is tempting to speculate that alloxan could attack the adrenals directly in a similar manner to that in which it destroys islet tissue. Since the adrenal gland has one of the highest glutathione contents of any tissue in the body and because of the fact that alloxan removes glutathione from the blood and attacks sulfhydryls in general, such a direct action is a definite possibility. If this was the case, the problem would be almost identical with the familiar adrenalectomy-salt experiments that are also concerned with stress.

The effect of the diet on the experimental animals receives some possible clarification by the results in groups PS-A, and S-D & E. The method of supplying the salt and protein in the diet of group A did not give the animals as much of these two components and as much adrenal hypertrophy as did the more accurate methods used in the later experiments, especially group G. There is a discrepancy between A & B that is difficult to explain. There is a possibility that the zinc contained in the insulin could have caused an allergic or poisoning action as it occasionally does in some human patients. On the other hand, the low values obtained in group F, in which the alloxan failed to produce diabetes, are probably only

due to a high level of circulating sulphhydryls in the animals at the time of injection of the alloxan. Groups S-D and S-E, in which salt was added but not protein, gave readings parallel with those obtained with protein included. The more important factor then is probably the salt.

Since it is an established fact that there is an increased supply and demand for cortical hormones during stress, and since it has been confirmed that there is a sustained overproduction of these hormones in this experiment, then there should be an increased amount of circulating D.C.A. type hormones. The action of these salt retaining hormones on the high salt content coming into the body must cause a critical increase in the Na Cl content of the blood. This rise is comparable to that seen in the resistance phase of the adaptation syndrome. If this sequence of events is prolonged, the hormone producing mechanism becomes exhausted and, as Selye points out, there may be a lack of these hormones when termination of life occurs. This exhaustion phase was evident, since some of the animals showed a complete absence of adrenal cholesterol and died a spontaneous death.

The method by which the salt and mineralo-corticoids produce the hypertension may involve one or both of the two following paths. The high sodium level in the blood might exert a direct action on the walls of the arteries due to the tendency of sodium to cause sustained muscular contraction therein. Again, the mineralo-corticoids could cause a nephritic

type of hypertension by their hyalinizing action on glomeruli.

There are important differences between the usual pathology of alloxan diabetes and that seen when salt is added to the diet. The conditions seen in the latter show an interesting and remarkable parallelism to typical, naturally occurring diabetes mellitus. Thus one finds arteriosclerosis, fatty degeneration of the liver, nephrosclerosis and fibrosis in both diabetes mellitus and in the alloxanized animals having the salt diet in this experiment, but not in alloxanized animals alone. Dr. Chute has made the clinical observation that diabetic children consume abnormally large quantities of salt. From this fact and from the results of this thesis it appears likely that the only reason that alloxan diabetes differs from diabetes mellitus pathologically is that lab animals kept after alloxanization do not normally get the chance to ingest extra salt as would human diabetics. In other words, the reason that alloxan diabetes is not the same as diabetes mellitus lies in the fact that the lab animals are fed a better diet than their human counterparts. Conversely, the complications of human diabetes must be due to a secondary hypertension caused largely by the craving of salt.

Summary.

The pituitaries and adrenals of alloxanized diabetic rats fed high protein and salt diets showed that the pituitary-adrenal systems of these animals were in a state of hyperactivity, as shown by adrenal hypertrophy, depleted adrenal cholesterol and increased pituitary acidophils. The use of insulin to remedy the diabetes and the omission of protein from the diet did not alter the extent of this response. The animals showed typical signs of hypertension such as arteriosclerosis, nephrosclerosis, fatty degeneration of the liver and hemorrhages. It was concluded that the animals were responding to alloxan as an agent of stress in conformity with the adaptation syndrome and that the high salt content of the diet aggravated and sustained the resulting hypertension.

Normally, the pathology of alloxan diabetes differs from that of diabetes mellitus. However on the salt diet the alloxan type of diabetes was the same as the naturally occurring variety. Since Dr. Chute has observed that diabetics consume abnormally large quantities of salt, it is proposed that the only reason that alloxan diabetes does not resemble diabetes mellitus pathologically is that lab animals are usually kept on regulation foods that do not permit the ingestion of excess salt, a doubtful luxury enjoyed by their human counterparts. Conversely, the hypertension that so often complicates human diabetes is probably largely due to the salt intake.

References:

1. Albright, F. & Elrick, H; An Attempt To Classify Hormone Disorders Of The Hypophysis.
Trans. Assoc. Am. Physicians., 61, 42, 1948.
2. Babes, U. & Joesco, U.; Compt. rend.soc. biol. 65, 67.
3. Bailey, C.C.; Alloxan Diabetes. Vit. & Hor. 7, 365, 1949.
4. Bailey, C.C. & Bailey, O.T.; The Production Of Diabetes Mellitus In Rabbits With Alloxan. J.A.M.A., 122, 1165, 1943.
5. Bailey, C.C. & Lecompte, P.M.; Alloxan Diabetes In Hypophysectomized Rats. Med.Clinics of N.A., 31, 427, 1947.
6. Bailey, C.C. et al; The Effect of Alloxan On Hypophysectomized Rats. P.S.E.B.M. 66, 271, 1947.
7. Bennett, H.S.; Steroid Properties Of Adrenal Lipids.
Am. J. Anat., 67, 151.
8. Bloch, K. et al; Biological Conversion Of Cholesterol To Cholic Acid. J. Biol. Chem. 149, 511, 1943.
9. Bloch, K.; Biological Conversion Of Cholesterol To Pregnanediol. J. Biol. Chem., 157, 661, 1945.
10. Briseno-Castrejon, B. & Finerty, J.C.; An Azocarmine Stain For Differential Cell Analysis Of The Rat Anterior Hypophysis. Stain Tech., 24, 103, 1949.
11. Bruckman, G. & Wertheimer, E.; Alloxan Studies;
The Action Of Alloxan Homologues And Related Compounds.
J. Biol. Chem., 168, 241, 1947.
12. Cannon, W.B.; The Wisdom Of The Body, Norton & Co., N.Y.
13. Catchpole, H.R.; Glycoprotein Hormones Of The Anterior Pituitary. Fed. Proc., 6, 88.

14. Cerecedo, L.R.; The Fate Of Parabanic Acid, Alloxan And Alloxantin In The Organism Of The Dog. *J. Biol. Chem.*, 93, 283, 1931.
15. Conn. J.H. et al; The Effect Of Alloxan On A Case Of Islet Carcinoma. *J. Lab. Clin. Med.* 32, 347.
16. Conn. J.H.; The Effect Of Desoxycorticosterone Acetate On The Retention Of Salts. *Arch. Int. Med.*, 83, 416.
17. Crooke, A.C. & Russell, D.S.; The Pituitary Gland In Addison's Disease. *J. Path. Bact.*, 40, 255, 1935.
18. Daughaday, W.H. et al; Hyperadrenalcorticism In Acromegaly With Insulin Resistant Diabetes. *J. Clin. Endo.*, 10, 410, 1950.
19. Deane, H.W. & Greep, R.O.; Cytochemical Evidence For The Cessation Of Hormone Production In The Zona Glomerulosa Of The Rat Adrenal Cortex After Prolonged Treatment With Desoxycorticosterone Acetate. *Endocrin.*, 40, 417, 1947.
20. Dempsey, E.W. & Bassett, D.L.; Observations On The Fluorescence, Birefringence And Histochemistry Of The Rat Ovary During The Reproductive Cycle. *Endocrin.* 33, 384, 1943.
21. Dempsey, E.W.; The Chemical Cytology Of Endocrines. *Rec. Prog. In Hor. Res.*, 3, 127, 1948.
22. Dempsey, E.W. & Wislocki, G.B.; The Identification Of Steroids. *Physiol. Rev.* 26, 1, 1946.
23. Dempsey, E.W. & Wislocki, G.B.; The Effect Of Ribonuclease On Cytoplasmic Basophilia In The Beta Cells Of The Anterior Pituitary. *Am. J. Anat.* 76, 277.

24. Desclin, L.; Compt. rend. soc. biol., 133, 457, 1940.
25. Dougherty, T.F. & White, A.; Influence Of Hormones On Lipid Tissue Structure & Function. Endocrin., 35, 1, 1944.
26. Duff, G.L.; The Comparative Pathology Of Alloxan Diabetes And Diabetes Mellitus. Am. J. Med. Sci., 210, 381, 1945.
27. Dunn, J.S. et al; Necrosis Of Islets Of Langerhans Produced Experimentally. Lancet, 121, 484, 1943.
28. Elftman, H.; Phosphatase Distribution In Adrenals Of Oestrogen Treated Mice. Anat. Rec. 97.
29. Finerty, J.C. & Briseno-Castrejon; Qualitative Studies Of Pituitary Cell Types In Unilateral Adrenalectomy. Endocrin., 44, 293, 1949.
30. Forsham, P.H. et al; The Effect Of Stress On The Eosinophil Count. J. Clin. Endo., 8, 15.
31. Gaarenstroom, J.H. et al; Abstract.
32. Giroud, A. & Leblond, C.P.; The Cytological Identification Of Ascorbic Acid. Arch. Anat. Micro., 31, 111.
33. Goldblatt, H.; Renal Hypertension Due To Renal Ischaemia. Ann. Int. Med., 11, 69.
34. Goldblatt, H.; Physiol. Rev., P. 120, 1947.
35. Goldman, M.L. & Schroeder, H.A.; The Pressor Effect Of D.C.A. Am. J. Med., 5, 33, 1948.
36. Goldner, M.G. & Gomori, G.; Alloxan Diabetes In The Dog. Endocrin., 33, 297, 1943.
37. Gomori, G., & Goldner, M.G.; The Acute Nature Of Alloxan Damage. P.S.E.B.M. 58, 232, 1945.

38. Harkins, H.N. & Long, C.N.H.; Metabolic Changes In Shock After Burns. *Am. J. Physiol.*, 144, 661, 1945.
39. Haynes, F.W. & Dexter, L.; Renin, Hypertensinogen & Hypertensinase Concentrations Of The Blood Of Dogs During The Development Of Hypertension By Construction Of The Renal Artery. *Am. J. Physiol.*, 150, 190, 1947.
40. Heinbecker, P. & Rolf, D.; Experimental Obeisity In The Dog. *Am. J. Physiol.* 141, 549, 1944.
41. Hune, D.; *J. Clin. Investigations* 28, 790; Abstract.
42. Jacobs, H.R.; Hypoglycemic Action Of Alloxan. *P.S.E.B.M.*, 37, 407, 1937.
43. Kempner, W.; *N. Carolina Med. J.* 6, 61, 117; Abstract.
44. Kraus, E.J.; *Path. Anat. u Allg. Path.* 78, 283, 1927; Abstract.
45. Labes, R. & Friesburger, H.; *Arch. Exptl. Path. Pharm.* 156, 226, 1930. Abstract.
46. Lazarow, A.; Protective Effect Of Glutathione & Cysteine Against Alloxan Diabetes In The Rat. *P.S.E.B.M.*, 61, 441, 1946.
47. Leech, R.S.; Blood Alloxan & Blood Glutathione In Rabbits Injected With Alloxan. *J.B. Chem.*, 157, 525, 1945.
48. Lehmann, H.; Factors Influencing The Formation Of The Robison Ester. *Biochem. J.*, 33, 1241, 1939.
49. Lieberman, S. & Dobriner, K.; Steroid Excretion In Health & Disease. *Rec. Prog. In Hor. Res.* 3, 91, 1948.
50. Long, C.N.H. & Fry, E.G.; Effect Of Epinephrine On Adrenal Cholesterol & Ascorbic Acid. *P.S.E.B.M.*, 59, 67, 1945.

51. Long, C.N.H.; The Relation Of Cholesterol & Ascorbic Acid To The Secretions Of The Adrenal Cortex.
Rec. Prog. In Hor. Res., 1, 99, 1947.
52. McDermott, W.V. et al; Release Of Adrenalcorticotrophic Hormone By Direct Application Of Epinephrine.
P.S.E.B.M., 73, 609, 1950.
53. Mason, H.L.; Urinary Steroids In Adrenal Disease & The Metabolism Of The Adrenal Hormones.
Rec. Prog. In Hor. Res., 3, 91, 1948.
54. Maximow & Bloom, Textbook Of Histology, P. 229.
55. Perera, G.A. & Blood, D.W.; The Effect Of Sodium Chloride Administration To Rats Injected With Desoxycorticosterone Acetate. Ann. Int. Med., 27, 401.
56. Perla, D. & Marmorstan, J.; Role Of Vitamin C In Resistance. J. Arch. Path., 23, 543 & 683, 1937.
57. Popjack, G.; Lipids Of The Rat Adrenal After Shock. J. Path. Bact., 56, 485, 1944.
58. Recant, L., Hume, D.M., Forsham, P.H. & Thorn, G.W.; Stress In The Addisonian. J. Clin. Endo., 9; 1949.
59. Reese, J.D. et al; Anterior Pituitary Changes Following Adrenalectomy In The Cat. Anat. Rec. 75, 373, 1939.
60. Rinderknecht, H.; Diabetes Insipidus. Lancet, 121, 384, 1943.
61. Rixon, R.H.; The Amelioration Of Experimental Chronic Hypertension By Vitamin C. M.A. Thesis, U.B.C., 1950.
62. Sayers, G. & Sayers, M.A.; Regulation Of Pituitary

cont'd

62. Adrenocorticotrophic Activity During Response Of The Rat To Acute Stress. *Endocrin.*, 40, 265, 1947.
63. Sayers, G. et al; The Cholesterol & Ascorbic Acid Content Of The Adrenal, Liver, Brain & Plasma Following Hemorrhage. *Endocrin.*, 37, 96, 1945.
64. Sayers, G. et al; The Effects Of Pituitary Adrenocorticotrophic Hormone On Cholesterol And Ascorbic Acid Content Of The Adrenal Of The Rat & Guinea Pig. *Endocrin.*, 38, 1, 1946.
65. Sayers, G. et al; Effect Of Adrenotrophic Hormone On The Ascorbic Acid & Cholesterol Content Of Rat Adrenal Glands. *P.S.E.B.M.*, 52, 200, 1943.
66. Sayers, G. et al; Effect Of Pituitary Adrenotrophic Hormone On Cholesterol Content Of The Rat Adrenal Glands. *P.S.E.B.M.*, 55, 238, 1944.
67. Sayers, G. et al; The Effect Of Adrenotrophic Hormone Of The Anterior Pituitary On The Cholesterol Content Of Adrenals. *Yale J. Biol. Med.*, 16, 361, 1944.
68. Sayers G. & Sayers, M.A.; Regulatory Effect Of Adrenal Cortical Extract On Elaboration Of Pituitary Adrenotrophic Hormone. *P.S.E.B.M.*, 60, 162, 1945.
69. Sayers, G. & Sayers, M.A.; The Pituitary-Adrenal System. *Ann. N.Y. Acad. Sci.*, 50, 522, 1949.
70. Salter, J.M.; Studies In Experimental Hypertension. M.A. Thesis, U.B.C., 1950.
71. Schonemann, A.; Cell Types Of The Anterior Pituitary. *Arch. Path. Anat. Physio.*, 129, 310.

72. Selye, F.L.; The Absorption Of Enteric-coated Ammonium Chloride.
73. Selye, H.; Hypertension As A Disease Of Adaptation.
Rec. Prog. In Hor. Res., 3, 343, 1948.
74. Severinghaus, A.E.; Sex & Internal Secretions, 1949.
75. Severinghaus, A.E.; The Cytology Of The Pituitary Gland.
Proc. Assoc. Res. Nervous & Mental Diseases, 17, 69, 1936.
76. Thorn, G.W. et al; J . A.M.A., 137, 1005.
77. Thorn, G.W. & Firor, W.M.; Desoxycorticosterone Acetate
Therapy In Addison's Disease. J.A.M.A. 114, 2517.
78. Thorn, G.W. et al; The Pathological Effects Of Excessive
Desoxycorticosterone Acetate Administration.
Ann. Int. Med., 16, 1053.
79. Thorn, G.W. & Forsham, P.H.; Metabolic Changes In Man
Following Adrenal & Pituitary Hormone Administration.
Rec. Prog. In Hor. Res., 4, 229.
80. Tyslowitz, R.; Effect Of Hypophysectomy On The Concen-
tration Of Ascorbic Acid In The Adrenals Of Rats.
Endocrin., 32, 103, 1943.
81. Venning, E.H. & Brown^e, J.S.L.; Fed. Proc., 4, 108.
82. Venning, E.H. & Kazmin, U.; Endocrin., 39, 131.
83. Vogt, M.; The Effect Of Chronic Administration Of Adrena-
line On The Suprarenal Cortex & The Comparison With That
Of Hexoestrol. J. Physiol., 104, 60, 1945.
84. Vogt, M.; The Output Of Cortical Hormone By The Mamma-
lian Suprarenal. J. Physiol., 102, 341, 1943.

85. Wyman, L.C. & tum Suden, C.; Functional Efficiency Of Transplanted Adrenal Cortical Tissue.

Endocrin., 21, 587, 1937.

86. Zwemer, R.L. et al, Experimental Studies Of Similarities Between Cortin & Some Cardiac Glucosides.

Endocrin., 27, 945, 1940.