NUTRITIONAL AND HORMONAL FACTORS INFLUENCING THE CORTEX OF THE ADRENAL GLAND

by

Marian H. Steele, B.Sc.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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INTRODUCTION

The objective of the researches described in this thesis has been to examine the influence of diet on the adrenal cortex, notably the actions of changes in intake of protein and cholesterol. Before describing these experiments, some aspects of adrenal cortical function are presented.

The adrenal is a member of the group of endocrine glands or glands of internal secretion. These glands have no ducts and secrete chemical substances, named hormones by Bayliss and Starling in 1902, directly into the blood. Claude Bernard in 1855 was the first to recognise the adrenal as being an organ of internal secretion, while in the same year the clinical studies of Addison drew attention to the importance of the gland. Further evidence that the adrenals are essential to life was given by Brown - Séquard (1858) who showed that removing the adrenal glands resulted in the death of the animal.

Since Addison's time much work has been done on the nature and functions of the secretory products of the adrenal glands. It is now recognised that they play an important part in the regulation of metabolic processes concerned with mineral, carbohydrate, and protein metabolism in the animal body. The release of hormones from the adrenal is under the control of another endocrine gland, the anterior pituitary. This gland regulates the action of the adrenal by the release of one of its hormones, the adrenocorticotrophic hormone (ACTH), which acts directly on the adrenal.

Nature of the Hormones of the Adrenal Cortex

There are a large number of hormones present in the adrenal cortex although only a few are of direct interest as many of the others are relatively inactive or are precursors of the active ones. They are all steroids and all have the same basic structure of a cyclopentenoperhydrophenanthrene ring system. Adrenocortical hormones were first isolated from the adrenal gland in 1936, (Mason, Meyers and Kendall, 1936a, 1936b; Reichstein, 1936; Wintersteiner and Pfiffner, 1936) and by 1938 21 steroids had been isolated. Over 40 steroids have now been isolated from extracts of adrenal glands of animals (Wettstein, 1959), but of these 7 are the most important ones with regard to adrenocortical function. They are hydrocortisone, cortisone 17 - hydroxy - 11 - deoxycorticosterone, corticosterone, 11 - dehydro-corticosterone, 11 - deoxycorticosterone, and aldosterone. These compounds all have two features in common, firstly the α, β - unsaturated carbonyl group in ring A, which is an essential characteristic of steroids with the properties of the adrenocortical hormones, and secondly, the presence of a 2 - carbon side chain at carbon atom 17.

The Secretion of Hormones by the Adrenal Cortex

All the hormones which are present in the adrenal cortex itself are not found in the adrenal venous blood as secretory products of the gland. Vogt in 1943 was the first to carry out analyses on the adrenal venous blood secreted by the dog. Investigations by Bush (1953) and Romanoff, Hudson and Pincus (1953) have shown, using chromatographic

techniques, that cortisol and corticosterone are the two steroids secreted in the greatest quantity by the adrenals of most mammals. The monkey, sheep, cat, dog and man have been shown to secrete primarily cortisol, while in the rabbit and the rat corticosterone is the most important secretory product, although there appears to be considerable individual variation within each species. (Hechter and Pincus, 1954). Various workers (Romanoff, Hudson, and Pincus, 1953; Sweat, 1955; Hudson and Lombardo, 1955; Bush and Sandberg, 1953; Morris and Williams, 1953) have found considerable variation in the ratio of the amounts of cortisol and corticosterone in the blood of human subjects. Grant, Forrest and Symington (1957) using patients undergoing two-stage adrenalectomy showed that the ratio of cortisol to corticosterone in human blood increases on treatment with ACTH and they conclude that the proportion of cortisol and corticosterone secreted in man appears to be related to the degree of stimulation of the adrenal cortex by ACTH. In the rabbit Kass, Hechter, Macchi and Hamm (1954) have shown that the ratio of cortisol to corticosterone present in the adrenal secretion alters with ACTH stimulation, cortisol becoming the predominant secretory product on prolonged treatment with ACTH. The other most important secretory product of the adrenal cortex is aldosterone. This hormone is secreted in very small amounts but is physiologically very active.

Biosynthesis of Adrenocortical Hormones

Cholesterol is present in the adrenal gland in a relatively high concentration, and various workers (Zaffaroni, Hechter and Pincus (1951),

Hechter, Solomon, Zaffaroni and Pincus, (1953)) have shown that the isolated adrenal gland is capable of converting it into corticosteroids; by perfusing isolated cow adrenal with radioactive cholesterol they showed that radioactive cortisol and corticosterone are produced. In this system they also showed that C^{14}- acetate could be transformed into corticosteroids. Similarly, incubation of C^{14}- acetate with hog (Haines, 1952), beef (Haynes, 1953), and rat (Heard, Jacobs, O'Donnell, Feron, Saffran, Solomon, Thompson, Willoughby and Yates, 1954) adrenal slices showed that radioactive cortisol and corticosterone were formed in each case. Cholesterol, however, has been shown to be a more efficient precursor of corticosteroids than is acetate (Hechter, Solomon, Zaffaroni and Pincus, 1953), and this, combined with the fact that treatment with ACTH causes a reduction in the adrenal cholesterol content (Long, 1947) would indicate that cholesterol is the more important precursor of corticosteroids in the adrenal gland. However, this is a controversial point, as it has been shown that corticosteroids may possibly be formed from acetate without the formation of cholesterol as an obligatory intermediate. (Hechter, 1952, 1953; Hechter, Solomon, Zaffaroni and Pincus, 1953.). More recent evidence that cholesterol may not be a necessary precursor comes from work done by Heard, Bligh, Cann, Jellinck, O'Donnell, Rao and Webb (1956) who, using C^{14}- acetate showed that the C^{14} was incorporated into the corticosteroids in adrenal preparations but they found no C^{14} incorporation in the cholesterol isolated from the system. However, Norbin and Chaikoff (1961) showed that cortisol excreted

in the urine of guinea pigs fed C^{14}-cholesterol had the same specific activity as the adrenal cholesterol, which would indicate that cholesterol is an obligatory intermediate for cortisol synthesis in the guinea pig. Work done by Caspi (1962) partly supports the theory that cholesterol is an intermediate in the biosynthetic pathway. Using perfused ox adrenals he established the origin of certain of the carbon atoms of C^{14}- cortisol from the methyl and carboxyl carbons of 1 - C^{14} - acetate and showed that the corresponding carbon atoms in cholesterol are also formed from the acetate carbon atoms in the same way, indicating that cholesterol is an intermediate in cortisol biosynthesis. However, some of the carbon atoms were found to be labelled unexpectedly which would indicate there may also be a cholesterol-independent pathway of synthesis of cortisol.

In the formation of adrenocortical steroids it is thought that the precursor cholesterol is converted by hydroxylation of carbons 20 and 22 and by scission of the side-chain to form the 21 carbon compound, pregnenolone, which is then converted to progesterone. Progesterone then undergoes various hydroxylations to give the individual corticosteroids.

Histology of the Adrenal Gland

The adrenal gland consists of two distinct regions, the inner region named the medulla and the outer region known as the cortex which is then enclosed in a capsule. The division of the adrenal into a central and a peripheral part was probably recognised from the seventeenth century. The terms "medulla" and "cortex" were introduced by Huschke in 1845,

Gray in 1852 and Kölliker in 1854. The cortex and the medulla are distinctly different both histologically and functionally. The adrenal medulla which secretes adrenalin and nor-adrenalin can be removed with no apparent effect on the animal and thus is not essential for life. The adrenal cortex, however, is indispensable and secretes the adreno-cortical hormones. Histological examination of the cortex shows it to consist of 3 concentric zones. Arnold (1866) named them the 'zona glomerulosa', or the outer zone, the 'zona fasciculata' which lies adjacent to the zona glomerulosa, and the 'zona reticularis', the inner-most zone. These three zones have markedly different appearances histologically. The zona glomerulosa consists of groups of cells lying beneath the capsule. The zona fasciculata has a regular appearance of long columns of cells which gradually emerge into the zona reticularis with an irregular appearance. The cells of this zone are compact and smaller than those of the rest of the cortex. A band of narrow cuboidal cells with darkly staining nuclei is sometimes observed lying between the zona glomerulosa and the zona fasciculata. This area is known as the zona intermedia or the 'transitional zone', and its presence and size varies according to the functional state of the gland. The zona glomerulosa is a very narrow zone, while the other two zones are much wider, the zona fasciculata usually being the widest one. The width of these zones and their structural appearance are found to vary as the activity of the gland alters.

Functions of the Different Zones of the Cortex

It was first suggested by Swann in 1940 that the different zones of the adrenal cortex may have different secretory functions. The first evidence showing that the glomerulosa and the fasciculata have different functions was given by Deane and Greep (1946) and Deane, Shaw and Greep (1948). Later other workers (Ayres, Gould, Simpson and Tait, 1956; Giroud, Stachenko and Filetta, 1958), by incubating ox adrenal slices, showed that aldosterone is preferentially produced by the zona glomerulosa and hydrocortisone by the zona fasciculata. The same functional zonation was found to exist in the human adrenal gland (Ayres, Garrod, Tait and Tait, 1958) and investigations on the rat adrenal (Giroud, Stachenko and Venning, 1956) showed that the principal site of secretion of aldosterone is the zona glomerulosa.

It is well known that the adrenal cortex is controlled by the secretion of ACTH from the anterior pituitary. However, in 1940 Swann suggested that all the zones of the cortex may not be under the control of this hormone. He drew his conclusions from the fact that adrenalectomy causes electrolyte imbalance but this does not occur after hypophysectomy when the zona fasciculata atrophies but the zone glomerulosa remains unchanged. The work done by Deane, Shaw and Greep (1948) supports this theory. Their experiments indicated that the glomerulosa undergoes morphological and cytochemical changes in physiological states that involve disturbance of the electrolyte balance of the body, and these changes occurred without any demonstrable alteration of the fasciculata.

Singer and Stack-Dunne (1954) provided direct evidence that both the glomerulosa and the fasciculata are not equally under the control of the pituitary. By analysis of the adrenal venous blood of rats they showed that hypophysectomy caused a much larger decrease in the blood level of corticosterone than it did of aldosterone. They also demonstrated that ACTH administration to hypophysectomized rats caused a larger increase in the secretion of corticosterone than of aldosterone. Using dogs as their experimental animals Farrell, Rauschkolf and Royce (1955) obtained the same results as did Singer and Stack-Dunne using rats. Thus the evidence provided by these various workers supports the hypothesis of Swann and indicates that aldosterone is preferentially produced by the zona glomerulosa, while the zona fasciculata produces hydrocortisone in the dog and corticosterone in the rat. The evidence provided also indicates that the zona fasciculata is under the control of the pituitary but that the secretion of the zona glomerulosa is influenced by extra-pituitary factors.

The Chemical Constituents of the Adrenal Gland

The adrenal cortex cells contain the usual constituents normally found in tissues - DNA, protein, RNA, phospholipid, etc. In our studies we have, in fact, estimated many of these substances and these will be discussed later; however, at this point some comments about special chemical features of the adrenal cortex will be made.

One of the substances which is present in a high concentration in the adrenal gland is cholesterol. The cholesterol content of the rat

adrenal cortex represents 4 - 5% of the wet weight of the gland. The presence of considerable amounts of cholesterol in the gland has been associated with the capacity of the adrenal to convert it into adrenocortical steroids (Zaffaroni, Hechter and Pincus, 1951; Hechter, Solomon, Zaffaroni and Pincus, 1953) and thus it is recognised as being an important constituent of the gland.

The vitamin ascorbic acid is found in higher concentrations in the adrenal gland than in any other tissue (Morgan, 1951). Its exact function in the adrenal is still unknown but Lowenstein and Zwemer (1946) have suggested that it plays a part in the synthesis of adrenocortical hormones. This suggestion is consistent with the fact that the concentration of ascorbic acid has been shown to fall in adrenals stimulated by ACTH (Sayers, Sayers, Fry, White and Long, 1944; Sayers, Sayers and Woodbury, 1948). More recently experiments on guinea pigs carried out by Eisenstein and Shank (1951) have shown that there is an inverse relationship between the vitamin C content of the diet and adrenal weight. They also showed that the adrenal gland of scorbutic guinea pigs still secretes steroid hormones on stimulation with ACTH and they suggested that vitamin C is not directly associated with the production of adrenocortical hormones. The findings of Prunty, Clayton, Medwiney and Mills (1955), from their experiments with scorbutic guinea pigs, that the production of adrenocortical hormones do not seem to be affected by a deficiency of vitamin C also supports this view. The more recent results of Guillemin, Clayton, Smith and Lipscomb (1956) have shown that treatment of rats with

ACTH results in a rapid increase in blood corticosterone before any appreciable decrease in adrenal ascorbic acid is observed. Thus these observations have shown that although the presence of ascorbic acid does not seem to be directly required for the synthesis of adrenocortical hormones its concentration does vary with the activity of the gland but the nature of its role in the adrenal cortex still remains obscure.

The nucleic acids are recognised as being important constituents of all animal tissues due to the fact that they play an important role in the synthesis of proteins. It is only of recent years that their presence in the adrenal gland has been investigated. In 1956 Symington and Davidson showed that the adrenal content of RNA in human adrenals increases with increased activity of the gland. Similar results have been obtained by other workers using rats (Fidia, Sproul and Fiala, 1956) and guinea pigs (Burns and Hale, 1959). These results would indicate that the amount of RNA present in the adrenal gives some indication of the functional state of the gland.

Control of the Adrenal Cortex

It has been realised since the 1920's that the pituitary gland exerts an influence over the adrenal cortex - (Smith, 1927; 1930). Its effect is produced by secretion of the trophic hormone, ACTH, which acts directly on the adrenal. This hormone when given to normal animals causes an increase in adrenal weight. This increase is due to stimulation of the glands with a consequent increase in the size of the adrenal cortex. Removal of the pituitary gland, or hypophysectomy, causes atrophy of the

adrenal cortex; this can be reversed by administration of ACTH. The effects produced by ACTH can also be produced in other circumstances which Selye (1950) called "stress", and the agent causing the stress be called the "stresser", which has been shown to produce its action by causing release of ACTH (Rochefort, Rosenberger and Saffran, 1956).

As a result of hypophysectomy, a decrease in the secretion of corticosteroids is observed, in particular those affecting carbohydrate metabolism, the glucocorticoids. The secretion of varying amounts of glucocorticoids according to the requirements of the body is controlled by changes in the rate of secretion of ACTH. The exact mechanism of control of ACTH is unknown but it is assumed that the control is humoral. It has been suggested that there are substances in the systemic blood which affect the anterior pituitary. A mechanism has also been proposed by Harris (1955) in which he considers that substances released in the hypothalamus as the result of nervous activity are carried in the blood to the anterior pituitary. This hormonal product released by the hypothalamus has been named the corticotrophin - releasing factor (CRF). The fact that stimuli which cause the release of vasopressin also release corticotrophin led to the idea that vasopressin may be the CRF (McCann, 1957). However, the most recent idea is that CRF is a polypeptide related in structure to vasopressin (Saffran, Schally and Benfey (1955); Guillemin, Hearn, Choak and Housholder, (1957).

The exact nature of substances which affect the release of CRF is unknown but it appears clear that the concentration of glucocorticoids in blood plays some part. Sydnor and Sayers (1954) showed that when they

observed a decrease of corticoids in the blood, there existed a high blood level of ACTH. Also the observations of Ingle and Kendall (1937) that injections of large doses of cortical extracts into rats causes adrenal atrophy, indicates that increased levels of blood corticoids suppress the release of ACTH.

Conditions of stress also seem to cause release of CRF but the nature of the stimulus which acts on the hypothalamus is unknown; it is thought that it may be a nervous one. Wells, Briggs and Munson, (1956) showed that general depressants of the brain, pentobarbitol, morphine and reserpine, cause suppression of release of ACTH in stress. This observation would indicate that the nervous system is involved in the control of responses of the adenohypophysis.

ACTH is also concerned in increasing the rate of secretion of androgens from the adrenal. It has some effect on the secretion of aldosterone but this effect is slight in comparison to the influence it exerts over the glucocorticoids (Singer and Stack-Dunne, 1955; Farrell, Reuschkolb and Royce, 1955). The secretion of aldosterone is controlled mainly by the amounts of sodium and potassium in the blood.

Mechanism of Action of ACTH

It is known that ACTH acts on the adrenal cortex increasing the output of corticosteroids, this effect being a rapid one. It also causes alterations in the gland itself which occur more slowly, namely an increase in adrenal weight accompanied by a decrease in adrenal cholesterol and ascorbic acid. (Sayers, Sayers, Fry, White and Long, 1944; Sayers, Sayers,

Liang and Long, 1946; Long, 1947; Sayers and Sayers, 1948). It is, however, unknown how these changes in the gland are connected with the stimulation of steroid secretion. It appears possible that there may be two or more independent effects produced by ACTH. Two apparently different effects of ACTH on corticosteroid production have been observed. The first is that it causes a rapid increase in steroid output as shown by Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus (1951), and Bush (1953). Secondly, it also appears to exert a slow action on the adrenal cortex. Kass, Hechter, Macchi and Mou (1954) showed that administration of ACTH to rabbits for 2 to 3 weeks caused an increase in the cortisol : corticosterone ratio in the blood. This would suggest that the increase in adrenal weight observed is associated with an increase in enzyme protein, in particular the 17-hydroxylating enzyme. Grant, Symington and Duguid (1957) showed there to be an increase in the 11-hydroxylating enzyme on stimulation of the human adrenal with ACTH, this may possibly also be a long term effect. The action of ACTH on the adrenal appears to be exerted directly on the gland. This is indicated by the fact that perfused adrenals (Bush, 1953; Macchi and Hechter, 1954) and adrenal slices (Haynes, Savard and Dorfman, 1954; Saffran, Grad and Bayliss, 1953) respond rapidly to the presence of ACTH.

The exact point at which ACTH acts in the production of steroids has been investigated by Stone and Hechter (1954) using perfused bovine adrenals. From their experiments, using C^{14}- cholesterol, C^{14}- acetate and C^{14}- progesterone, they suggested that ACTH acts by stimulating the conversion of cholesterol to pregnendlone. They also postulated that the conversion of

acetate to corticosteroids takes place mainly via a non-cholesterol pathway which is independent of ACTH. It has been observed that in ACTH stimulated adrenal slices there is an increase in adrenal phosphorylase (Haynes and Berthet, 1957.). Haynes and Berthet (1957) also found that addition of reduced triphosphopyridine nucleotide (TPNH), fumarate, glucose - 6 - phosphate and TPN, or glucose - 1 - phosphate and TPN to incubated bovine adrenal homogenates caused an increase in corticosteroid production. The most recent theory of the action of ACTH postulated by Haynes and Berthet (1957) is that it stimulates adrenal phosphorylase which by the breakdown of glycogen causes the formation of glucose - 1 - phosphate and so of glucose - 6 - phosphate. This, in the presence of glucose - 6 - phosphate dehydrogenase and TPN is converted to 6 - phosphogluconic acid and TPNH. The TPNH so formed is utilised in the synthesis of corticosteroids. This view is substantiated by the findings of Kelly, Nielson, Johnson, and Vestling (1955) that adrenal tissue has a high level of glucose - 6 - phosphate dehydrogenase. More recently it has been shown that ACTH increases the production of 3', 5'- adenosine monophosphate cyclic ester (3', 5'- AMP) in adrenal slices and that this stimulates adrenal phosphorylase (Haynes, 1958; Haynes, Koritz and Peron, 1959). It would therefore seem that the most immediate action of ACTH is to increase the 3', 5'- AMP present in the adrenal. In adrenals stimulated by 3', 5' - AMP the output of corticosteroids cannot be further increased by ACTH which suggests that 3', 5'- AMP and ACTH both stimulate adrenals in the same way. (Birmingham, Kurlents, Lane,

Muhlstock and Traikov, 1960). Koritz and Peron (1958) using quartered rat adrenals confirmed the findings of Haynes and Berthet, but their results led them to the conclusion that there was also another mechanism of action of ACTH, namely that it converted unavailable corticosteroid precursors into available ones.

Assessing adrenocortical function.

There are a considerable number of different methods by which the activity of the adrenal cortex may be assessed. Earlier workers relied mainly on observations of change in weight of the adrenal gland and on morphological and histochemical changes in the adrenal cortex (Moon, 1936). Houssay (1936) determined a decrease in lipids histochemically and Selye (1936) observed a decrease in neutral fat and they considered these decreases to be proportional to the increase in activity of the adrenal cortex.

However, increase in adrenal weight is not a satisfactory indication of the state of activity of the adrenal cortex as the gland may increase in size due to reasons other than hyperactivity. When the adrenals of an animal are in a hyperactive state, the increased release of cortical steroids produces some changes in the carbohydrate and protein metabolism of the animal. Thus the functional state of the adrenal cortex may be evaluated by the effect that the hormones released produce on other tissues of the animal. The adrenal cortex is closely associated with protein, carbohydrate and mineral metabolism (Long, Katzin and Fry, 1940; Long, 1942) and Britton and Silvette in 1937 showed that adrenocortical steroids

with an oxygen atom at carbon 11 cause the deposition of glycogen in the liver. This fact has been used by various workers (Sayers, Sayers, Liang and Long, 1946; Gemzell, 1948,) who have used increased deposition of liver glycogen of fasting animals as an indication of increased output of adrenal cortical hormones. Adrenal cortical hormones also cause a decrease in the number of circulating eosinophils in the blood and this fact has been used as an indication of adrenal activity. (Hills, Forsham and Finch, 1948; Spairs and Meyer, 1949; Eisenstin and Shank, 1951).

Direct chemical estimations of the various substances present in the adrenal cortex have also been employed to estimate gland activity. In adrenals stimulated by ACTH both the cholesterol and the ascorbic acid content of the glands have been shown to fall. (Sayers, Sayers, Fry, White and Long, 1944; Sayers, Sayers, Liang and Long, 1946; Long, 1947; Sayers and Sayers, 1948.) These changes are specific for the adrenal gland as the levels of these substances in other tissues remain unchanged, and alterations in the concentrations of these substances in the adrenal are recognised as an indication of the activity of the gland. An alteration in the lipid content of the gland also appears to accompany variations in adrenal activity. These alterations have been studied using chemical methods (Symington and Davidson, 1956), and by using histochemical methods (Houssay, 1936; Bergner and Deane, 1948; Schönbaum and Casselman, 1958; Burns and Hale, 1959) changes in the distribution of the lipids in the different zones have been shown.

More recently it has been shown that the level of RNA in endocrine glands is related to the activity of the gland (Mantovinovic and Vickery, 1959; Fiala, Sproul and Fiala, 1956a; Hoss, Corrigan and Hodak, 1959). Various workers have shown that in cases of increased adrenal activity there is an increase in the content of RNA in the adrenal gland of humans, (Symington and Davidson, 1956); rats (Fiala, Sproul and Fiala, 1956a, 1956b), guinea pigs (Burns and Hale, 1959), and dogs (Bransome and Reddy, 1961). The changes in the RNA content and distribution in the adrenal have also been studied using histochemical techniques (Symington and Davidson, 1956; Symington, Duguid and Davidson, 1956; Burns and Hale, 1959). Thus the functional state of the adrenal gland could be assessed biochemically by observations of changes in the RNA content of the gland.

All these methods of assay are, however, indirect ones and in the past few years a more direct approach has been taken as methods to estimate directly the adrenal cortical hormone content of the blood have been developed. There are various methods available for the estimation of adrenal steroids in blood. They can be estimated by the use of colour reactions based on the fact that these compounds possess ketone groupings (Nelson and Samuels, 1952; Silber and Porter, 1954). Analyses of cortical steroids in the adrenal vein blood or systemic blood have been carried out by various workers (Eik-Nes, Sandberg, Nelson, Tyler and Samuels, 1954; Tyler, Migeon and Castle, 1955; Forsham, Di Raimondo, Island, Rinfret and Orr, 1955; Querido, Kassenaar and Cats, 1955). Adrenal cortical steroids are catabolised in the liver and excreted as

17 - hydroxyketosteroids or 17 - ketosteroids and assays of these substances in the urine have been used by Sayers, (1950) and Mason and Engstrom (1950) to demonstrate alterations in adrenal cortical activity. Another method of estimation is by the use of a fluorescence technique, which makes use of the fact that certain steroids form a compound with sulphuric acid which fluoresces. Sweat in 1955 published a method for the measurement of corticosteroids in human blood using a fluorescence technique. The advantage of using fluorescent techniques is that very small quantities of steroids can be detected.

Another direct approach is by using in vitro techniques by which the steroid output of the adrenal is estimated. Tissue slices from hyperactive glands have an increased capacity to synthesize hormones and Schönbaum and Casselman (1958) have shown that results obtained by this method are in good agreement with those obtained by chemical and histochemical methods. Estimations of the levels of certain enzymes in the adrenal involved in corticoid biosynthesis such as 11β - hydroxylation have also been used to demonstrate alterations in activity (Grant, Symington and Duguid, 1957).

Influence of diet on the adrenal gland

The present investigations record studies made on the influence of diet on adrenal composition and function. A short discussion of published work on this topic is therefore included here.

1. Protein Intake

The possibility that dietary protein may exert some influence on the

adrenal gland has been suggested by the investigations of many workers. Interest in the subject was first aroused by Fahr in 1912 who showed that when rabbits were fed a diet of milk and eggs hypertrophy of the adrenal cortex occurred. Since then, the effect of the protein content of the diet on the weight of the adrenal gland has been investigated by a number of workers, but conflicting results have been obtained.

Much of the published work relates to experiments in which excessive amounts of protein were added to the diet. Tepperman, Engel and Long (1943) observed an increase in the adrenal weight of rats fed ad libitum on diets containing from 60 to 75% casein for periods ranging from 2 to 7 weeks. Tuchman - Duplessis, Aschkenasy - Lelw and Aschkenasy (1948) also found an increase in the adrenal weights of rats fed a 90% casein diet compared to those fed a 15% diet. Other workers have also noted adrenal enlargement on high intakes of protein (Ingle, 1945; Leathem, 1945, 1947, 1951; Kaunitz, Slanetz, Johnson and Guilmain, 1956). However, Ingle, Ginther and Nezamis (1943) found only a very slight difference, which was not significant, in the adrenal weights of rats fed ad libitum a 67% casein diet for 1 to 4 weeks. Benua and Howard (1945) using young mice also found no changes in adrenal weight with diet.

Although excessive intake of protein has been sometimes found to cause an increase in adrenal size and activity, prolonged administration ad libitum of protein deficient diets to young rats has not consistently resulted in a diminution in the weights of the adrenal glands relative to the final body weights (Limson and Jackson, 1932; Guggenheim and Hegsted, 1953; Leathem, 1958). However, in these experiments, comparison

is difficult due to extensive differences in body weight between depleted and control groups over the long duration of the experiments, and Munro, Hutchison, Ramaiah and Neilson (1962) therefore later examined the effects of short periods of protein deficiency on the adrenal glands of mature rats. Feeding a protein-free diet for 11 days caused a significant decrease in adrenal weight and in the total adrenal content of phospholipid, protein, RNA and DNA, the effects being much greater than the small loss of body weight caused by feeding the protein-free diet. Their observation of a decrease in the DNA content of the gland indicates a loss of adrenal cells. However, the decreases observed in the other adrenal constituents were proportionately much greater than the decrease in DNA, indicating there is also a decrease in the cell size and contents as well as in cell number.

Although some doubt exists whether protein level affects adrenal size, various workers have shown that the adrenals only respond maximally to stimuli when an adequate amount of protein is fed. This suggests that there is increased sensitivity of the adrenal glands to stress when the intake of protein is high. Rats previously on a high intake of protein are better able to maintain liver glycogen levels during subsequent starvation for 24 to 48 hours (Mirski, Rosenbaum, Stein and Weertheimer, 1938; Newburger and Brown, 1942) suggesting adrenal hyperactivity. An increase in the protein content of the diet from 16 to 22% caused a decrease in the cholesterol content of the adrenal glands of male rats (Dumm, Laken and Ralli, 1955) and this effect was taken to

indicate increased activity of the gland on the higher protein intake although no change in the adrenal weight was observed. Moya, Prado, Rodriguez, Savard and Selye (1948) showed that in rats which have undergone unilateral adrenalectomy there is a greater degree of compensatory adrenal hypertrophy in those fed a 30% protein diet than those fed a 15% protein diet. Also the reduction in adrenal ascorbic acid was significantly greater 1 hour after exposure to cold in the rats on the high protein diet than in those fed the low protein diet. Leathem (1957) using immature female rats had similar findings. He observed that those fed 3% or less casein showed no adrenal hypertrophy after unilateral adrenalectomy and that the response was related to the level of protein fed. He also showed that proteins of lower nutritional value such as wheat gluten and cottonseed caused a smaller increase in adrenal weight. Handler and Bernheim (1950) showed that the ability of the rat to respond to stress is diminished when fed a low protein diet. They observed that the decrease in eosinophil count 4 hours after epinephrine administration was significantly less in rats fed a low protein diet than in those fed a high protein diet. The work of Constantinides (1950) and of Kovacs and Korpassy (1952) has also shown that a maximum response to stress is observed only when adequate protein is fed. From these observations it is apparent that the pituitary-adrenal mechanism as a whole is sensitive to dietary protein level. The experiments described above however, do not reveal whether protein level directly changes the sensitivity of the adrenal cortex to ACTH or

changes the rate of secretion of ACTH from the pituitary. Several experiments suggest the latter explanation is correct. Using purified ACTH, Ingle, Prestrud, Li and Evans (1947), and Moya, Prado, Rodriguez, Savard and Selye (1948) failed to demonstrate any influence of dietary protein level on the response of gland size to this stimulus. This would indicate that the dietary protein level does not affect the sensitivity of the adrenal cortex to ACTH but acts directly on the rate of secretion of ACTH from the pituitary. Munro et al (1962) also showed that on ACTH administration to rats fed a protein-free diet the adrenals were still able to respond normally. They thus concluded that the decrease in gland constituents in the protein depleted animals was not due to a decreased capacity of adrenal synthesis but to a decrease in the secretion of endogenous ACTH by the anterior pituitary. This conclusion is supported by studies made on animals with a low protein intake and subjected to the stress of subtotal nephrectomy (Handler and Bernheim, 1950) or chronic undernutrition (Selye, 1950). In both investigations it was concluded that in states of protein deficiency the pituitary gland secretes less ACTH.

Apart from variations in protein level in the diet there is evidence that single amino acids can influence adrenocortical function. The work of Vartiainen and Apalajahti (1953) showed that eosinopenia followed the administration of casein or tyrosine to rats, which would indicate an increase in adrenal activity. Góth, Tengyel, Benczo, Savély and Majsay (1954) investigated this effect of proteins and amino acids more fully.

They administered 0.02g of various amino acids to normal fasting rats and found that leucine and methionine caused a very significant eosinopenia, and a significant effect was found after the administration of valine, phenylalanine and tryptophan. Glycine and isoleucine caused a decrease in the eosinophils but this did not attain significance, while histidine showed a tendency to cause an increase in the eosinophil level. In adrenalectomized rats 0.04g leucine caused no eosinopenia. These workers also showed that administration of valine or leucine to rats caused depletion of adrenal ascorbic acid compared to the effect produced by pure saline solution.

The work of Góth et al on the effect of amino acids on the circulating eosinophils may be correlated to some extent with the findings of Munro and Mukerji (1958, 1962), who administered various single amino acids to rats and observed the uptake of ^{32}P by liver RNAP and the deposition of glycogen in their livers 18 hours after feeding. Glycine, methionine and leucine each caused a significant increase in the uptake of ^{32}P by liver RNAP, and also in the total amount of RNAP in the liver. They interpreted this increased incorporation of ^{32}P into liver RNAP to be associated with protein synthesis in the liver and considered that this might depend on increased secretion of hormones from the adrenal cortex. This is consistent with the findings of Trémolières, Dorache and Lowy (1955) that cortisone causes an accumulation of protein and RNA in the liver. Comparing these with the results of Góth et al (figure 1) it is seen that in each case methionine and leucine cause a considerable

Figure 1. Comparison of the results of Munro and Mukerji with those of Góth et al.

Addition to Diet	Uptake of ^{32}P by liver RNAP: Change in total relative activity. (Munro and Mukerji (1958))		% Decrease in eosinophils (Góth et al. (1954))	
No protein	-			
Methionine	+ 95	$P < 0.01$	- 55	very significant
Glycine	+ 80	$P < 0.01$	- 29	
DL-Leucine	+ 68	$P < 0.01$	- 57	very significant
L-Cystine	+ 56	$P < 0.05$		
L-Tryptophan	+ 47	$P < 0.05$	- 38	significant
L-Valine	+ 36		- 36	significant
L-Phenylalanine	+ 30		- 55	significant
L-Tyrosine	+ 27			
L-Histidine	+ 23		- 34	
L-Arginine	+ 19			

effect. Glycine, however, causes a small and insignificant eosinopenia compared to methionine and leucine whereas Munro and Mukerji found it produced considerable effects on the incorporation of ^{32}P into liver RNAP. This may be explained by the fact that Munro and Mukerji found that a certain minimum amount of glycine had to be fed to obtain a response and Goth et al fed only very small amounts of glycine which may not have been sufficient to produce an effect. In later experiments, Munro and Mukerji (1962) showed that feeding glycine, methionine or leucine caused a deposition of glycogen in the liver, suggesting that the influence of these amino acids on the liver was connected with increased adrenal activity. They also observed that, when adrenalectomized rats were used, feeding these amino acids caused no deposition of liver glycogen. The uptake of ^{32}P by liver RNAP also showed no increase on feeding methionine or leucine to adrenalectomized rats but on feeding glycine an increased uptake was still observed. Thus the results obtained by both groups of workers indicate that methionine and leucine each cause an increase in adrenocortical activity, while the action of glycine, although having some influence on the adrenal cortex, appears to differ in action from these two amino acids.

From these observations it is apparent that the protein level in the diet can, at least under some circumstances, affect the size and activity of the adrenal gland. From the available evidence this would appear to depend on the rate of ACTH secretion, since the adrenal of the protein depleted animal can still respond normally to ACTH. Its smaller

size implies that it is receiving less ACTH from the pituitary. In addition the literature demonstrates some instances in which single amino acids are capable of changing adrenocortical function.

2. Cholesterol Feeding.

It has been known for a considerable time that feeding rabbits on diets containing a large amount of cholesterol produces hypercholesterolaemia and also later atherosclerosis, (Anitschow, 1913; Wacker and Hueck, 1913). Since adrenocortical hormones raise blood cholesterol level in normal subjects, the increase in blood cholesterol found in cholesterol-fed rabbits has aroused interest as to whether cortisone or ACTH has any influence on atherosclerosis and its consequent hypercholesterolaemia. Administration of large doses of ACTH or cortisone produces hypercholesterolaemia and elevations of serum lipid fractions in normal man (Adlersberg, Schaefer and Drachman, 1950; 1951). Similar results have been found in the rabbit (Kobernick and Moxe, 1950; Rich, Cochran and McGoon, 1951; Gordon, Kobernick, McMillan and Duff, 1954). In studying the effects of these steroids on plasma lipids in the cholesterol fed rabbit, further increases in the hypercholesterolaemia and hyperphospholipaemia were observed (Oppenheim and Bruger, 1952; Adlersberg, Wang and Schaefer, 1953; Adlersberg, Schaefer and Wang, 1954;; Stumpf and Wilens, 1954; Wang, Schaefer and Adlersberg, 1955; Adlersberg, 1959). However, Cook, Ray, Davison, Feldstein, Calvin and Green, (1952) and Gordon et al, (1954) obtained conflicting results, that rabbits fed cholesterol and treated with cortisone had lower levels of serum cholesterol than those receiving cholesterol only. However, all the investi-

gators found that cortisone-treated, cholesterol-fed animals showed a diminished degree of atherosclerosis than those fed cholesterol alone. Thus the role, if any, played by adrenal hormones in atherosclerosis appears difficult to evaluate. Although both cholesterol feeding and adrenocortical hormones raised blood cholesterol level, they have opposite effects on arteriosclerotic lesion production.

In view of the conflict of action between cholesterol and adrenocortical secretion it is therefore of interest that various workers have observed changes in the adrenal cortex of animals fed diets containing excessive amounts of cholesterol. Sternberg (1915), Krylow (1914) and McMillan, Klatzo and Duff (1954) fed rabbits with cholesterol dissolved in an oily vehicle and observed that the cortex increased in breadth with enlargement and fusion of cells and increased lipid content. Cholesterol without added oil was used by Reinock (1926) and by Kay and Whitehead (1935) who drew similar conclusions about adrenal changes. Bernick and Patek (1961) have observed the effects produced by feeding cholesterol and cottonseed oil to rats on various endocrine glands. Their investigations were carried out on rats fed for varying time intervals from 3 days to 5 months. They noticed an increase in the lipids in the adrenal glands and this became more marked with an increased time of cholesterol feeding. This increase in stainable lipids was observed in all zones of the cortex. It will be noted that in some of these experiments the adjuvant consisted of cholesterol plus an oily vehicle which may have contributed to the adrenocortical hypertrophy and in all

experiments food intake was uncontrolled. We have noted earlier that adrenocortical size is sensitive to diet and in consequence an action of cholesterol on diet acceptance could well induce a change in the adrenal gland by such an indirect mechanism.

Purpose of Present Investigations

The work undertaken in this thesis may be divided into two parts. First, an attempt has been made to investigate further the influence of dietary protein on the adrenal cortex. Experiments have been carried out to investigate whether the quality of the protein exerts any effect on adrenal composition. This has been done by feeding rats diets which vary in protein value, namely containing either no protein, an adequate level of good quality protein namely casein, an inadequate protein, zein, or zein to which tryptophan and lysine have been added to make it nutritionally adequate. To investigate further the results of Munro and Mukerji (1958; 1962) diets containing single amino acids have also been fed. To give a direct assessment of adrenal function corticosterone analyses have been performed on the blood and adrenals of rats fed these diets. The effect produced by these diets on liver composition have also been observed.

The second series of investigations is concerned with the changes produced in the adrenal glands and liver of the cholesterol-fed rabbit. Rabbits received a controlled diet containing 1% cholesterol and adrenal analyses were carried out to investigate whether the increase in gland size is due to hypertrophy or to hyperplasia. The adrenals of ACTH treated

rabbits have also been analysed and the nature of the increase in size compared with that of the cholesterol-fed rabbit. Observations have also been made on ACTH-treated cholesterol-fed rabbits to determine whether cholesterol feeding alters the sensitivity of the adrenal gland to ACTH. Experiments have also been carried out to find out if there is any relationship between the effects produced by cholesterol feeding and (i) the sex of the rabbit and (ii) the dietary intake of protein.

On the basis of experiments, the role of dietary protein level in regulating adrenocortical function is discussed.

EXPERIMENTAL

AND

METHODS

A. Rat Experiments.

The animals used were albino rats. In some investigations female rats were used while in others males were used. They were obtained from the departmental colonies and housed in individual cages under thermostatic conditions. The rats were provided with their diets in individual feeding dishes.

Diets.

The object here was not only to provide various protein levels, but also to train the rats to each at given times. In all dietary experiments the rats received two meals per day, at 9 a.m. and at 5 p.m. At 9 a.m. they received 1g of a vitamin - mineral - roughage mixture (V.M.R.) (Munro, 1949) and 2.8g glucose (Dextrosol). Details of the V.M.R. composition are given in tables I to III. The evening meal at 5 p.m. contained all the protein of the diet. In experiments where single amino acids or proteins were fed for 11 days, there was a preliminary feeding period of 3 days. During these 3 days the meal at 5 p.m. contained 4.2g of the protein diet, particulars of which are shown in table IV. One of the following diets was then given at 5 p.m. for the subsequent 11 days:-

1. 2.2g. protein-free diet + 2g. casein.
2. 4.2g. " " " - no protein added to meal.
3. 3.7g. " " " + 0.5g. glycine.
4. 4.0g. " " " + 0.2g. DL-methionine.

5. 3.7g. protein-free diet + 0.5g. L-leucine.

6. 2.2g. " " " + 2g. gelatin.

7. 2.2g. " " " + 2g. zein.

8. 2.05g. " " " + 2g. zein + 0.05g. L-tryptophan + 0.1 L-lysine.

Particulars of the protein-free diet are given in table IV.

In some experiments the rats were fed on the preliminary diet containing casein, as described above, for a week, and then received a final meal containing casein or an amino acid added to a protein-free diet. This was fed to the rats at 9 a.m. and they were killed at time intervals varying from 2 to 24 hours later. The final meals fed were one of the following:-

1. 2g. protein-free diet.

2. " " " " + 1g. casein.

3. " " " " + 1g. glycine.

4. " " " " + 1g. DL-methionine.

5. " " " " + 1g. L-leucine.

6. " " " " + 1g. DL-alanine.

7. " " " " + 1g. L-aspartic acid.

8. " " " " + 1g. L-glutamic acid.

In cholesterol-feeding experiments using rats, the morning meal was as described above for the control animals while the cholesterol-fed animals received 1g. V.M.R. and 2.7g. glucose to which 0.1g. cholesterol was added. The meal at 5 p.m. consisted of 4.2g. of either the protein-free

diet, the protein diet or the high protein diet particulars of which are given in table IV. This feeding was continued for 2 weeks.

B. Rabbit Experiments

Mature rabbits of both sexes were selected weighing 1.8 - 2.2 Kg. at the commencement of the experiment. Equal proportions of males and females were included in the control and cholesterol-fed groups. Individual cages were used and the animals housed under thermostatically controlled conditions.

Diets.

By trial and error, it was found that 100g. of stock diet (Diet 18) per day was an adequate food intake for animals of this size. Rabbits were fed this amount daily for 10 to 16 weeks, the cholesterol-fed groups receiving in addition 1g. of cholesterol to 99g. of stock diet. The diets were ground and pelleted for feeding. Diet 18 is given in Table V.

One experiment was carried out in which cholesterol was fed at different levels of dietary protein. In this experiment one group of rabbits received a protein-free diet and a second group received a high protein diet, particulars of which are given in table VI. Another two groups of rabbits each received one of these diets to which 1% cholesterol was added.

ACTH.

The ACTH used was Cortrophin ZN, Organon Laboratories Limited, and was injected intramuscularly.

Particulars of Diets used in Rat Experiments

TABLE I. Salt Mixture "446"

NaCl	243.198g.	$CaCl_2.6H_2O$	0.2g.
Potassium citrate	533.0 g.	$K_2Al_2(SO_4)_4.24H_2O$	0.2g.
KH_2PO_4	174.0 g.	NaF	0.002g.
$CaHPO_4$	800.0 g.	$MgCO_3$	92.0 g.
$CaCO_3$	366.0 g.	$MnSO_4$	2.8 g.
Ferric citrate.$3H_2O$	36.0 g.	KI	0.1 g.
$CuSO_4.5H_2O$	0.4 g.	$ZnCO_3$	0.1 g.

TABLE II. Vitamins in Starch

Pyridoxine hydrochloride	25 mg.	Calcium pantothenate	0.2 g.
Riboflavin	25 mg.	Para-amino benzoic acid	0.5 g.
Thiamine hydrochloride	25 mg.	Inositol	1.0 g.
Nicotinic acid	100 mg.	Choline Chloride	10.0 g.
Menaphthone	5 mg.	Folic acid	trace.
Biotin	5 mg.	Potato starch to 500 g.	

TABLE III. Vitamin : mineral : roughage Mixture

Wool	32.5 g.	Vitaminized Margarine	
Salt Mixture	130.0 g.	1 g. Tocopheryl acetate in	
Vitamins in Starch	250.0 g.	14 ml. radiostoloum 0.9 ml. of	
Agar powder	62.5 g.	this mixed with margarine, then	
Vitaminized Margarine	77.5 g.	with the rest.	

TABLE IV. Protein Diets

Protein-free Diet		Protein Diet		High Protein Diet	
Margarine	42 g.	Casein	240 g.	Casein	340 g.
Glucose	189 g.	Margarine	42 g.	Margarine	42 g.
Potato Starch	189 g.	Glucose	69 g.	Glucose	19 g.
		Potato Starch	69 g.	Potato Starch	19 g.

Particulars of diets
used in Rabbit Experiments

TABLE V

The Composition of Diet 18. (Bruce and Parkes, 1 47)

Bran	15%	Ground nut meal	15%
Barley meal	20%	Linseed cake	10%
Dried grass meal	30%	Calcium carbonate	1%
Dried meat and bone meal	8%	Sodium chloride	1%

Theoretical Analysis of Diet 18

Crude digestible protein	16.5%	Soluble carbohydrate	33.7%
Fat	4.6%	Minerals	6.7%

TABLE VI

Protein-Free Diet	High Protein Diet
50 g. Diet 18	50 g. Diet 18
50 g. Starch	25 g. Starch
	25 g. Casein

General Analytical Procedures

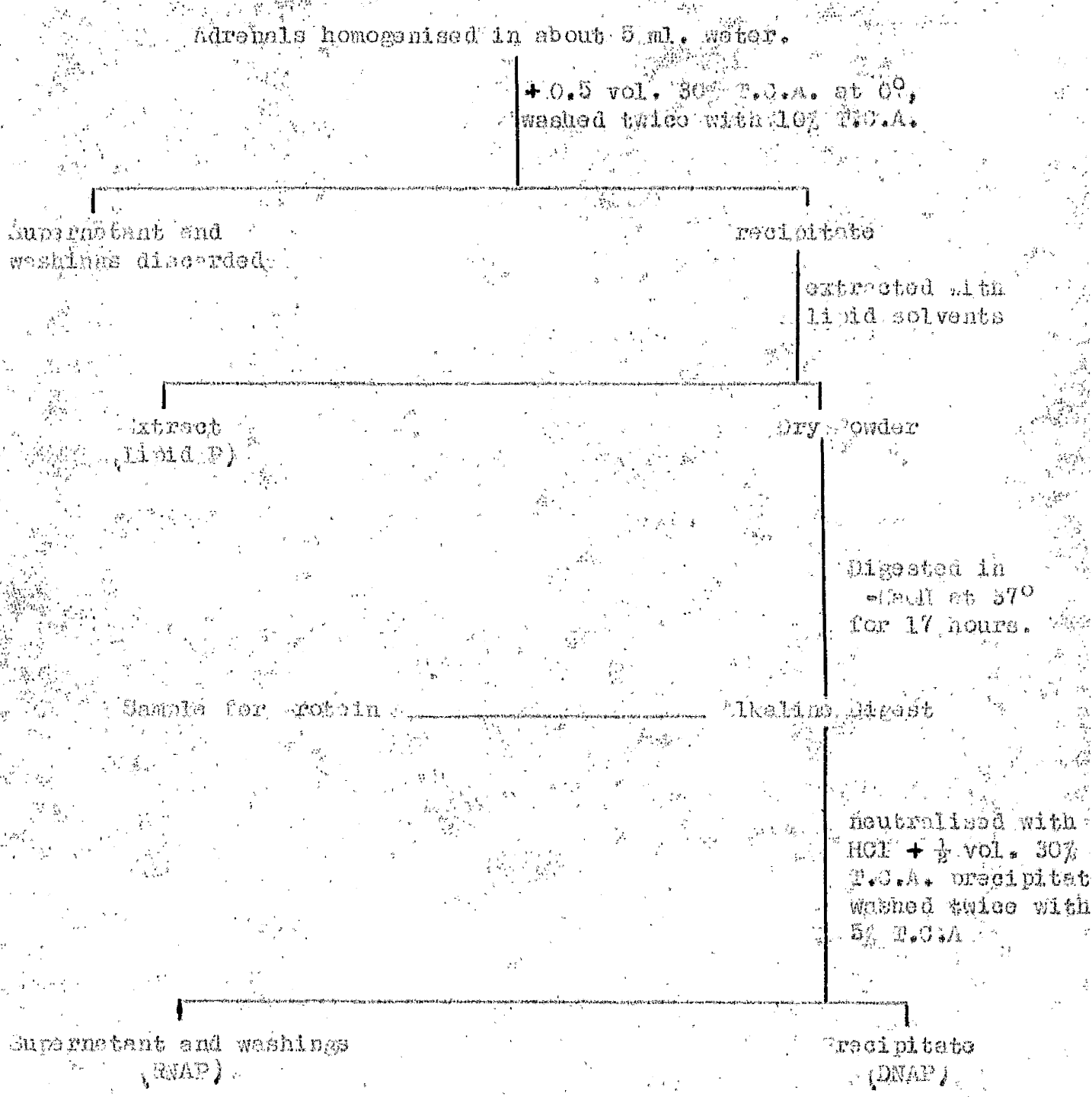
Analysis of Rat Adrenals

The animals were killed by stunning and exsanguination, the adrenals removed, trimmed of connective tissue and fat and weighed on a torsion balance. Homogenisation was carried out in ice-cold distilled water using a Potter-Elvehjem (1936) homogeniser such that the final volume was approximately 5 ml. ½ volume 30% (w/v) trichloroacetic acid (TCA) was added and the precipitate formed spun down at 0°C at 2,000 r.p.m. for 10 minutes. The residue was washed twice with 1 ml. 10% TCA and the supernatant and washings discarded.

The precipitate was then extracted successively with approximately 5 ml. portions of ethanol, ethanol:chloroform (3:1), ethanol: ether (3:1) (twice) and ether. The lipid extracts were combined and the volume made up to 40 ml. with ethanol for estimation of lipid phosphorus (lipid P).

The tissue residue was then subjected to a modified Schmidt-Thannhauser (1945) separation. It was incubated at 37°C for 17 hours in 1 ml. N-NaOH. The alkaline digest thus obtained was diluted to 5 ml. with distilled water and 3.5 ml. was removed for the analysis of RNA and DNA. This was neutralised with N-HCl and ½ volume 30% TCA added. This was spun at 2,000 r.p.m. for 10 minutes at 0°C. The DNA precipitate was washed twice with 1 ml. 5% (w/v) TCA and the combined supernatants made up to 10 ml. with distilled water for the estimation of RNA. The precipitate was dissolved in 1 ml. N-NaOH and diluted to 10 ml. with distilled water for estimation

Separation Procedure for Rat Adrenals



of DNA.

The remainder of the alkaline digest was used for the estimation of protein nitrogen.

Analysis of Rabbit Adrenals

This procedure was essentially the same as for the rat adrenals. The final volume of the homogenate was 10 ml. 5 ml. of this was removed for estimation of RNA, DNA, protein nitrogen and lipid P as described for the rat adrenals. Aliquots of the remainder of the homogenate were used for cholesterol estimation.

Analysis of Livers

After weighing, the rat liver, or part (about 4g) of the rabbit liver was homogenised in ice-cold distilled water in a Nelco blender and the final volume adjusted to 80 ml. 5 ml. samples were removed and the analysis of these performed essentially as described for adrenals. Nucleic acids and proteins were precipitated with ½ volume 30% TCA and the precipitate washed twice with 2 ml. 10% TCA. The precipitate was extracted with 5 ml. of each of the lipid solvents as described for the adrenals and the supernatants combined and the volume made up to 50 ml. with ethanol for the estimation of lipid P.

After incubating the residue with 3 ml. N-NaOH for 17 hours at 37°C, the alkaline digest was diluted to 10 ml. with distilled water. 5 ml. of this was removed, neutralised with N-HCl, and the DNA precipitated with ½ volume 30% TCA and the precipitate washed twice with 2 ml. 5% TCA. The combined supernatants and washings were diluted to 100 ml. for estimation

of RNAP. The precipitate was redissolved in 3 ml. 1-NaOH and diluted to 25 ml. with distilled water for DNAP estimation.

Protein nitrogen estimations were carried out on the remaining alkaline digest.

Estimation of RNAP

This was estimated as ribose by the orcinol colour reaction by a modification of the method of Mejbaum (1939). A standard solution of D-ribose (10 μg/ml) was also carried through the procedure and the amount of RNAP present in the tissue extracts calculated on the assumption that 10 μg ribose is equivalent to 4.13 μg RNAP.

To a 2 ml. aliquot (diluted to 3 ml. with distilled water) or a 3 ml. aliquot of the supernatant from the Schmidt-Thannhauser separation, 3 ml. orcinol reagent (600 mg. orcinol in 100 ml. 0.02% (w/v) $FeCl_3 . 6H_2O$ in concentrated HCl) were added. The tubes were capped and heated in a boiling water bath for 30 minutes. After rapid cooling the optical densities of the solutions were read in a Unicam SP 600 spectrophotometer at 665 mμ against a reagent blank (3 ml. distilled water and 3 ml. orcinol reagent), and compared with the colour of the standard (1 ml. standard ribose solution, 2 ml. distilled water and 3 ml. orcinol reagent).

Estimation of DNAP

DNAP was estimated by the method of Ceriotti (1952). To 2 ml. aliquots of the dissolved and diluted Schmidt-Thannhauser residue in glass stoppered tubes, 1 ml. 0.04% (w/v) indole in distilled water, and 1 ml. concentrated HCl were added. The tubes were capped and heated in a

boiling water bath for 10 minutes. After rapid cooling the solutions were extracted 3 times with about 4 ml. portions of chloroform, shaking for 30 seconds after each chloroform addition. The chloroform layers after the first and second extractions were removed with a pasteur pipette and after the third extraction the tubes were spun at about 500 r.p.m. for 5 minutes to separate the liquid layers. The colour intensity of the aqueous layer was measured in a Unicam SP 600 spectrophotometer at 490 mμ. Blanks and standards were also carried through this procedure. For the blanks 2 ml. distilled water and for the standard 2 ml. of a standard solution of DNA were used. The DNA standard was a purified sample of the sodium salt of calf thymus DNA prepared by the method of Kay, Simmons and Dounce (1952). A stock solution of DNA was prepared by dissolving an accurately weighed amount (about 20 mg.) of pure, dry DNA in distilled water, with a drop of alkali to help solution, and making up to 50 ml. 1 ml. of this solution was diluted with 0.5 N perchloric acid (PCA) and heated to 70°C for 20 minutes to redissolve any precipitated DNA and then made up to 50 ml. thus giving a standard solution. The amount of DNAP in the standard was estimated on 1 ml. portions of the solution by the method of Griswold, Humeller and McIntyre (1951) as described later, and was 2.66 μg DNAP/2 ml.

Estimation of Protein Nitrogen

Protein nitrogen estimations were carried out on the alkaline digest by the Nesslerization method of Paul (1958). 0.5 ml. aliquots of the alkaline digest were digested with 0.5 ml. 1% $CuSO_4$ (w/v) in 50% (v/v)

H_2SO_4 until the solutions were clear. The digest was washed into a measuring cylinder and the volume made up to 10 ml. with distilled water. To 2 ml. of this, 2 ml. Nessler's reagent (3.5g. gum acacia in 750 ml. distilled water added to a solution of 4g. KI (Analar) and 4g. HgI_2 (B.P.) in 25 ml. distilled water and then made up to 1 litre) was added and 3 ml. 2 N-NaOH. Blanks and standards consisting of 1 ml. of water and 1 ml. of a solution of ammonium sulphate (containing 100 μg N/ml.) respectively were carried through the same procedure. After allowing the colour to develop for 15 minutes the intensity was read in the Unicam SP 600 spectrophotometer at 490 mμ against the blank.

Estimation of Lipid P

The method of Criswold, Humoller and McIntyre (1951) was used. 1 ml. aliquots of the pooled lipid extracts in the case of adrenal analysis and 0.4 ml. in the case of liver analysis were evaporated to dryness in a graduated test-tube. To this 0.5 ml. 10 N-H_2SO_4 and 0.5 ml. 4N ~~perchloric acid~~ (PCA) were added and the mixture digested until the solutions were clear. 1 ml. of diluted standard solution (2.193g. KH_2PO_4 in 500 ml. distilled water; 1 ml. of this diluted to 500 ml. with water gives 2μ g. P/ml.) and 1 ml. of water were treated in the same way to give standard and blank readings. The tubes were sealed and the solutions diluted to approximately 3 ml. To this 0.5 ml. reducing agent (13.6g. sodium metabisulphite, 1g. Na_2SO_3. $6H_2O$ and 0.25g. 2 - naphthol - 1 amino - 4 sulphonic acid (B.D.H.) in 250 ml. distilled water) followed by 0.5 ml. 2.5 (w/v) ammonium molybdate were added with careful mixing after each

addition. The solutions were made up to 5 ml. and the tubes heated in a boiling water bath for 10 minutes. The intensity of the colour was read in a Unicam SP 600 spectrophotometer at 820 mμ against the blank solution.

Estimation of Cholesterol

This was carried out by the method of Zlatkis, Zak and Boyle (1953) as described by Knobil, Hagnay, Wildon and Briggs (1954). 0.5 ml. of homogenate was added to 10 ml. glacial acetic acid. To 3.5 ml. of this solution 3.5 ml. of colour reagent (1 ml. of a solution of $FeCl_3$ [10g. $FeCl_3$ dissolved in 100 ml. glacial acetic acid] made up to 100 ml. with concentrated H_2SO_4, and 2 ml. water added to prevent cloudiness) were added and the solution mixed and allowed to come to room temperature. Blanks consisting of glacial acetic acid and standards containing 20 μg cholesterol in glacial acetic acid were used. The colour was read in a Unicam SP 600 spectrophotometer at 560 mμ against the blank.

Estimation of Corticosterone

The fluorometric method of Silber, Busch and Oslapas (1958) was used. The rats were killed by the use of a guillotine and blood collected from the neck vessels using heparin in the collecting vessel as an anticoagulant, and the adrenals removed. For the analysis of blood corticosterone the plasma was used, this being separated from the red cells as soon as possible after collection. 0.2 to 0.5 ml. plasma was pipetted into a glass stoppered centrifuge tube and the volume diluted to 1 ml. with distilled water. 3 ml. redistilled 2,2,4-trimethylpentane was added and the tubes shaken for 30 seconds. After centrifugation the solvent layer was removed, the volume

made up to 5 ml. with distilled water and 15 ml. redistilled methylene chloride added. The corticosterone was extracted by shaking for 30 seconds and the tubes were then spun. The bulk of the aqueous layer was removed and discarded and 1 ml. 0.1 N-NaOH added and the tubes shaken again for 10-15 seconds. After centrifugation the alkaline layer was discarded. A 10 ml. aliquot of the methylene chloride extract was placed in a tube containing 2 ml. 30 N-H_2SO_4. The tube was then shaken for 30 seconds and after centrifugation the supernatant solvent layer was removed and discarded. After standing at room temperature for 30 to 90 minutes the fluorescence of the acid was determined using an Aminco Bowman spectrophotofluorometer at an exciting wavelength of 465 mμ and a fluorescent wavelength of 530 mμ. An Ilford filter No. 109 was used as recommended by Braunsberg and James (1960). The following slit arrangement was found to be the most satisfactory, where the numbers 1 to 6 are the standard numberings of the cell slits of the fluorometer:-

1	2	3	4	5	6
1/16"	1/32"	1/16"	1/16"	1/32"	1/16"

with a slit of 3/16" in the photomultiplier tube slit. All readings were taken at a meter multiplier reading of .001 and a sensitivity of 10.

A standard solution of corticosterone was prepared by dissolving 30 mg. corticosterone in 5 ml. absolute ethanol and diluting to 1 litre with distilled water to give a standard stock solution of 20 μ g/ml. which was diluted before use to give a solution containing 0.5μ g/ml. With each estimation 3 standards containing 0.1, 0.3 and 0.5μ g corticosterone and

and a blank consisting of 1 ml. of water were carried through the procedure, and the amount of corticosterone in the plasma calculated from the readings obtained from the standards.

For determination of the amount of corticosterone in the adrenal glands, the glands were first homogenised in 2 ml. 33% ethanol (redistilled), using a Potter Elvehjem homogeniser (1936), and the volume diluted to 10 ml. with distilled water. 3 ml. aliquots were used for estimation which was performed as described for plasma, except washing with 2, 3, 4-trimethylpentane was omitted. For correct comparison purposes the standards also contained the same amount of ethanol as did the unknowns.

Tests of Statistical Significance

In the present series of experiments the results obtained have been analysed statistically using an analysis of variance (Snedecor, 1946). Two types of approach have been used. Where only two groups of data are compared the variance ratios (F) are given which indicate the significance of the results. The expressions $P < 0.05$ and $P < 0.01$ are used in the conventional sense to indicate significance at the 5% and 1% levels respectively. Where more than two groups of data are compared simultaneously and significance is shown, the fiducial limits were calculated thus indicating which differences between individual treatments are significant ones.

RESULTS

SECTION I

Investigation of Techniques

During the course of this thesis some problems connected with the techniques used arose. Since the evaluation of all the experimental data depends on these technical investigations they are discussed now.

A. Investigation of lipid solvent treatment on the amount of protein nitrogen extracted from rat adrenals

Downie (1962) in experiments using rat livers found that part of the protein nitrogen of the liver is extracted during treatment of the tissue with lipid solvents prior to protein nitrogen estimation. A similar investigation has been carried out here to find how much of the adrenal protein nitrogen is lost during this procedure, and also how constant this loss is between different animals. It has also been desirable to investigate if the amount of nitrogen lost in the lipid solvents varies according to the treatment of the animal. For this purpose the experiment was carried out on both control and ACTH treated animals. To give an assessment of the amount of nitrogen lost, nitrogen analyses were carried out on the combined lipid extracts and compared with the total amount of nitrogen present in the tissue. As phospholipid present in the lipid extract contains nitrogen which is also estimated by the nitrogen assay method used, this has to be allowed for in the final calculation. Thus phospholipid estimations were also performed on the lipid extract and 0.45 of this value subtracted from the amount of

nitrogen present in the lipid solvents to give a true value of the amount of protein nitrogen extracted. This factor assumes that all the phosphorus in the lipid extract is phospholipid P and thus the protein nitrogen of the lipid solvents = total nitrogen of the lipid solvents - phospholipid nitrogen, where phospholipid nitrogen = total P of the extract x 0.45.

The analysis of rat adrenals for protein nitrogen and lipid phosphorus was carried out as described in the methods section. 5 ml. aliquots of the lipid extract were evaporated to dryness. 0.5 ml. SeO_2/H_2SO_4 reagent was added and digested for 2 hours. The volume was made up to 10 ml. and nitrogen estimated by nesslerization as described for the case of alkaline digests. 0.45 of the lipid phosphorus value was subtracted from the amount of nitrogen in the lipid extract to allow for the phospholipid nitrogen. In this investigation 3 rats were treated with 5 units ACTH twice daily for 3 days prior to killing and 3 rats were used as controls. Table 1 shows the amount of nitrogen in the alkaline digest and the amount in the lipid extract. From these results the percentages of the total nitrogen lost in the lipid extract is found to give an average of 26.3% in the control animals and 25.9% in the ACTH treated animals. Thus the loss of nitrogen into the lipid solvents is quite a considerable one but as this loss is not too variable from animal to animal it does not invalidate any results obtained subsequently in which this fraction is discarded. Also, the average loss is almost the same for both control and ACTH treated animals, indicating that this

TABLE 1.

The amount of protein nitrogen extracted from adrenal tissue by lipid solvents.

Treatment	Protein Nitrogen in alkaline digest. (µg/ 100g. body weight).	Protein Nitrogen in lipid solvents. (µg/ 100g. body weight).	% Protein Nitrogen lost in lipid solvents.
Control	240	98	28.9
	331	70	17.4
	249	121	32.7
			Average 26.3
ACTH	493	196	28.4
	459	159	25.7
	543	159	22.6
			Average 25.9

treatment of the animals has no effect on this loss and thus results obtained from two such groups may be directly compared even after lipid treatment. These results are similar to those obtained by similar investigations on liver (Downie, 1962) where it was found that about 22% of the protein nitrogen of the liver tissue used was lost into the lipid solvents.

B. Investigations of the method of RNA estimation

(1.) The effect of tissue extraction with lipid solvents on the estimation of the RNA content of the tissue

From their investigations on the estimation of RNA in liver, Hallinan, Fleck and Munro (1963) have shown that after extraction of the tissue with 10% TCA and subsequently with lipid solvents only about 80% of the RNA is recovered in comparison to tissue which is not extracted with lipid solvents. Similar investigations have been carried out using adrenal tissue to determine how much adrenal RNA is lost by extracting with lipid solvents. In this case, however, RNA estimations cannot be performed directly on the lipid solvents and thus the amount of RNA lost in the lipid solvents has to be obtained by difference. Therefore, RNA estimations have been carried out on tissue samples which have been treated with lipid solvents and also on samples which have not been treated with lipid solvents. As in the investigation of the amount of protein lost during lipid extraction, both control and ACTH treated animals have been used to determine whether this loss of RNA varies in treated animals compared to non-treated animals.

The analysis of rat adrenals for RNA was carried out as described previously except the adrenal homogenate was divided into two portions and one portion carried through the usual procedure while with the second portion the lipid extractions were omitted. The adrenals from 3 control rats and 3 ACTH treated rats were used as described in the previous section. In table 2 the amount of RNAP present in the adrenal tissue treated with and without lipid solvents is shown. From these observations it was found that an appreciable percentage is lost due to lipid extraction, there being an average loss of 17.4% in the control animals and 17.1% in ACTH treated animals. As in the case of the nitrogen loss in the lipid solvents some variation in the extent of the loss is observed. However, the fact that the average loss between the two groups of animals is constant indicates that treatment of the animals has had no effect on this loss and results obtained between the two groups may be directly compared. These results also agree with those of Hallinan et al (1963) who found that 16 - 22 % of the RNAP was lost into the lipid solvents.

(2) Comparison of estimation of RNA by orcinol and ultra-violet (U.V.) after 1 hour and 18 hours incubation periods.

The work done by Fleck and Munro (1962) on rat liver has shown that after a 1 hour incubation period prior to a Schmidt-Thannhauser separation the values obtained for the amount of RNA in the tissue estimated by orcinol and U.V. procedures agree very well. However, if the period of incubation is extended to 18 hours the value for RNA estimated by

TABLE 2

The loss of tissue RNAP by lipid extraction

The results given are for individual animals and are expressed as ug RNAP/100g. body weight.

Treatment	Tissue extracted with lipid solvent ug/100g. body wt.	Tissue not extracted with lipid solvents ug/100g. body wt.	% RNAP lost in lipid solvents
Control	8.3	8.8	5.9
	9.0	10.9	16.9
	8.1	11.5	29.3
			Average 17.4
ACTH	16.8	20.7	18.9
	17.0	20.6	17.5
	18.2	21.3	14.8
			Average 17.1

the U.V. method is greater than that obtained using the orcinol colour reaction. Thus the values obtained for the amount of RNA in adrenal tissue by both U.V. and orcinol procedures are compared in the present investigation.

Twelve pairs of rat adrenals were homogenised to a total volume of 50 ml. and 4 ml. aliquots taken for analysis. Prior to the Schmidt-Thannhauser separation half the tubes containing tissue were incubated for 1 hour and the other half for 18 hours. RNA analyses were carried out as described previously except 0.6N perchloroacetic acid (PCA) and 0.2N PCA were used instead of 30% and 10% TCA respectively in the Schmidt-Thannhauser separation. The volume of the combined supernatant and washings was made up to 10 ml. 0.1N with respect to PCA. The optical density of this solution was read at 260 mμ in a Beckman double-beam spectrophotometer. The amount of RNA present was calculated on the basis that an optical density of 1.00 is equivalent to 3.412 μg. RNAP/ml. RNA estimations were also carried out by the orcinol procedure. The values obtained for the amount of RNAP in the aliquot of tissue used, estimated by the two procedures, are given in table 3. These investigations showed that after 1 hour incubation the values obtained for RNAP estimated by orcinol and U.V. procedures agreed very well. However, after 18 hours incubation in alkali the U.V. estimation always gave a larger amount of RNA present than did the orcinol reaction. This is thus in agreement with the results of Fleck and Munro (1962), who concluded that the larger value obtained by the U.V. method after 18 hours

TABLE 3

Comparison of RNAP estimation by orcinol and ultra-violet methods after both 1 hour and 18 hours incubation

Extraction procedure	µg RNAP in tissue aliquot			
	1 hour incubation		18 hours incubation	
	Orcinol	U.V.	Orcinol	U.V.
Lipid extraction	22.8, 20.0 — Average 21.4	23.6, 19.8 — Average 21.2	23.1, 23.4 — Average 23.3	25.3, 25.2 — Average 25.3
No lipid extraction	24.2, 24.6, 24.3, 23.5 — Average 24.2	24.6, 24.6, 24.5, 23.9 — Average 24.4	25.9, 25.5, 24.4, 24.6 — Average 25.1	29.0, 29.0, 28.9, 29.0 — Average 29.0

incubation is due to breakdown of protein with the prolonged incubation period with release of U.V.- absorbing material. As the RNAP values estimated by the two different methods agree well after incubation of the tissue for 1 hour only, this may be taken as a suitable time of incubation. Comparison of the orcinol results for 1 hour and 18 hours incubation shows that there is a trivial increase in the orcinol value with the longer incubation, while using the U.V. method this increase with 18 hours incubation is considerably larger. Thus, if RNAP is estimated by the orcinol method, as was done in experiments described in this thesis, no great difference is incurred by using either a 1 hour or an 18 hour incubation period - in fact, we chose an 18 hour period.

In this investigation of the orcinol and U.V. procedures, we took the opportunity of confirming the action of lipid solvent treatment on RNA recoveries. 4 aliquots of the tissue homogenate were treated by the usual procedure of extracting with lipid solvents while 8 aliquots were not lipid extracted. This showed, as had been observed previously, that an amount of RNA is lost in the lipid solvents. Also DNAP estimations were carried out on the precipitate from the Schmidt-Thannhauser separation as described previously. The results of this investigation are presented in table 4. They show that a ~~lower~~ higher DNAP value is obtained after extracting the tissue with lipids compared to non-lipid extracted tissue. Thus although a lower estimate of RNAP is obtained by the method employed in this thesis due to loss in the lipid solvents, a higher value for DNAP is obtained when lipid solvent extraction is carried

TABLE 4

Effect of tissue extraction with lipid solvents on DNAP estimation

Extraction Procedure	µg DNAP in tissue aliquot	
	1 hour incubation	18 hours incubation
Lipid extraction	16.2	18.4
	17.2	18.7
No lipid extraction	17.5	17.6
	16.3	17.0
	15.3	-
	16.0	-

out. Thus the values given for DNAP in the subsequent investigations are probably more correct than if extraction with lipid solvents had been omitted.

In conclusion, these studies demonstrate that the data to be presented for the protein and RNA content of the adrenal gland are somewhat low, but that the effect is a constant one from animal to animal and irrespective of treatment. Consequently, we can accept comparisons between groups as being valid evidence of the correct picture.

C. Investigations of the method of estimating blood corticosterone

(1) Conditions of killing the rats

To obtain a true assessment of blood corticosterone levels it is necessary that the rat should be in an unstressed state at the time of killing. In the present series of investigations considerable difficulty was experienced in finding a suitable method of killing the animals which caused the minimum of stress. At first the rats were killed with ether anaesthesia and blood collected from the inferior vena cava. Both male and female rats had higher blood corticosterone levels than those found by other workers (Silber, Busch and Oslapas, 1958; Guillemin, Clayton, Smith and Lipscomb, 1958; Moncloa, Péron and Dorfman, 1959; Lipscomb and Nelson, 1960; Yates, Leeman, Glenister and Dallman, 1961; Kitay, 1961). For example, two female rats gave values of 74 and 81 μ g/100 ml. plasma for the individual rats, and the male rats 53 and 36 μ g/100 ml. plasma for two individual rats whereas other workers obtained values ranging from 9 to 28 μ g/100 ml. plasma. Ether has been shown to exert

a stressful influence on rats, causing a rapid increase in blood corticosterone (Kitay, 1961) and also in adrenal corticosterone (Holzbauer, 1957), and thus does not provide a suitable method of killing.

Killing the rats by means of nembutal was investigated to see if the stressing effect could be lessened. However, using female rats, considerably elevated results ranging from 60 to 116 μg./100 ml. plasma with a mean value of 93 μg were still obtained. Thus the use of nembutal did not provide a satisfactory method of killing as the rats were still being stressed, presumably due to the injecting and handling.

Corticosterone levels were also investigated in female rats which had been killed by stunning and blood collected from the blood vessels of the neck. This method also stressed the rats as shown by blood corticosterone levels of 112 and 114 μg./100 ml. plasma for two individual rats.

The use of nitrogen anaesthesia also provided an unsatisfactory method of killing as the corticosterone levels in female rats was found to range from 64 to 112 μg./100 ml. plasma with a mean of 90 μg./100 ml. plasma.

As the results obtained by these various methods of killing are all considerably higher than those of the other workers as mentioned above it was concluded that these killing procedures were all stressing the rat considerably.

It has been noted above that ether causes stress to the animals. Other workers have also shown that nembutal (Beigelman, Slushery Slater

and Roberts, 1956; Moncloa et al, 1959; Yates et al, 1961; Guillemin et al, 1958) causes an increase in corticosterone levels. Moncloa et al showed that there was a 77% increase compared to decapitated animals. Guillemin et al also showed that handling of the animals causes an increase in blood corticosterone. Thus from these observations it was concluded that killing the rats rapidly by means of a guillotine was the only means by which to obtain a true resting value of the corticosterone level thus preventing stressing the animal. Male rats killed by this method were found to have blood corticosterone levels ranging from 17 to 42 μg./100 ml. plasma with an average value of 25.8 μg./100 ml. plasma. Thus these values are comparable with those of other workers and therefore it was concluded that the use of the guillotine provides the least stressful method of killing. 3 individual female rats gave values of 42, 58 and 60 μg./100 ml. plasma, but Kitay (1961) has shown that female rats have corticosterone levels 2.5 times that of male rats, and thus these values are also comparable with those of other workers. A comparison of the blood corticosterone levels produced by the different methods of killing is given in table 5.

To assess the effects of handling as a stressing agent on rats an experiment was carried out in which the blood and adrenal corticosterone levels of handled rats could be compared with rats which had not been handled. Four groups of rats were used and all were fed on rat cake. Three groups were each kept in communal cages with four rats in each group for 4 days. Of these, one group was fed ad lib. for the duration

TABLE 5

The effects of different killing procedures on blood corticosterone levels

Method of killing	Sex of rat	Blood corticosterone (µg./100 ml. plasma)
Ether anaesthesia	Female	74 ; 81
	Male	53 ; 36
Nembutal	Female	Ranging from 60-116. Average 93
Stunning	Female	112 ; 114
Nitrogen anaesthesia	Female	Ranging from 64-112. Average 90
Guillotine	Male	Ranging from 17-42. Average 29.6
	Female	Ranging from 42-50. Average 53

of the experiment, one group fasted overnight prior to killing and the third group were handled twice daily for the 4-day period. The fourth group were kept in individual cages and fed on rat cake for the same length of time. At the end of the four-day period the rats were killed by means of a guillotine and the blood and adrenals analysed for corticosterone as described previously. Examination of the results of this experiment, given in table 6, shows that the handled rats have considerably higher corticosterone levels in both the blood and the adrenal glands. Overnight fasting and the keeping of the rats in individual cages as compared to communal cages was not found to have any effect on the blood and adrenal corticosterone levels. Thus from these results it is concluded that repeated handling of the animals produces a considerable stressing effect resulting in an increase in both blood and adrenal corticosterone. This occurred even although the animals were killed 18 hours after the last time of handling and thus the stressing effect produced by handling would seem to have a lasting influence causing elevated blood and adrenal corticosterone levels. Thus during the course of an experiment the animals should be handled as little as possible in order to ensure a low resting level of corticosterone. These results in table 6 are compared with the levels of corticosterone we found in rats on synthetic diets in the experiments described in section 2. The rats on the synthetic diet received a controlled amount of diet twice daily for a week and were found to have blood and adrenal corticosterone levels which exceeded those of control animals on a stock diet, even when these animals were handled twice daily. As it has been shown above that

Table 6

Effect of handling and of diet on

blood and adrenal corticosterone levels

Cage	Handling	Diet	Blood corticosterone (μg/100 ml.)	Adrenal corticosterone (μg/100 mg.)
Communal	None	Stock ad lib.	26	2.1
Separate	None	Stock ad lib.	23	2.0
Communal	None	Fasted 18 hr.	26	2.5
Communal	Twice daily for 4 days	Stock ad lib.	64	6.6
Separate	Twice daily for 7 days	Fed twice daily fixed intake of synthetic diet	107	10.4

Mean data from 4 rats in each group

handling, and so disturbance of the rats considerably increased blood and adrenal corticosterone levels, the high levels found in the rats on a fixed synthetic diet may be partly accounted for by the disturbance caused to the animals in the opening of cages and the removal and replacement twice daily of feeding dishes. Also the fact that the diet is restricted and the rats are perhaps slightly underfed would also account for the high corticosterone levels obtained in these experimental animals. Thus the high corticosterone levels found in the experiments subsequently described in section 2 may be attributed to disturbance of the animals and to the fact that food intake was restricted.

(2) Investigation of validity of corticosterone estimation

To test the validity of the method recovery experiments were carried out in which a known amount of corticosterone was added to plasma and carried through the usual procedure as described previously. Both plasma and plasma with added corticosterone were estimated for corticosterone. The results obtained are shown in table 7 and the method considered satisfactory.

TABLE 7

Recovery of Corticosterone when added to plasma

Initial amount of corticosterone in plasma. (µg)	Amount added. (µg)	Theoretical Total (µg)	Actual Total. (µg)	Recovery %
0.36	0.25	0.61	0.58	95
0.25	0.35	0.60	0.61	102
0.29	0.35	0.64	0.72	112
0.32	0.35	0.67	0.61	91
0.21	0.35	0.56	0.57	102
0.097	0.25	0.347	0.418	120
0.179	0.35	0.529	0.628	118
0.119	0.40	0.519	0.480	93

SECTION II

It had previously been demonstrated that feeding single amino acids to normal rats can cause an increased uptake of ^{32}P by liver RNAP (Munro and Mukerji, 1958) and increased deposition of liver glycogen (Munro and Mukerji, 1962). These effects were, however, abolished when the amino acids were fed to adrenalectomized rats. Also, Munro et al (1962) demonstrated that the adrenal gland is sensitive to ACTH even at low protein intakes. On administration of ACTH to protein deficient rats they observed a large increase in gland size and in the content of phospholipid protein and RNA.

The question thus arises, do the individual amino acids which increase liver metabolism in normal but not in adrenalectomized rats act by release of extra ACTH. The objective in these present experiments was to examine the effect of feeding these single amino acids on the size and composition of the adrenal glands of rats not receiving dietary protein. If the individual amino acids stimulate output of ACTH from the pituitary gland, they will cause an enlargement of the adrenal gland since, as pointed out above, the data of Munro et al (1962) show that the gland remains sensitive to ACTH, even in protein-depleted animals. Also, an attempt has been made to obtain a direct estimate of adrenal function by assaying the corticosterone content of the blood and adrenals of rats fed these single amino acids.

Since significant changes in adrenal composition take a few days to appear it was thought desirable to feed these amino acids for a length of time, in this case 11 days was the time chosen. Also, as control procedures

the incomplete proteins, gelatin and zein, and the complete proteins casein and zein to which tryptophan and lysine were added, were used. A technical difficulty in these experiments is the problem of dosage of these proteins and amino acids. Sufficient quantities must be fed to ensure that any effect which they may produce is large enough to be observed, while at the same time if the quantities of amino acids and incomplete proteins given are too large the animal will lose its appetite and so an insufficient amount of amino acid and food will be consumed. A special and additional problem is present in the case of methionine as Munro and Mukerji (1958) observed that if doses larger than 0.2 g. are fed the rats showed gastric retention when killed 18 hours later. Thus in these present experiments the amount of methionine fed was limited to 0.2 g. as over a duration of 11 days this probably represents the maximum amount of this amino acid which the animals will eat and absorb. In the case of glycine, however, Munro and Mukerji (1958) observed that at least 0.4 g. must be fed to obtain any effect - this is the threshold dose (see fig. 2). Thus in these experiments quantities of 0.5 g. were given which thus exceeds th threshold dose for a response.

The effect of the presence of single amino acids in the diet on the chemical composition of the rat adrenal gland and liver

Due to the fact that variations in the protein content of the diet cause only small changes in adrenal weight and composition it has been necessary to carry out these observations on a large number of animals to obtain a true assessment of any changes that occur. Thus in this investi-

Figure 2 shows the uptake of ^{32}P by liver RNA after administration of different doses of glycine, methionine or leucine to rats.

gation 10 rats have been fed on each diet and the adrenal composition studied.

Female rats of body weight 150 to 180 g. were used in these experiments. They were fasted overnight before commencing the experiment and then were fed for 3 days on a preliminary diet. In the morning they each received 1 g. of V.M.R. and 2.8 g. glucose (Dextrosol), and in the evening 4.2 g. of the protein diet. For the following 11 days the rats received the same morning meal as during the preliminary 3 day period but in the evening they received one of the following diets:-

1. 2.2 g. protein-free diet + 2 g. casein.
2. 4.2 g. " " "
3. 3.7 g. " " " + 0.5 g. glycine
4. 4.0 g. " " " + 0.2 g. DL-methionine
5. 3.7 g. " " " + 0.5 g. L-leucine
6. 2.2 g. " " " + 2 g. gelatin
7. 2.2 g. " " " + 2 g. zein
8. 2.05 g. " " " + 2 g. zein + 0.05 g. L-tryptophan + 0.1 g. L-lysine.

At the end of the 11 days the rats were killed by stunning and the adrenals and livers removed for analysis.

Table 8 shows the effect of feeding the above diets to rats on the chemical composition of the adrenal gland. Table 9 shows the results of Table 8 expressed relative to the value for the protein-free diet as 100%.

All the animals on these diets lost weight. This decrease in weight

was smallest in the rats receiving casein where an average of only 8 g. was lost. Those receiving the other complete protein diet, namely zein plus tryptophan and lysine, lost slightly more weight. The loss of weight in animals receiving these diets is presumably due to the fact that the amount of food eaten was restricted and thus they did not receive as much food as was required for continued growth. The rats receiving the other diets which contained either no protein, an incomplete protein or a single amino acid lost more weight. This larger loss may be attributed to the incomplete nature of the diets fed, which were therefore inadequate for growth and tissue maintenance, and thus in rats fed on diets of this nature a loss in body weight may be expected. The largest losses in weight occurred in rats fed the diets containing methionine and leucine; this is also due to the fact that a portion of these diets are left uneaten resulting in a larger loss in body weight. It will be noted that the rats fed methionine or leucine tended not to consume the diet completely - an average of 1 g. and 2.5 g. of diet per day respectively remained uneaten out of a total of 8 g., and thus this would account for the fact that these rats lost more weight than other groups, the leucine-fed rats having lost the most weight corresponding to the most food being left uneaten. All the rats receiving an inadequate protein intake, that is, those diets except casein and zein plus tryptophan and lysine, showed some considerable loss in weight but the rats receiving methionine or leucine had a larger loss in weight which may be attributed to their failure to consume all the food given. When these losses in body weight are compared

TABLE 8

The effect of the presence of single amino acids in the diet on adrenal weight, RNAP, DNAP, protein nitrogen and lipid phosphorus

The values given are the mean data of 10 replicates.

All diets were fully consumed except for those containing methionine and leucine.

Addition to protein-free Diet	Decrease in Body wt. (gm.) (6 w)	Adrenal wt. mg./ 100g. BW	DNAP µg/100g BW	RNAP µg/100g BW	Protein N µg./ 100g. BW	Lipid P µg/100g BW	Total Constituents (µg/100g.BW)	Adrenal wt. / DNAP	RNAP / DNAP	Protein N / DNAP	Lipid P / DNAP	Total constituents / DNAP
Casein	- 8	26.4	10.7	12.1	383	47.7	433.5	2.53	1.15	35.8	4.52	38.9
None	- 21	20.8	9.4	8.9	269	35.8	313.7	2.21	0.95	28.6	3.78	27.4
Glycine	- 22	24.6	10.4	10.4	335	40.2	385.8	2.32	0.99	31.4	3.81	29.3
Methionine	- 27	22.4	9.9	9.7	311	39.4	360.1	2.27	0.98	30.9	4.05	29.4
Leucine	- 40	20.6	9.6	10.1	284	32.0	326.1	2.18	1.06	27.7	3.41	27.4
Gelatin	- 31	21.1	9.4	9.1	293	36.3	338.3	2.26	0.96	30.4	3.85	25.7
Zein	- 21	23.8	10.0	10.7	329	43.0	382.6	2.40	1.07	32.7	4.32	31.2
Zein + tryptophan + lysine	- 13	24.2	9.9	11.1	335	41.9	388.2	2.51	1.13	33.9	4.30	32.1

TABLE 9

The results below are taken from table 8 expressed relative to the value for the protein-free diet as 100%.

Addition to Protein-free diet	Adrenal wt.	DNAP	RNAP	Protein N	Lipid P	Adrenal wt. / DNAP	RNAP / DNAP	Protein N / DNAP	Lipid P / DNAP	Total constituents / DNAP
None	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Casein	127.0	113.9	135.9	142.2	133.0	114.3	121.0	125.0	119.3	123.8
Glycine	118.2	110.6	116.8	124.5	112.0	104.8	104.0	109.8	100.8	107.0
Methionine	107.8	105.3	109.0	115.5	110.0	102.5	103.0	108.0	107.0	107.2
Leucine	99.0	102.1	113.3	105.5	115.0	93.5	111.5	96.8	90.2	100.0
Gelatin	101.5	100.0	102.1	108.8	101.3	102.0	101.0	106.3	101.8	104.7
Zein	114.5	106.2	120.1	122.2	120.0	108.5	112.7	114.3	114.0	113.8
Zein + tryptophan + lysine	116.2	105.2	124.7	124.3	117.0	113.5	119.0	118.5	113.8	117.0

TABLE 10

Statistical analysis of the data of table 8. The values given below are the variance ratios.

For $P < 0.05$ $F = 4.54$ indicated in the table by +.

$P < 0.01$ $F = 8.68$ indicated in the table by ++

Treatment (versus P.F.)	Adrenal wt.	DNAP	RNAP	Protein N	Lipid-P	Total constituents	Adrenal wt / DNAP	RNAP / DNAP	Protein N / DNAP	Lipid-P / DNAP	Total constituents / DNAP
Casein	9.1++	-	12.0++	11.5++	3.7	8.1+	9.3++	10.3++	14.3++	2.5	10.9++
Glycine	2.7	2.3	2.1	2.6	-	2.4	-	-	1.3	-	-
Methionine	0.8	-	-	1.6	-	-	-	-	-	-	-
Leucine	-	-	-	-	-	-	-	2.5	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-	-
Zein	-	-	-	2.5	1.04	-	-	2.9	5.49+	-	-
Zein + tryptophan + lysine	5.6+	-	7.7+	4.43	1.2	4.44	5.8+	8.0+	5.50+	-	4.1

with those observed by Munro et al (1962) on rats receiving an adequate protein or a protein-free diet at two levels of caloric intake, it is seen that they do not compare directly with any of the groups considered by those authors. This is because their diets gave a caloric intake of either 21 or 41 K cal per rat per day, while in the present series of experiments the caloric intake was 31 K cal per rat per day. Although the methionine and leucine fed rats failed to consume some of the diet this does not represent too large a portion of the individual amino acids to invalidate any results obtained as at least 87.5% of the methionine and 69% of the leucine given will have been eaten.

Adrenal Changes

Examination of the results of table 8 shows that a diet containing casein or zein plus tryptophan and lysine caused an increase in the weight of the adrenal gland as compared with the protein-free diet alone. The variance ratios obtained from an analysis of variance are shown in table 10. These indicate that the increase in weight of the adrenal glands is statistically significant at the 5% level of significance in the case of feeding the zein plus tryptophan and lysine diet, and at the 1% level in the case of the casein-containing diet. Glycine caused some increase in adrenal weight but this was not significant.

None of the diets caused a significant alteration in adrenal DNA content, although both casein and glycine showed a tendency for this constituent of the gland to increase. Munro et al (1962) found that rats fed an adequate protein diet had significantly more DNA in the adrenal than

those fed a protein-free diet. Although the present results show a tendency for this constituent to increase in rats fed an adequate protein diet, complete agreement with the results of Munro et al (1962) was not obtained, since the present results fail to attain statistical significance.

The presence of casein in the diet and of zein plus tryptophan and lysine caused a statistically significant increase in the RNAP content of the gland. ($P < 0.01$ in the case of casein and $P < 0.05$ in the case of zein plus tryptophan and lysine.) Although the glycine and zein diets tend to show some increase in RNAP, these were not significant.

The nitrogen content of the adrenal glands was significantly increased in the rats receiving a casein-containing diet ($P < 0.01$). An increase which just failed to attain statistical significance was observed in the adrenals of the rats receiving a zein with tryptophan and lysine diet. The presence of the other amino acids and proteins in the diet caused varying smaller non-significant increases in the amount of nitrogen in the gland.

The diet containing casein caused an increase in the lipid phosphorus content of the gland but this did not attain statistical significance. The diets containing zein or zein plus tryptophan and lysine also showed an increase which was not significant.

The "total" constituents of the gland (the sum of protein + RNA + phospholipid) also increased when an adequate protein diet, casein or zein plus tryptophan and lysine, was fed; this increase was significant in the case of the casein diet but the results for the zein plus tryptophan and lysine

diet just failed to attain significance.

These results are in agreement with those of Munro et al (1962) who found that rats receiving an adequate protein diet, namely one containing casein, had increased amounts of RNAP, protein and phospholipid in their adrenal glands, as compared to rats fed a protein-free diet.

Since the DNAP content of an organ can be taken as a measure of cell number (Davidson and Leslie, 1950; Thomson, Heagy, Hutchison and Davidson, 1953), expressing individual gland constituents in relation to DNAP would indicate whether the amount of individual constituents per cell have increased. If the ratio of adrenal weight to DNAP has increased, this may be taken as indicative of an increase in cell size and thus that hypertrophy has occurred. If hyperplasia occurs, the total amount of DNAP will also increase. Thus the present results have been expressed in relation to the DNAP content of the adrenal gland in order to determine whether changes in diet have affected the size and composition of the average adrenal cell. These results (table 8) showed that the presence of casein in the diet, and of zein with tryptophan and lysine, caused a statistically significant increase in the ratio of adrenal weight to DNAP, ($P < 0.01$ for the casein diet and $P < 0.05$ for the zein plus tryptophan and lysine diet), and this can thus be taken as an indication of adrenal hypertrophy. Similarly, these diets caused a statistically significant increase in the amount of RNAP and of protein nitrogen in the adrenal cell ($P < 0.01$ for the casein diet and $P < 0.05$ for the zein plus tryptophan and lysine diet). A diet containing zein alone also caused a significant increase in the protein nitrogen per

cell ($P < 0.05$). The diets containing casein, zein, or zein plus tryptophan and lysine showed increases in the amount of lipid phosphorus per cell but these were not significant. The addition of casein or zein plus tryptophan and lysine to the diet caused an increase in the total constituents per cell. This increase was significant in the case of the casein diet ($P < 0.01$) but the results failed to attain significance in the case of the zein plus tryptophan and lysine diet.

Thus the effect of feeding various diets to rats on adrenal size and composition is related to the nutritional completeness of the protein fed. Feeding the diets containing an adequate protein, namely casein, or zein plus tryptophan and lysine, caused significant alterations in adrenal size and in its individual constituents, compared to feeding a protein-free diet. Those diets which contained an inadequate protein or a single amino acid had no significant influence on the size and composition of the adrenal (table 10).

Liver Studies.

Since the experiments of Munro and Mukerji (1958) showed that feeding single doses of certain amino acids caused an increase in the uptake of ^{32}P by liver RNA and that this depended on intact adrenal glands, it was felt desirable to extend the present work to investigate the effect of prolonged administration of these amino acids on liver composition. The results of these analyses are given in tables 11 and 12. An analysis of variance was carried out on these results and the variance ratios are tabulated in table 13.

TABLE 11

The effect of the presence of single amino acids in the diet on liver weight, RNAP, DNAP, protein nitrogen and lipid phosphorus

The values given are the mean data of 10 replicates.

Addition to protein-free diet	Liver wt. g./100g body wt.(BW)	DNAP mg./100g BW	RNAP mg./100g BW	Protein N mg./100g BW	Lipid P mg./100g BW	Total constituents mg./100g BW	Liver wt. / DNAP	RNAP / DNAP	Protein N / DNAP	Lipid P / DNAP	Total constituents / DNAP
Casein	3.19	0.96	3.02	98.2	3.96	105.3	3360	3.20	102	4.17	110
None	2.94	0.98	2.55	64.2	2.83	69.7	3040	2.65	65	2.91	70
Glycine	3.35	1.01	2.85	77.2	3.20	83.2	3350	2.86	76	3.21	82
Methionine	3.23	0.90	2.46	69.7	2.81	75.1	3650	2.80	78	3.13	84
Leucine	2.77	0.84	2.41	63.3	2.59	68.3	3350	2.94	76	3.11	82
Gelatin	3.09	0.96	2.81	73.9	3.04	79.7	3250	2.99	77	3.18	82
Zein	2.86	0.90	2.56	76.8	3.14	82.8	3260	2.92	85	3.53	91
Zein + tryptophan + lysine	3.17	0.94	2.93	86.1	3.93	103.1	3390	3.16	102	4.21	109

TABLE 12

The results below are taken from table 11 expressed relative to the value for the protein-free diet as 100%.

Addition to protein-free diet	Liver wt.	DNAP	RNAP	Protein N	Lipid P	Total constituents	Liver wt. / DNAP	RNAP / DNAP	Protein N / DNAP	Lipid P / DNAP	Total constituents / DNAP
None	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Casein	108.5	97.8	118.5	153.0	140.0	160.3	110.5	120.6	157.0	143.6	160.2
Glycine	114.0	103.0	111.8	120,5	113.0	121.2	110.0	107.3	116.8	110.3	117.9
Methionine	109.5	91.7	96.5	108.5	99.3	111.3	120.0	105.8	120.0	107.5	121.3
Leucine	94.0	85.6	94.5	98.7	91.5	102.1	110.0	111.0	116.8	106.9	119.2
Gelatin	105.0	97.8	110.0	115.0	107.5	115.0	107.0	112.8	118.5	109.2	117.9
Zein	97.0	91.7	100.5	119.6	111.0	122.0	107.2	110.2	131.0	121.2	131.0
Zein + tryptophan + lysine	107.8	95.8	115.0	149.8	139.0	155.1	111.5	119.2	157.0	144.4	159.6

TABLE 13

Statistical analysis of the results of table 11

The values given below are the variance ratios.

For $P < 0.05$ $F = 4.54$ +

$P < 0.01$ $F = 8.68$ ++

Treatment (versus Protein free diet)	Liver wt.	RNAP	DNAP	Protein N	Lipid P	Total constituents	Liver wt. / DNAP	RNAP / DNAP	Protein N / DNAP	Lipid P / DNAP	Total constituents / DNAP
Casein	2.4	11.1++	-	21.7++	37++	22.5++	9.4++	32.8++	61.5++	51.5++	73.3++
Glycine	3.0	2.1	-	4.8+	3.0	5.0+	7.0+	5.8+	11.4++	3.1	55.9++
Methionine	-	-	3.1	-	-	1.3	25.6++	-	13.0++	-	65.0++
Leucine	-	-	12.8++	-	-	-	7.7+	4.2	6.5+	-	7.0+
Gelatin	-	-	-	5.1+	-	5.2+	4.0	6.2+	6.9+	-	7.0+
Zein	-	-	-	8.8++	-	8.9++	3.4	9.1++	29.1++	15.9++	31.0++
Zein + tryptophan + lysine	-	8.6+	-	31.1++	29.9++	33.6++	8.0+	14.7++	41.1++	33.8++	42.7++

Examination of table 11 shows that some variation in liver weights is observed with the different diets fed but none of the differences was statistically significant, showing that variations in the protein content of the diet had no effect on liver size. Very little change in the DNAP content of the liver was observed with the different diets fed except in the case of leucine where the DNAP content has decreased. This decrease is significant at the 1% level which would indicate a loss of liver cells in the rats fed this diet.

The diets containing casein or zein plus tryptophan and lysine caused a significant increase in the RNAP content of the liver. ($P < 0.01$ for the casein diet and $P < 0.05$ for the zein plus tryptophan and lysine diet.) Thus only the adequate proteins caused an increase in this constituent, since no change was observed on feeding the inadequate proteins or single amino acids. Munro and Mukerji (1958) showed that feeding glycine, methionine or leucine caused a significant increase in the RNAP of rat liver. However they observed an increase 18 hours after a single dose of the amino acid had been fed, whereas the present results show the effect of prolonged feeding. Thus the two sets of results are not directly comparable as, in the present investigations, the rats were in a protein-depleted state. All the diets fed, except methionine and leucine, caused a significant increase in the protein content of the liver. Methionine also caused some increase which did not attain statistical significance. Munro and Mukerji (1958) also showed an increase in the protein content of liver after feeding leucine. The effect was also observed 18 hours after feeding a single dose and so may

not be directly compared with the present results. A significant increase in the liver lipid phosphorus was observed on feeding the diets containing casein or zein plus tryptophan and lysine ($P < 0.01$). All the diets except leucine and methionine showed a significant increase in the total constituents; this increase is very large in the case of the adequate protein diets.

These results have also been expressed in relation to the DNAP content of the liver to indicate whether there are any changes in the average liver cell size and constituents. An increase in the ratio of liver weight to DNAP content is observed with each diet fed and, except in the cases of feeding gelatin or zein, these increases attained statistical significance, indicating an increase in the average size of the liver cell. The amount of RNAP per cell was increased after feeding each of the diets. This increase is statistically significant in each case except in those of methionine and leucine, although in the case of leucine the increase just fails to attain statistical significance. The nitrogen content per cell was significantly increased by feeding each of the different diets. Increases in the amount of lipid phosphorus per cell which were statistically significant were observed after feeding the diets containing casein, zein, or zein plus tryptophan and lysine. The total constituents per cell also show large significant increases after feeding each of the diets.

Thus a large number of changes in liver composition have been observed after feeding diets with varying nitrogen sources. Marked changes occurred in the size of the liver cell and in RNAP and protein content after feeding

many of the diets. In all cases the largest changes have occurred where an adequate protein is fed, and these diets have caused increases in every constituent except DNAP. The single amino acids, glycine, methionine and leucine caused large increases in both cell size and the amount of nitrogen per cell, and glycine also induced a significant increase in the cell content of RNAP. Gelatin and zein produced significant increases in the cell content of RNAP and protein but not in cell size. All the diets caused considerable increases in the total constituents per cell.

From these results it is apparent that the picture of ACTH stimulation of the adrenal is not obtained after feeding individual amino acids, as evidenced by lack of increase in adrenal size or in its constituents. Yet the liver shows distinct alterations in its main constituents. We are thus faced with a dilemma. Do the effects of single amino acids on liver RNA metabolism observed by Munro and Mukerji (1958; 1962) depend on adrenal function? It would seem that they must, since they are absent in adrenalectomized rats (Munro and Mukerji, 1962). Yet continued administration of these amino acids produces no evidence of ACTH stimulation. We know of course from the work of Munro et al (1962) that the adrenal of the protein-depleted rat is sensitive to ACTH and would thus show a recognisable change if amino acid action is mediated through ACTH. Consequently, it has been necessary to examine blood and adrenal corticosterone levels after giving individual amino acids. First, studies were made with single doses, in amounts known to cause changes in liver RNA metabolism. This yielded positive results, in the form of increases in blood and adrenal corticosterone levels, and the

question then was , do repeated doses of amino acids continue to stimulate the adrenals in the same way? Therefore, a second series of experiments were carried out to investigate the effect of prolonged amino acid feeding on blood and adrenal corticosterone. The results obtained here also showed increased corticosterone levels indicating stimulation of the adrenals, despite the absence of a change in cell size. These data are presented in the next section.

The effect of the presence of single amino acids in the diet on the level of corticosterone in the blood and in the rat adrenal gland.

The results in tables 14 and 15 show the effect of feeding diets containing various single amino acids on the concentration of corticosterone in the blood of the rat. In the first two experiments female rats of 150 to 180 g. body weight were used, but in subsequent experiments male rats were used in the same weight range. The rats were fed on a basal diet for a week, in the morning they received 1 g. V.M.R. and 2.8 g. glucose (Dextrosol) and in the evening 4.2 g. of the protein diet. On the eighth day they were fed 2 g. of the protein-free diet plus 1 g. of casein or of one of the following amino acids:- glycine, DL-methionine, L-leucine, DL-alanine, L-aspartic acid, L-glutamic acid. These amino acids were chosen from the group used by Munro and Mukerji (1958) in their investigation of the effect of feeding amino acids on the metabolism of liver RNA. Glycine, methionine and leucine were the ones they found to stimulate significantly incorporation of P^{32} into liver RNAP, whereas alanine, aspartic acid and glutamic acid showed the least effect. Thus the amino acids which showed the greatest and

TABLE 14

The effect of the presence of single amino acids in the diet on the level of blood corticosterone in the rat

The results given for the rats killed after two or four hours are the mean values of two experiments. Those for eight hours are the mean values of three to five animals and those for twenty-four hours are the mean values of seven to nine animals.

Results are expressed as ug. corticosterone/100 ml. plasma.

Time of Killing	Addition to Protein-Free Diet								Fasting
	None	Casein	Glycine	Methionine	Leucine	Alanine	Aspartic Acid	Glutamic Acid	
2 hrs.	72	-	83	105	125	-	-	-	44
4 hrs.	108	-	92	50	105	-	-	-	-
8 hrs.	99	83	93	104	102	91	118	86	85
24 hrs.	117	105	109	155	139	99	100	96	74

TABLE 15

The results below are taken from table 14 expressing all the values relative to a protein-free value of 100%.

Time of Killing	Addition to Protein-Free Diet								Fasting
	None	Casein	Glycine	Methionine	Leucine	Alanine	Aspartic Acid	Glutamic Acid	
2 hrs.	100	-	114	147	174	-	-	-	64
4 hrs.	100	-	95	49	91	-	-	-	-
8 hrs.	100	91	101	111	120	104	141	116	109
24 hrs.	100	86	85	125	107	80	82	76	82

the least effect on liver RNA were chosen, to determine whether a similar degree of response was present in the adrenal. The rats were killed at intervals of 2, 4, 8 and 24 hours, after receiving the amino acid meal, by means of a guillotine and the blood collected and the adrenals removed for analysis of corticosterone.

The results (tables14 and 15) obtained, show that the presence of methionine or leucine in the diet causes an increase in the blood corticosterone 2 hours after feeding, but there seems to be no increase at the 4 hour time interval. The animals killed after 8 hours show some increase in blood corticosterone after feeding methionine, leucine or aspartic acid, and after 24 hours considerable increases are seen in the blood corticosterone levels of those rats fed methionine or leucine, whereas other amino acids caused a depression at 24 hours. An analysis of variance was carried out on the values obtained for the rats killed 24 hours after feeding using the values expressed relative to a protein-free value of 100. By analysis of variance, the treatments were found to have a significant effect at the 1% level with fiducial limits of 28. This means that the presence of methionine in the diet causes the blood corticosterone to be raised significantly above the level of fasted rats and of those which are fed diets containing casein, glycine, alanine, aspartic acid or glutamic acid. Feeding leucine also causes a statistically significant increase in the concentration of blood corticosterone compared to feeding glutamic acid (figure 3).

The results in tables 16 and 17 show the effect of feeding single amino acids on the concentration of corticosterone in the rat adrenal gland.

TABLE 16

The effect of the presence of single amino acids in the diet on the concentration of corticosterone in the rat adrenal gland

The results given for the rats killed after two or four hours are the mean values of two experiments. Those for eight hours are the mean values of three to five animals and those for twenty-four hours are the mean values of seven to nine animals.

Results are expressed as ug. corticosterone/100 gm. body weight.

Time of Killing	Addition to Protein-Free Diet								
	None	Casein	Glycine	Methionine	Leucine	Alanine	Aspartic Acid	Glutamic Acid	Fasting
2 hrs.	2.46	-	2.74	2.71	2.66	-	-	-	1.23
4 hrs.	1.96	-	2.52	2.36	2.18	-	-	-	-
8 hrs.	2.03	1.74	2.27	2.56	1.91	2.46	1.84	1.85	1.12
24 hrs.	2.37	2.42	2.62	2.88	2.46	2.04	2.08	1.89	1.80

TABLE 17

The results below are taken from table 16 expressing all the values relative to a protein-free value of 100%.

Time of Killing	Addition to Protein-Free Diet								Fasting
	None	Casein	Glycine	Methionine	Leucine	Alanine	Aspertic Acid	Glutamic Acid	
2 hrs.	100	-	112	110	108	-	-	-	51
4 hrs.	100	-	142	121	102	-	-	-	-
8 hrs.	100	104	108	156	150	157	151	173	122
24 hrs.	100	106	129	143	142	121	116	95	58

Figure 3 illustrates the increases produced in blood and adrenal corticosterone levels 24 hours after feeding the single amino acids, methionine or leucine.

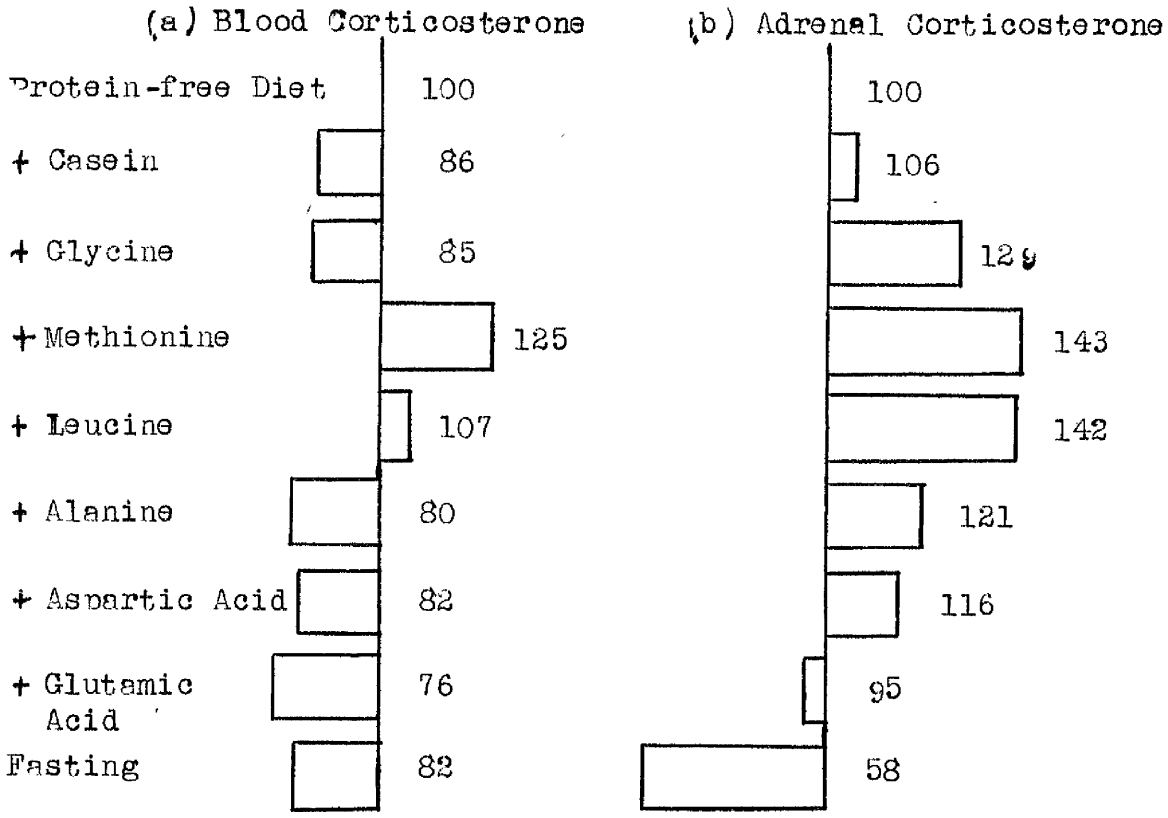

(a) Blood Corticosterone
(b) Adrenal Corticosterone
Protein-free Diet
100
100
+ Casein
86
106
+ Glycine
85
129
+ Methionine
125
143
+ Leucine
107
142
+ Alanine
80
121
+ Aspartic Acid
82
116
+ Glutamic Acid
76
95
Fasting
82
58

Figure 3

After 2 hours glycine, methionine and leucine have each caused some increase in the concentration of corticosterone in the gland compared with the protein-free level. After 4 hours the increase due to feeding glycine or methionine is larger. 8 hours after feeding, all diets have caused some increase in the adrenal corticosterone level, methionine, leucine, alanine, aspartic acid and glutamic acid having caused the largest increases. 24 hours after feeding, the results obtained show that each diet except glutamic acid caused some increase in the adrenal corticosterone, methionine and leucine having caused the largest increases. However, an analysis of variance shows that none of these changes are statistically significant.

Although the data obtained in this experiment show considerable variation at different times after feeding, it is apparent that feeding methionine or leucine has a stimulating action on the adrenal causing a significant increase in blood corticosterone levels at 24 hours after feeding. These amino acids also appear to be exerting some effect on the adrenal corticosterone levels although the increases observed did not attain statistical significance. Other amino acids caused changes in blood corticosterone levels but they were irregular and only the effects of methionine and leucine were sustained throughout the 24 hour period.

The effect of prolonged administration of diets containing single amino acids on the level of corticosterone in the blood and adrenal gland of the rat.

In these experiments, rats were fed for 11 days on diets containing various single amino acids or proteins and the effect on the concentration

of corticosterone in the blood and adrenals examined. Male rats of about 130 g. body weight were used. They were fed for 3 days on a preliminary diet containing casein and for the subsequent 11 days were fed on the same diets as described in the experiments in which the adrenal composition was examined, when individual amino acids and proteins were added to a protein-free diet. After this 11-day feeding period, the rats were killed by means of a guillotine and the blood collected, and adrenals removed for analysis of corticosterone.

Examination of the results of this experiment, given in table 18, shows that the rats receiving any of the amino acids or proteins in addition to the protein-free diet showed some elevation in their blood corticosterone levels in comparison to the group receiving the protein-free diet alone. The rats receiving casein show a very large elevation in the blood corticosterone level and considerable increases are also seen when glycine, methionine, leucine or zein plus tryptophan and lysine are added to the diet. An analysis of variance was carried out on these results but, due to the variation between animals, these differences were not statistically significant.

The levels of corticosterone in the adrenal gland also increased markedly in rats which had received casein, glycine, methionine, leucine or zein plus tryptophan and lysine added to the diet. Analysis of variance of these results showed them to be statistically significant at the 5% level, fiducial limits, being ± 1.08. Thus the adrenal corticosterone of the casein, methionine and leucine fed rats is significantly higher than that of the protein-free animals, while that of zein plus tryptophan and lysine fed

TABLE 18

The effect of prolonged administration of diets containing single amino acids or proteins on the level of blood and adrenal corticosterone in the rat

The results given are the average of 6 investigations.

Addition to protein-free diet	Blood corticosterone ug./100 ml. plasma	Adrenal corticosterone ug./100 g. body weight
None	70	1.29
Casein	126	2.40
Glycine	99	2.28
Methionine	97	2.41
Leucine	101	3.54
Gelatin	77	1.23
Zein	83	1.26
Zein + tryptophan + lysine	91	2.35

animals just fails to attain statistical significance. Also, casein, methionine, leucine and zein plus tryptophan plus lysine-fed animals show a significant increase in adrenal corticosterone concentrations compared to the rats on the diet containing added gelatin or zein (figure 4).

Thus both the blood and the adrenals of rats receiving the diets containing casein, glycine, methionine, leucine and also zein to which tryptophan and lysine have been added, show an increase in the corticosterone levels, although statistical significance is not attained in all cases. Consequently, these results suggest that the capacity of the adrenal gland to build up corticosterone does not depend on increase in cell constituents, as no change in cell constituents above the levels on a protein-free diet, was observed in the previous experiments when these amino acids were fed. The significance of these findings is as follows. ACTH causes an outpouring of cortical hormones, followed later by enlargement of the adrenal cortex. This latter occurs even in animals on a protein-free diet (Munro et al, 1962). Here, we observe the action of single amino acids on adrenal corticosterone formation but no effect on gland size; i.e., one part of the action of ACTH without the other. It seemed therefore desirable to complete this series of experiments by demonstrating under our experimental conditions, that adrenal corticosterone level is sensitive to ACTH administration.

<u>The effect of protein deficiency and ACTH administration on the concentration of corticosterone in blood and adrenals.</u>

Munro et al (1962) showed that, although the adrenal weights and adrenal constituents decreased when rats were fed on a protein-free diet, the glands

Figure 4 compares the effect of feeding single amino acids or proteins for 11 days on blood and adrenal corticosterone levels with the effect produced on the average adrenal cell weight. This demonstrates how a complete protein, namely casein, or zein plus tryptophan and lysine, can cause increases in both adrenal cell size and in blood and adrenal corticosterone levels. On the other hand, the single amino acids, glycine, methionine and leucine show changes in blood and adrenal corticosterone levels but no change in adrenal cell size.

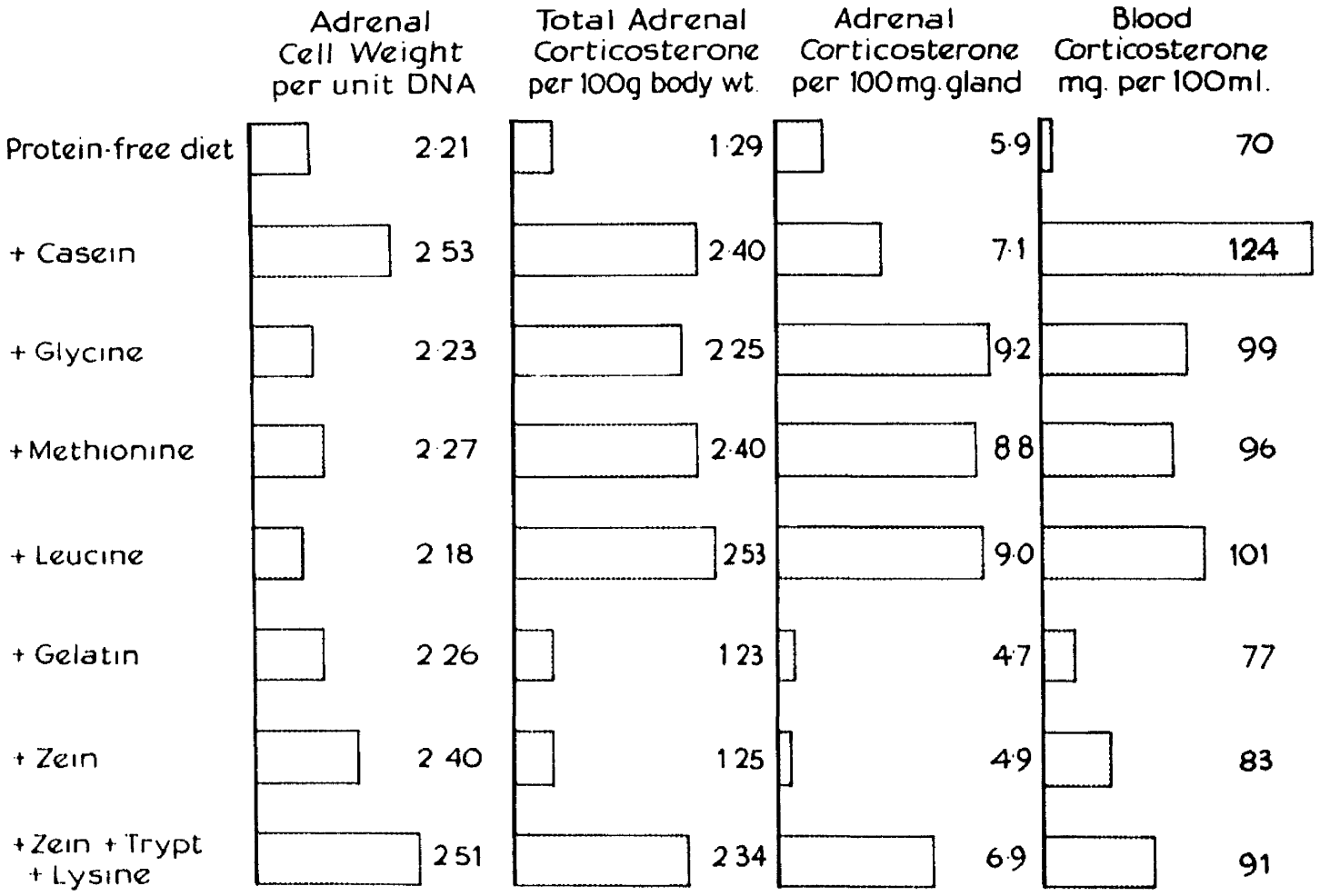

Adrenal Cell Weight per unit DNA
Total Adrenal Corticosterone per 100g body wt.
Adrenal Corticosterone per 100mg. gland
Blood Corticosterone mg. per 100ml.
Protein-free diet
2·21
1·29
5·9
70
+ Casein
2·53
2·40
7·1
124
+ Glycine
2·23
2·25
9·2
99
+ Methionine
2·27
2·40
8·8
96
+ Leucine
2·18
2·53
9·0
101
+ Gelatin
2·26
1·23
4·7
77
+ Zein
2·40
1·25
4·9
83
+ Zein + Trypt + Lysine
2·51
2·34
6·9
91

Figure 4.

were still able to respond normally to ACTH as shown by an increase in gland size and constituents. Therefore we examined the corticosterone levels of the blood and adrenal glands of rats under these experimental conditions. Rats were fed on a protein-free diet or on a protein-free diet to which casein was added for 11 days as in the previous experiments. For the last 3 days of the experiment they were injected with 5 units ACTH twice daily. The blood and adrenals were analysed for corticosterone and the results compared with those obtained from control animals on the same diets. The results are presented in table 19.

ACTH administration caused considerable increases in the adrenal weights of rats fed on both a protein-free diet and on a casein containing diet, but those receiving casein showed a somewhat larger increase in adrenal size after ACTH administration. The total amount of corticosterone in the gland also shows a large increase in both dietary groups in response to ACTH. The concentration of corticosterone in the adrenal glands is dependent on the relationship of increase in corticosterone to increase in gland size caused by ACTH; and in the present investigation the concentration of corticosterone in the adrenal glands of the casein-fed rats shows a decrease after ACTH treatment. This is due to the fact that the gland size has increased to a greater extent than has the adrenal corticosterone thus resulting in a decrease in adrenal corticosterone concentration. The corticosterone concentration of the rats receiving a protein-free diet shows an increase. However, this may not be a true assessment, as only one value has been obtained for these ACTH treated animals on the protein-free diet and

TABLE 19

The effect of protein deficiency and ACTH administration on the level of corticosterone in blood and adrenals

The figures given are results from individual rats.

Diet	Adrenal weight mg/100g initial body weight		Adrenal corticosterone				Blood Corticosterone µg/100ml. plasma	
			µg/100g initial body weight		µg/100mg. adrenal weight			
	Control	ACTH	Control	ACTH	Control	ACTH	Control	ACTH
Protein-Free	23.4	42.2	2.5	4.2	10.4	10.0	82	78
	17.7	-	1.3	-	7.5	-	83	-
	20.5	42.2	1.9	4.2	9.0	10.0	83	78
Protein-Free + Casein	30.4	65.7	3.4	4.3	11.2	6.5	(307)?	88
	26.9	64.0	2.9	4.9	10.8	7.7	63	104
	28.7	64.9	3.2	4.6	11.0	7.1	185	96

as this value is lower than one of the control values and higher than the other control value no definite conclusions can be drawn from this particuler result.

The blood corticosterone levels after ACTH treatment show a decrease in both dietary groups of animals. This decrease is small in the case of the rats receiving a protein-free diet but quite considerable in the rats fed casein. As only one result has been obtained from the rats fed a protein-free diet and treated with ACTH, this decrease observed in blood corticosterone may not be taken as a true assessment of the picture. The large decrease in blood corticosterone levels observed in the casein fed animals treated with ACTH is due to the presence of a very high control value of 307 for one of the rats. This result may be treated with suspicion due to its unusually high value; it is also possible that it may be incorrect as in this particular case insufficient blood was obtained to perform the assay in duplicate. If this value is neglected, an increase would be shown in the blood corticosterone after ACTH treatment which would seem to be a more acceptable picture. Thus no conclusion can be safely drawn from this result. However, the experiment has shown conclusively that the rats responded to ACTH by an increase in adrenal corticosterone content as well as in gland size when either a protein-free or a casein containing diet is fed.

The general picture thus shows that ACTH increases both total gland weight and total corticosterone content. This contrasts with the action of single amino acids, which induce an increment only in corticosterone.

SECTION III

Various workers have observed changes in the adrenal cortex of animals fed excessive amounts of cholesterol (Krylow, 1914; Sternberg, 1915; McMillan et al, 1954; Reinecke, 1928; Kay and Whitehead, 1935; Bernick and Patek, 1961). Their observations were, however, confined to increases in the size of the gland and in its lipid content. In the present series of investigations chemical analyses have been performed to examine the type of change that occurs in the adrenals of cholesterol-fed rabbits. Histological and histochemical investigations have been carried out.

The effect of the presence of cholesterol in the diet on the chemical composition of the rabbit adrenal gland

In the first series of experiments, rabbits were fed on a diet containing 1% cholesterol for 14 weeks in one experiment and 15 weeks in another experiment. Table 20 shows the changes observed in the adrenal size and chemical constituents of rabbits fed on this cholesterol diet, compared with a control group of animals fed on the same amount of diet without cholesterol. The data presented are the average of the 14 week and 15 week experiments.

A large increase in adrenal weight (+ 146%) was observed in the cholesterol-fed group. Statistical analysis (table 22) showed this increase to be significant at the 1% level. The cholesterol content of the glands showed an even larger increase (+ 403%). The RNAP and protein content of the glands also increased significantly ($P < 0.01$,

TABLE 20

The effect of cholesterol feeding on adrenal gland weight, cholesterol, DNAP, DNAP, protein nitrogen and lipid phosphorus.

The values given are the mean of 10 replicates and are expressed per Kg. final body weight.

Gland Constituent	Control Series	Cholesterol-fed	Difference
Gland wt. (mg.)	78.0	192.0	+ 146%
Cholesterol (mgm.)	6.7	33.8	+ 403%
DNAP (µg.)	41.7	61.2	+ 47%
DNAP (µg.)	24.9	27.7	+ 11%
Protein nitrogen (mg.)	1.00	1.52	+ 52%
Lipid P (µg.)	144.0	176.0	+ 22%

$P < 0.05$ respectively). Smaller insignificant increases occurred in the DNAP and phospholipid. The fact that the DNAP content of the gland has not markedly increased indicates that the increase in gland size is not due to an increase in the number of cells, and therefore that the increase is due to enlargement of the existing cells - i.e. hypertrophy.

The gland size and constituents have also been expressed in relation to its DNAP content (table 21) and this substantiates the conclusion that gland hypertrophy has occurred. The ratio of adrenal weight to DNAP shows a large increase which is statistically significant ($P < 0.01$), indicating an increase in cell size. The amount of cholesterol per cell also shows a very large increase (+ 356%). The RNAP and protein content per cell showed smaller but significant increases ($P < 0.01$). A considerably smaller increase in lipid phosphorus was observed but this was not significant.

Histological examination of the glands (carried out by Dr. W. Forbes) showed that the adrenal cortex of the cholesterol-fed animals was about twice as broad as normal, this increase being confined to the zona fasciculata. The glomerulosa was not notably increased while the reticularis had shrunk to a thin layer of cells incompletely surrounding the medulla.

The cells of the zona fasciculata were enlarged to about four times the area of the fasciculata cells in the control group, as seen in cross section. These enlarged cells stained weakly with haemalum and eosin,

TABLE 21

The effect of cholesterol feeding on the constituents of the adrenal gland expressed relative to the DNAP content of the gland

Gland Constituent	Control Series	Cholesterol-fed	Difference
Gland wt. (mg.)	3.7	7.2	+ 94%
Cholesterol (µg.)	276.0	1277.0	+ 356%
DNAP (µg.)	1.7	2.2	+ 33%
Protein nitrogen (µg.)	40.0	53.0	+ 33%
Lipid P (µg.)	6.0	6.4	+ 7%

TABLE 22

Statistical Analysis of the result of tables 20 and 21

The values given below are the variance ratios.

For $P < 0.05$ $F = 4.49^{*}$

$P < 0.01$ $F = 8.53^{**}$

Gland constituent	Variance Ratio
Adrenal weight	25.0 **
Cholesterol	12.4 **
RNAP	10.9 **
DNAP	0.9
Protein nitrogen	5.2 *
Lipid phosphorus	2.5
$\frac{\text{Adrenal weight}}{\text{DNAP}}$	14.6 **
$\frac{\text{Cholesterol}}{\text{DNAP}}$	30.0 **
$\frac{\text{RNAP}}{\text{DNAP}}$	88.0 **
$\frac{\text{Protein nitrogen}}{\text{DNAP}}$	21.5 **
$\frac{\text{Lipid P}}{\text{DNAP}}$	0.9

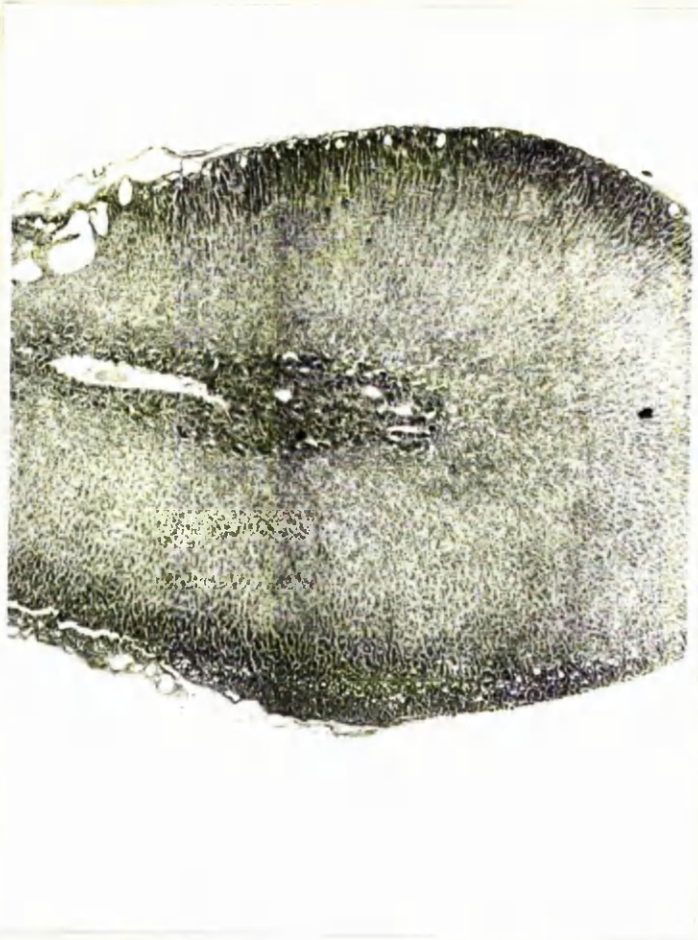
Figure 5. Sections of rabbit adrenals stained with haematoxylin and eosin and magnified 30 times.

(a) Section from a control animal fed on stock diet.

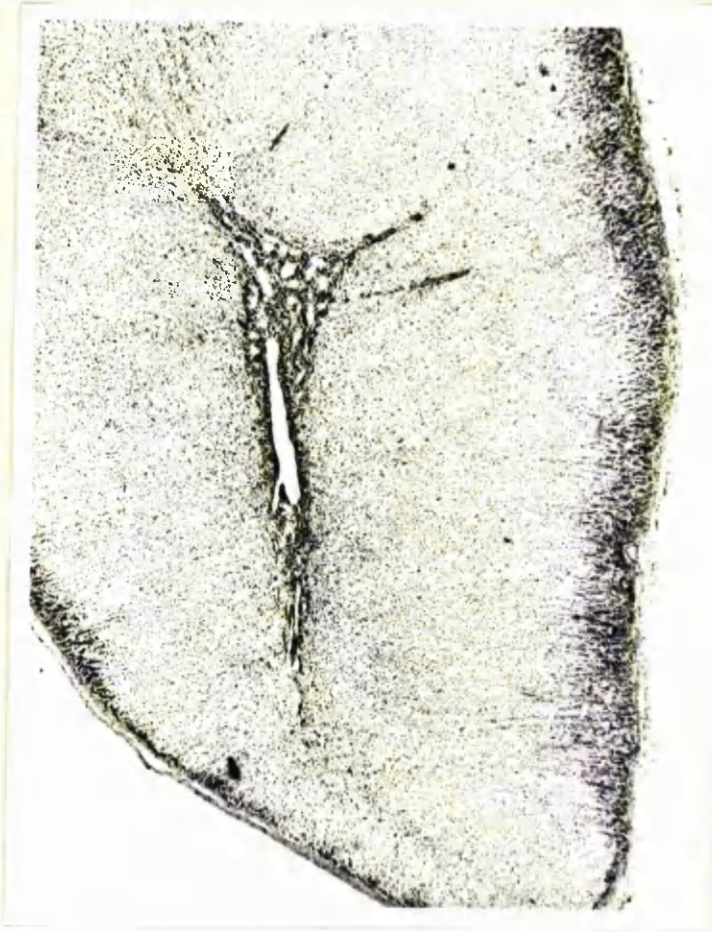
(b) Section from a cholesterol-fed animal showing increase in width of zona fasciculata.

(c) Section from ACTH treated animals fed on stock diet showing increase in width of both zona fasciculata and zona reticularis.

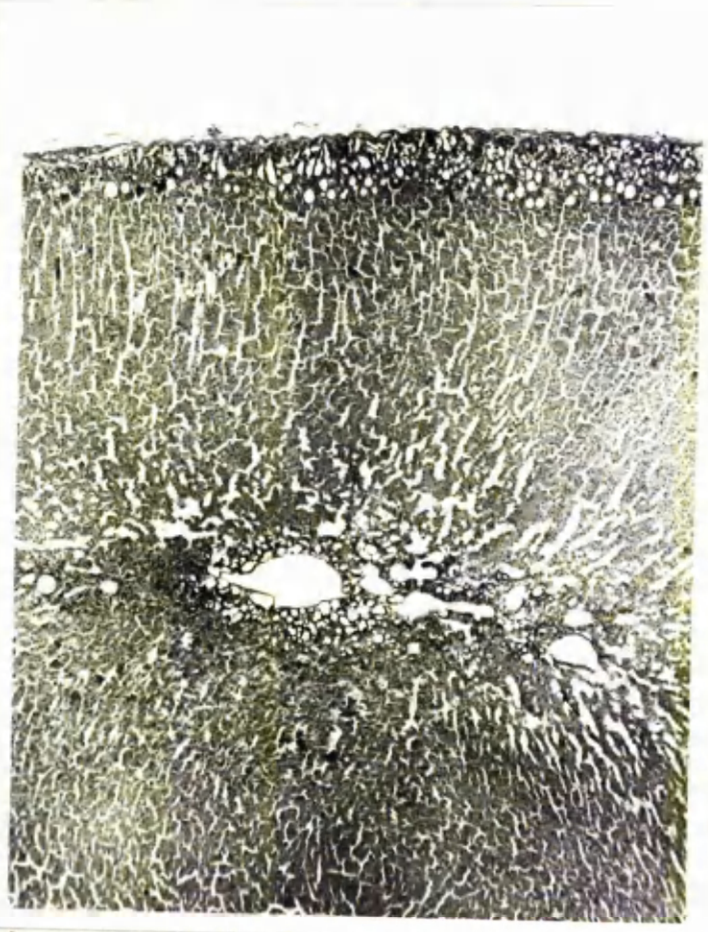
(d) Section from ACTH treated cholesterol-fed animal showing similar changes to those produced by treating a control animal with ACTH.



(a)



(b)



(c)

(d)

Figure 5.

Figure 6. Sections of rabbit adrenals stained with haematoxylin and eosin showing the zona fasciculata cells magnified 500 times.

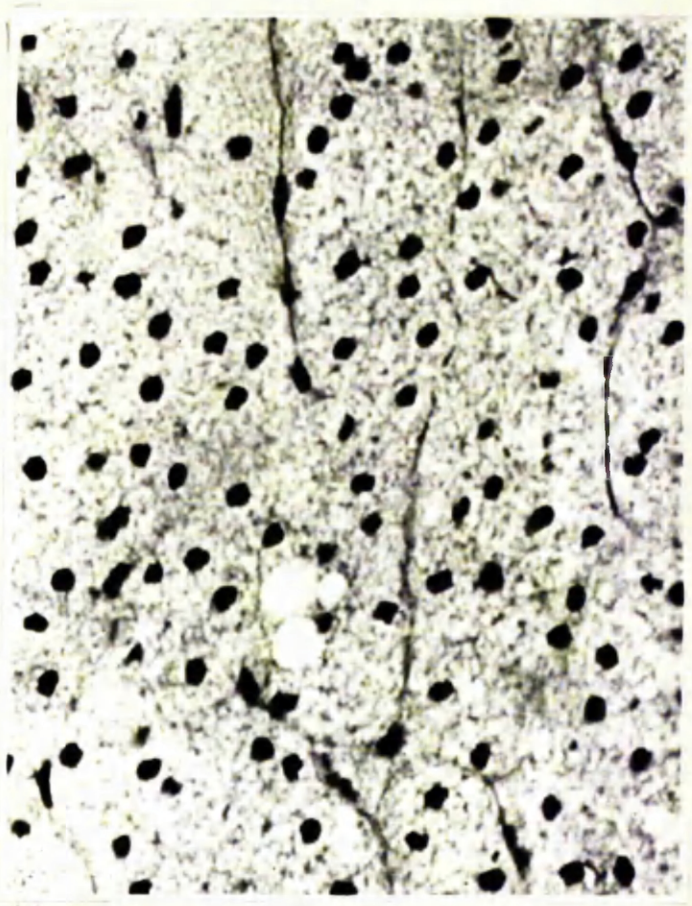
(a) Cells from a control animal on stock diet, showing usual size of cells arranged in typical columns.

(b) Cells from a cholesterol-fed animal showing enlarged cells with transparent, foamy cytoplasm and loss of columnar arrangement.

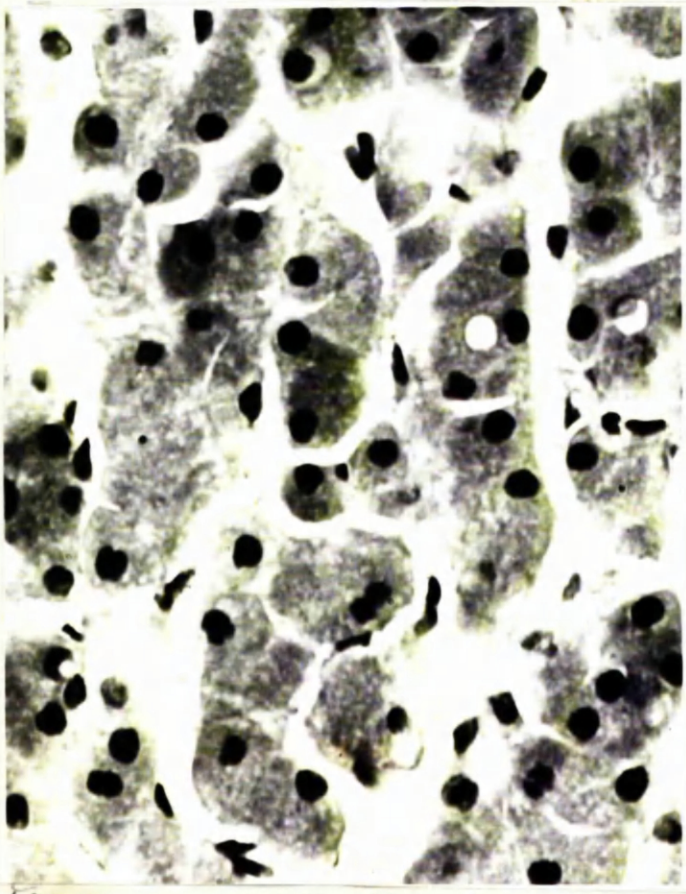
(c) Cells from an animal on stock diet treated with ACTH, showing cell enlargement and dark-staining cytoplasm, with partial loss of columnar arrangement and prominent blood vessels between the cells.

(d) Cells from a cholesterol-fed animal treated with ACTH, showing cell enlargement and dark-staining cytoplasm. The changes caused by ACTH treatment of the cholesterol-fed rabbit are essentially similar to those observed in the control animal after ACTH administration.

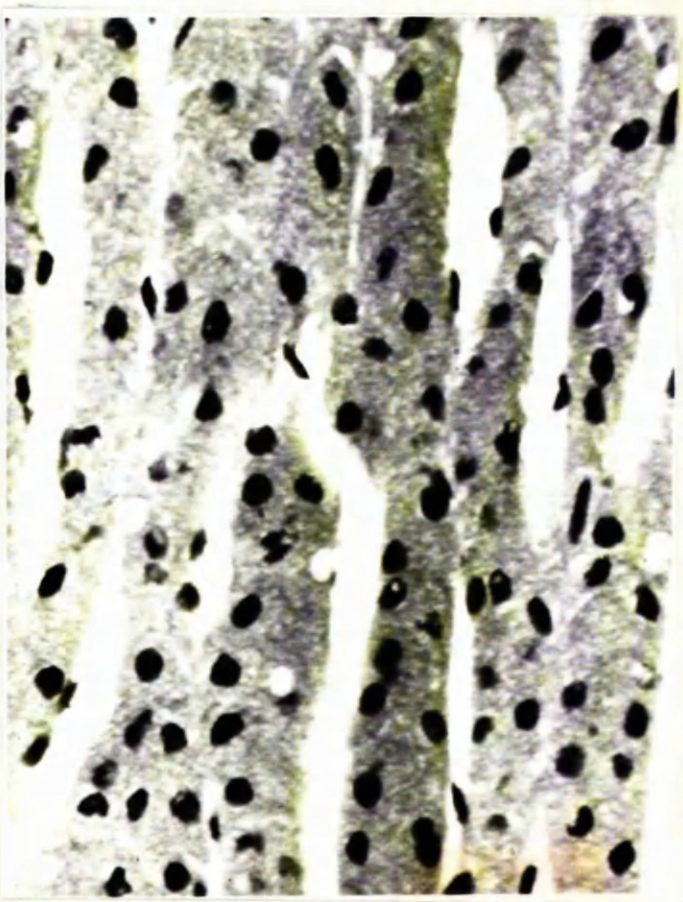
(a)



(b)



(c)



(d)

Figure 6.

Figure 7. Sections of rabbit adrenals stained with Sudan III to show the distribution of neutral fat; magnified 30 times.

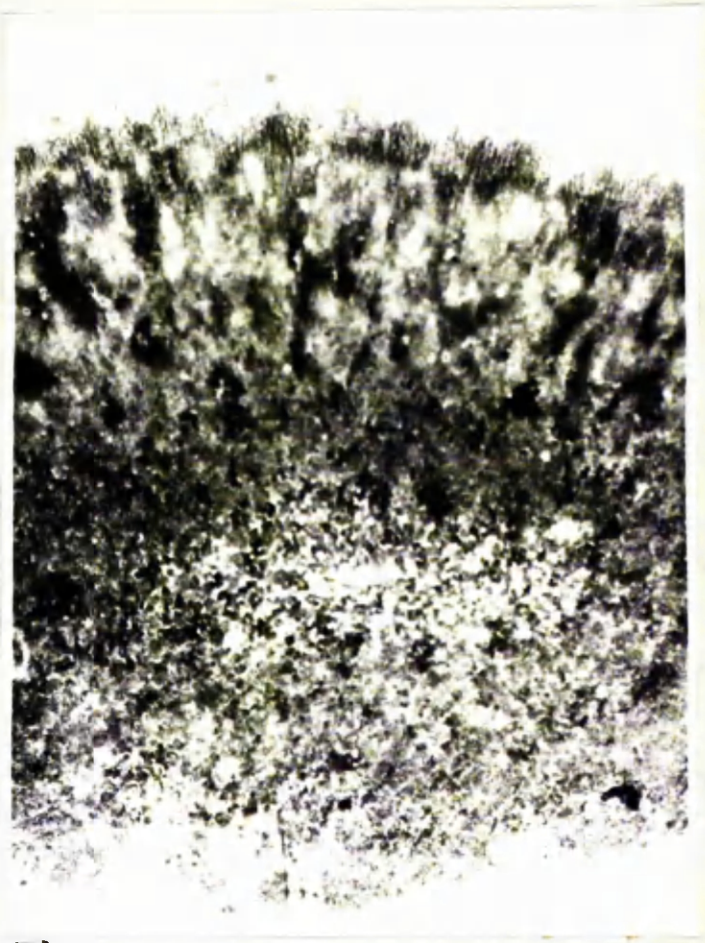
(a) Section from a control animal on stock diet showing very little neutral fat in the zona glomerulosa with a larger amount in the zona fasciculata and zona reticularis.

(b) Section from a cholesterol-fed animal showing changes in the fat distribution.

(c) Section from an ACTH-treated rabbit fed on stock diet showing increase in neutral fat in the zona glomerulosa with a decrease in the zona fasciculata.

(d) Section from an ACTH-treated rabbit fed cholesterol showing changes in distribution of fat which are essentially similar to those produced by treating a control rabbit with ACTH.

(a)



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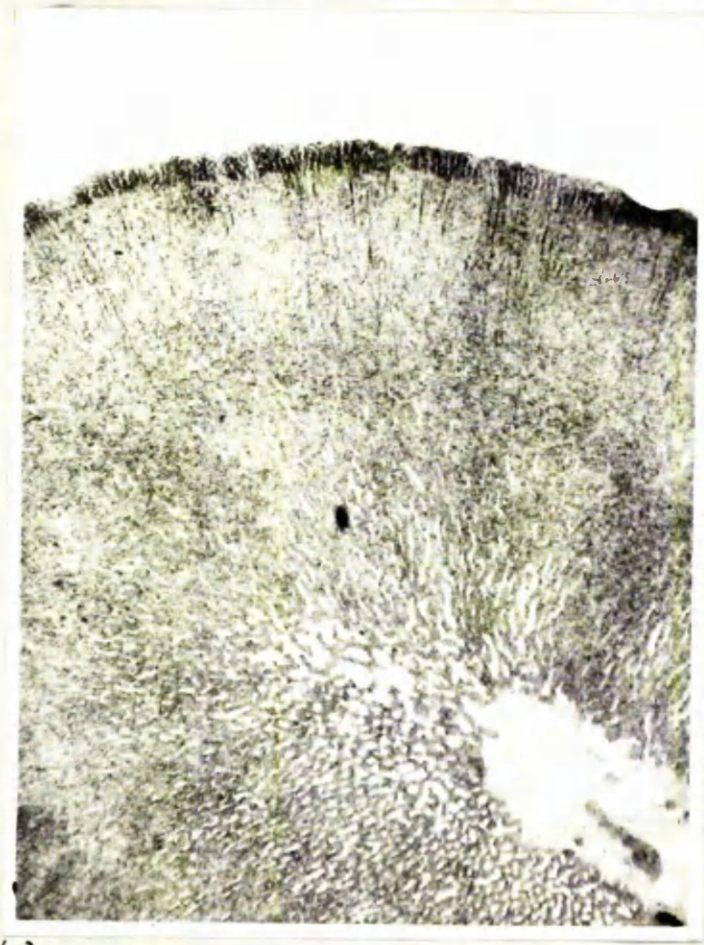
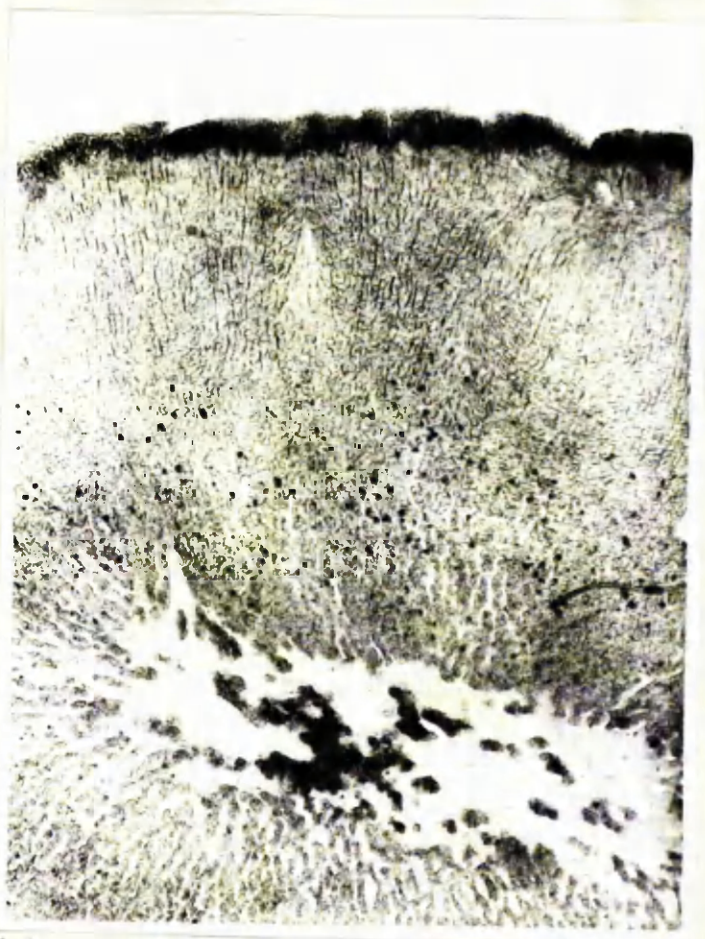


(c)



(d)

Figure 7.

contained many vacuoles and were often binucleate. Some very large cells showed by the presence of broken cell membranes that they had arisen by confluence of adjacent cells. There were also large areas of the zone in which the cells had very clear cytoplasm. By contrast, the only changes noted in the cells of the zona glomerulosa was an increase in the number of cytoplasmic vacuoles. The cells of the zona reticularis also showed a number of vacuoles which were rarely observed in this zone in the control animals; the cells of the reticularis of the cholesterol-fed group were moderately enlarged.

Histochemical tests (performed by Dr. W. Forbes) showed that changes had occurred in all zones after feeding cholesterol, but these were most pronounced in the zona fasciculata (figures 5 and 6). Total fat, neutral fat (figure 7) and phospholipid content were increased in all parts of the cortex. The cholesterol content was also increased, almost completely due to deposition in the esterified form, although some free cholesterol was noted in every zone of many sections taken from the animals fed cholesterol. A marked rise was noted in the fatty acid content of the zona glomerulosa, but a fall in the content of the fasciculata. RNA was increased in both the glomerulosa and fasciculata and ascorbic acid was decreased in all zones.

Thus examination chemically and histologically of the adrenals of cholesterol-fed rabbits has thus shown that an increase in cell size has occurred and that this increase is confined to the zona fasciculata. Large increases in cholesterol and less striking increments in RNAP and

protein were observed in the adrenal cells. The chemical changes were found to occur in all zones of the cortex but were most pronounced in the zona fasciculata.

The effect of the presence of cholesterol in the diet on the chemical composition of the rabbit liver

It was observed that, after feeding rabbits with cholesterol, the livers were also increased in size compared to those of control animals. Thus the chemical composition of the livers of these animals has also been investigated to observe any changes that occurred.

Tables 23 and 24 show the effect of dietary cholesterol on the chemical composition of the liver. The variance ratios obtained by statistical analysis of these results are given in table 25. The presence of cholesterol in the diet was found to cause a significant increase in the weight of the liver ($P < 0.01$). The cholesterol content of the liver also showed a very large significant increase ($P < 0.01$). The rabbits fed cholesterol had significantly more RNAP in their livers than the control animals ($P < 0.05$), but no significant changes occurred in the DNAP, protein nitrogen and lipid phosphorus. The fact that the DNAP shows no change indicates that hypertrophy of the liver cells has occurred with no increase in cell number. Expressing the results in relation to DNAP (table 24) also indicates this. The ratio of liver weight to DNAP was significantly increased ($P < 0.01$) indicating an increase in cell size. Similarly the amounts of RNAP and cholesterol per cell showed significant increases ($P < 0.05$) for RNAP and $P < 0.01$

TABLE 23

The effect of cholesterol feeding on liver weight, cholesterol, RNAP, DNAP, protein nitrogen and lipid phosphorus

The values given are the mean data of 10 replicates and are expressed per Kg. final body weight.

Liver constituent	Control Series	Cholesterol-fed	Difference
Total weight (g.)	23.9	31.7	+ 33%
Cholesterol (mg.)	160.0	634.0	+ 296%
RNAP (mg.)	12.3	15.7	+ 28%
DNAP (mg.)	4.7	5.0	+ 6%
Protein nitrogen (mg.)	586.0	653.0	+ 11%
Lipid P (mg.)	28.4	34.1	+ 20%

TABLE 24

The effect of cholesterol feeding on the constituents of the liver expressed relative to the DNAP content of the liver

Liver constituent	Control Series	Cholesterol-fed	Difference
Total weight (g.)	12.1	15.1	+ 25%
Cholesterol (mg.)	35.0	122.0	+ 248%
RNAP (mg.)	2.7	3.2	+ 18%
Protein nitrogen (mg.)	126.0	132.0	+ 5%
Lipid P (mg.)	6.2	6.8	+ 10%

TABLE 25

Statistical Analysis of the results of tables 23 and 24

The values given below are the variance ratios.

For $P < 0.05$ $F = 4.49^{*}$

$P < 0.01$ $F = 8.53^{**}$

Liver constituent	Variance Ratio
Liver weight	13.1 **
Cholesterol	8.8 **
RNAP	7.0 *
DNAP	0.5
Protein nitrogen	1.2
Lipid phosphorus	2.6
Liver weight / DNAP	12.1 **
Cholesterol / DNAP	10.5 **
RNAP / DNAP	6.5 *
Protein nitrogen / DNAP	0.3
Lipid P / DNAP	1.9

for cholesterol). No significant changes occurred in the amount of protein nitrogen and phospholipid per cell.

Thus feeding rabbits on a diet containing cholesterol has caused an increase in liver size due to hypertrophy of the liver cells. These cells show increases in their content of RNAP and of cholesterol, an increase which is particularly large in the case of the cholesterol content. Thus in both the adrenal gland and the liver, the feeding of cholesterol induces a proportionately much greater increase in cell size than in its constituents, namely RNA, protein and phospholipid.

The effect of ACTH on the chemical composition of the rabbit adrenal gland

As cholesterol feeding caused increases in adrenal size and composition, it was felt desirable to compare these changes with those caused by administration of ACTH which also produces adrenal enlargement.

Eight rabbits were divided into two groups, each consisting of 4 animals. The first group were used as control animals and were injected with saline for 9 days. The other group of rabbits were injected with 40 units ACTH per day for 9 days. On the tenth day the animals were killed, the adrenals removed and analysed as described previously. The results of this experiment are presented in tables 26 and 27, and the variance ratios obtained by a statistical analysis of these results is shown in table 28.

Following intramuscular injection of ACTH for nine days to rabbits on the stock diet, adrenal enlargement occurred which was of a magnitude

TABLE 26

The effect of ACTH on the composition of the rabbit adrenal gland

The results are the mean of 4 replicates and are expressed per kg. final body weight.

Gland constituent	Control Series	ACTH-Series	Difference
Total weight (mg.)	65.4	172.5	+165%
Cholesterol (mg.)	3.6	1.9	- 48%
RNAP (µg.)	27.9	81.2	+191%
DNAP (µg.)	15.0	22.8	+ 52%
Protein nitrogen (µg.)	584.0	1854.0	+218%
Lipid P (µg.)	83.5	306.1	+266%

TABLE 27

The effect of ACTH on the composition of the rabbit adrenal gland expressed relative to the DNAP content of the gland

Gland constituent	Control Series	ACTH Series	Difference
Total weight (mg.)	4.4	7.6	+ 73%
Cholesterol (µg.)	237.0	84.4	-64.6%
RNAP (µg.)	1.9	3.5	+ 84%
Protein nitrogen (µg.)	39.0	80.0	+105%
Lipid P. (µg.)	5.6	12.9	+130%

TABLE 28

Statistical Analysis of the results of tables 26 and 27

The values given below are the variance ratios.

For $P < 0.05$ F = 5.99 *

$P < 0.01$ F = 13.74 **

Adrenal constituent	Variance Ratio
Adrenal weight	10.8 *
Cholesterol	8.9 *
RNAP	9.1 *
DNAP	5.3
Protein nitrogen	13.0 *
Lipid phosphorus	7.3 *
$\frac{\text{Adrenal weight}}{\text{DNAP}}$	16.5 **
$\frac{\text{Cholesterol}}{\text{DNAP}}$	2.7
$\frac{\text{RNAP}}{\text{DNAP}}$	18.3 **
$\frac{\text{Protein nitrogen}}{\text{DNAP}}$	63.0 **
$\frac{\text{Lipid P}}{\text{DNAP}}$	10.1 **

similar to that observed in rabbits fed cholesterol for 14 or 15 weeks. However, the pattern of change differed in the two cases. After ACTH administration, the protein, RNAP and phospholipid content of the gland underwent an increase which was proportional to the change in gland size (table 26), whereas hypertrophy induced by feeding cholesterol was accompanied by a proportionately smaller increase in protein, RNAP and phospholipid (table 20). Furthermore, administration of ACTH caused a considerably larger increase in the DNAP content of the gland than occurred after feeding cholesterol. This increase just failed to attain statistical significance. The fact that this increase was almost significant, however, indicates that some multiplication of cells had occurred after ACTH administration, a feature which was absent from the cells of animals fed on cholesterol. The data of table 27 in which cell constituents are expressed in relation to DNA show that significant enlargement of the adrenal cells had also occurred after ACTH administration ($P < 0.01$) and that the amount of RNAP, protein nitrogen and lipid phosphorus per cell also showed significant increases ($P < 0.01$ for RNAP and protein nitrogen and $P < 0.05$ for lipid phosphorus). Thus after ACTH administration both hypertrophy and hyperplasia had occurred, whereas after cholesterol feeding the change in adrenal size was due to hypertrophy alone. In agreement with much published data on other species, the cholesterol content of the glands was significantly reduced ($P < 0.05$) by administration of ACTH.

On histological examination (Dr. A. Forbes), the adrenal cortex of

the ACTH-treated animals was broader than normal, due to enlargement of both the zona fasciculata and zona reticularis, which now consisted of cells of the same type, namely "compact" type cells with highly eosinophilic cytoplasm and few vacuoles. There were no changes in the cells of the zona glomerulosa (figures 5 and 6).

On histochemical examination (Dr. W. Forbes), the zona glomerulosa showed a marked increase in all lipids including esterified cholesterol, whereas the fasciculata and reticularis exhibited a decrease in these constituents, except that the neutral fat content of the zona reticularis was unaltered by ACTH administration (figure 7). All zones showed increments in free fatty acids, in phospholipid content and in RNA content. In all parts of the cortex there was a reduction in ascorbic acid content.

These observations show that the giving of cholesterol in the diet induced a different pattern of chemical, histological and histochemical change in the adrenal cortex from that caused by stimulation with ACTH. The enlargement in cell size induced by ACTH affects many cell constituents uniformly (mean weight, RNA, protein, phospholipid); whereas cholesterol administration causes mainly an increase in cell weight and a proportionately much smaller effect on the cell constituents.

The effect of administering ACTH to cholesterol-fed rabbits

In view of the differences noted in the response of the rabbit adrenal gland to cholesterol and to ACTH, it was of interest to determine whether the action of ACTH on the gland could be modified by prior treatment with cholesterol. Rabbits were given the 1% cholesterol diet for

10 to 12 weeks, during the last 9 days of which they received intramuscular injections of 40 units ACTH (Acthar gel) per day. Table 29 shows the effect of cholesterol feeding and ACTH treatment on adrenal composition, and table 30 shows the effect of the treatment on the ratio of the individual constituents of the gland to DNAP. In spite of the increase in adrenal weight caused by cholesterol feeding these results show that the administration of ACTH induced a further enlargement in gland size. Furthermore, the change in individual chemical constituents and in adrenal cell size and composition caused by ACTH administration to the cholesterol-fed rabbits was similar to that induced in control animals fed the stock diet without cholesterol. The increases in the individual constituents (or decrease in the case of cholesterol) after ACTH treatment were found to be relatively constant in relation to control animals, irrespective of whether the animals were pretreated with cholesterol or if they were ordinary control animals. Thus prolonged administration of cholesterol does not alter the specific pattern of response to injection of ACTH.

Histological examination of the glands confirmed that the response of animals to ACTH was not grossly altered by feeding a diet rich in cholesterol (figures 6 and 7). The lipid, phospholipid, fatty acids and ascorbic acid content and distribution underwent changes similar to those observed in animals receiving ACTH along with the stock diet. There were, however, minor differences from the changes induced by ACTH in animals on the stock diet; there were areas of very clear

TABLE 29

Effect of Cholesterol Feeding and ACTH Administration on Adrenal Composition

The values given were obtained from rabbits which were fed cholesterol for 10 or 12 weeks, during the last 8 days of which 40 units ACTH was administered per day.

All values are expressed per Kg. final body weight, and are the mean of 4 animals for the control and control + ACTH groups, and mean of 7 animals for the cholesterol and cholesterol + ACTH groups. The figures in brackets represent the increase, or decrease, caused by ACTH treatment over the corresponding group of animals which were not treated with ACTH.

Gland constituent	Treatment			
	Control Group	Control + ACTH Group	Cholesterol Group	Cholesterol + ACTH Group
Gland weight (mg.)	110	268 (+158)	150	304 (+154)
Cholesterol (mg.)	6.6	3.8 (-2.8)	8.6	5.7 (-2.9)
[illegible] (µg.)	48	162 (+114)	63	187 (+124)
[illegible] (µg.)	24	46 (+22)	28	45 (+17)
Protein nitrogen (mg.)	1.4	4.1 (+27)	1.6	4.5 (+2.9)
Lipid P (µg.)	124	459 (+335)	154	462 (+308)

TABLE 30

Effect of Cholesterol Feeding and ACTH Administration on the Composition of the Rabbit Adrenal Gland Expressed Relative to the DNAP content of the Gland

The figures in brackets represent the increase or decrease over the control animals due to ACTH treatment

Gland Constituent	Treatments			
	Control Group	Control + ACTH Group	Cholesterol Group	Cholesterol + ACTH Group
Gland weight (mg.)	4.6	5.7 (+1.1)	5.5	6.7 (+1.2)
Cholesterol (µg.)	266	77 (-189)	335.6	122.6 (+214)
RNAP (µg.)	2.0	3.4 (+1.4)	2.4	4.1 (+1.7)
Protein nitrogen (µg.)	56.1	86.4 (+30.3)	60.0	98.3 (+38.3)
Lipid P (µg.)	5.2	9.8 (+4.6)	6.3	10.3 (+4.0)

cells in the zona fasciculata similar to those seen in the adrenals of animals given cholesterol alone, rather more esterified cholesterol was deposited in the zona glomerulosa and more RNA was found in the outer part of the zona fasciculata.

The influence of the sex of the rabbit on the effect produced by cholesterol feeding

An experiment was carried out using both male and female rabbits as in previous experiments. These rabbits were fed on the diets for 16 weeks and the results of this experiment are summarized in tables 31 and 32. It would appear from these results that cholesterol feeding causes a greater increase in the weight of the adrenal gland of male rabbits than in female rabbits. However, the individual results showed considerable variation and no real difference according to the sex of the animal was observed. The concentration of RNAP in the gland does not show any increase in this experiment with cholesterol feeding but here again considerable variation was present within individual groups so that no true conclusion can be drawn and certainly there is no obvious difference in the amount of RNAP according to the sex of the animal. The DNAP, protein nitrogen and lipid phosphorus also show very little change with cholesterol feeding although considerable variation between individual animals was observed and no differences are noticed according to the sex of the animal. The results would indicate that there is a considerably greater increase in the amount of cholesterol in the adrenal in the male animal than there

TABLE 31

The Influence of the Sex of the Rabbit on the Effect produced by Cholesterol Feeding on the Composition of the Adrenal Gland

The results shown are the mean values obtained from 2 control males and 1 control female, 4 cholesterol-fed males and 3 cholesterol-fed females, and are expressed per Kg. final body weight.

Gland Constituent	Male		Female	
	Control	Cholesterol-fed	Control	Cholesterol-fed
Adrenal weight (mg.)	167	264	183	183
RNAP (µg.)	93	90	74	66
DNAP (µg.)	58.5	63.0	49.0	40.0
Protein nitrogen (µg.)	2760	2369	1755	1868
Lipid P (µg.)	334	314	284	225
Cholesterol (mg.)	10.9	41.2	20.5	23.5

TABLE 32

The Influence of the Sex of the Rabbit on the Effect produced by Cholesterol Feeding on the Composition of the Adrenal Gland expressed relative to the DNAP content of the Gland

Gland Constituent	Male		Female	
	Control	Cholesterol-fed	Control	Cholesterol-fed
Gland weight (mg.)	3.3	4.1	3.7	4.6
RNAP (µg.)	1.6	1.5	1.5	1.6
Protein nitrogen (µg.)	45	38	36	47
Lipid P (µg.)	5.8	5.1	5.8	5.8
Cholesterol (µg.)	190	500	417	637

is in the female animal after cholesterol feeding but once again the individual results show considerable variation and no significant difference in the amount of cholesterol in the gland according to the sex of the animal is observed. Comparison of the ratios of the various constituents of the gland to the amount of DNAP in the gland shows that no differences are apparent between the two sexes except in the case of the cholesterol: DNAP ratio. As before, this may be attributed to the large individual variations. In this experiment only one control female animal was used, so the possibility exists that lack of additional controls may mean that a true assessment of the situation was not obtained.

When data obtained from all the cholesterol feeding experiments carried out are considered, that is data from 19 male and 11 female rabbits, they showed that the adrenal glands underwent increases in weight of 94% and 68% respectively when compared with control animals of the same sex. The RNAP content increased 27% in male rabbits and 35% in female while the cholesterol content increased 444% and 282% in male and female animals respectively. However, none of the differences observed was significant.

Histological and histochemical examination also revealed no differences between male and female rabbits after feeding cholesterol. Thus the present results indicate that no differences were detected according to the sex of the animal after cholesterol feeding, though the considerable variation in response in successive animals makes fine distinctions impossible.

The influence of different dietary levels of protein on the effect produced by cholesterol feeding on the adrenal gland of the rabbit

An experiment was carried out in which rabbits were fed cholesterol at different levels of dietary protein to investigate whether variations in the amounts of protein in the diet influenced the effects produced by cholesterol feeding on the rabbit adrenal gland. The rabbits used in this experiment were divided into four groups, one group receiving a low protein diet consisting of 50 g. diet 18 and 50 g. starch, (i.e. 8.3% protein as against 16.5% in normal diet); the second group received the high protein diet which contained 50 g. diet 18, 25 g. starch and 25 g. casein, (i.e. 33% protein), while the third and fourth groups were fed the low protein and high protein diets respectively, to each of which 1% cholesterol was added. Each rabbit received 150 g. of its particular diet per day and the feeding was continued for 10 weeks.

The results of this experiment are presented in Tables 33 and 34. Cholesterol feeding is shown to cause an increase in the constituents of the gland in a similar manner as was observed in previous experiments, the amount of cholesterol in the adrenal is found to increase more in rabbits fed cholesterol when their diet contains a high level of protein than when less protein is present in the diet. The cholesterol to DNA ratio shows that the amount of cholesterol per cell is greater in rabbits fed a high-protein cholesterol diet than in those fed a low protein cholesterol diet. None of the other gland constituents were found

TABLE 33

The influence of different dietary levels of protein on the effect produced by cholesterol feeding on the composition of the adrenal gland of the rabbit

The results given below are the mean values obtained from 3 control animals on the 8% protein diet and 4 control animals on the 33% protein diet, 4 cholesterol-fed animals receiving an 8% protein diet and 2 cholesterol-fed animals receiving a 33% protein diet. They are expressed relative to per Kg. final body weight.

Gland Constituent	Diet			
	8% Protein	33% Protein	Cholesterol + 8% Protein	Cholesterol + 33% Protein
Adrenal weight (mg.)	118	137	320	334
RNAP (µg.)	57.0	57.0	77.6	77.5
DNAP (µg.)	34.2	33.3	43.2	42.0
Protein nitrogen (µg.)	1511.0	1517.0	2270.0	1623.0
Lipid P (µg.)	216	212	274	238
Cholesterol (mg.)	9.6	7.3	40.9	58.9

TABLE 34

The influence of different dietary levels of protein on the effect produced by cholesterol feeding on the composition of the adrenal gland of the rabbit expressed relative to the DNAP content of the gland

Gland Constituent	Diet			
	8% Protein	33% Protein	Cholesterol + 8% Protein	Cholesterol + 33% Protein
Adrenal weight (mg.)	3.5	4.1	7.2	8.8
DNAP (µg.)	1.7	1.8	1.9	1.9
Protein nitrogen (µg.)	44.8	46.6	52.5	46.0
Lipid P (µg.)	6.5	6.5	6.4	6.3
Cholesterol (µg.)	271	211	880	1644

to alter with the protein content of the diet when the rabbits received a diet with or without cholesterol. The data thus suggest that a high intake of protein sensitises to deposition of cholesterol, but more work is needed to validate this statistically.

Histological examinations of these glands by Dr. W. Forbes showed that the changes caused by cholesterol feeding were more marked when a high protein diet was fed. The cells were much larger and very pale staining and there was an increase in the number of degenerating cells. Histochemical examination showed that the increase in cholesterol deposition after cholesterol feeding was greater in the zona glomerulosa of the high protein-fed animals than in the low protein-fed animals, whereas the deposition in the other zones was the same. The increase in fat after cholesterol feeding was also greater in the rabbits fed a high protein diet than in those on the low protein diet.

The Icholesterol content of the livers of rabbits fed cholesterol and cholesterol content of the livers of rabbits fed cholesterol

As it was observed that feeding rabbits cholesterol combined with an increased level of dietary protein produced an increased deposition of adrenal cholesterol compared to rabbits receiving a lower amount of protein in the diet, the cholesterol content of the livers of these animals was also examined. The liver weights, and cholesterol content are shown in table 35. Cholesterol in the diet caused an increase in liver weight, and liver cholesterol as was observed previously. An increase in the protein content of the diet caused an increase in liver weight

TABLE 35

The influence of different dietary levels of protein on the weight and cholesterol content of the livers of rabbits fed cholesterol

The results given below are the mean values obtained from 3 control animals on an 8% protein diet and 6 control animals on a 33% protein diet, 4 cholesterol-fed animals receiving an 8% protein diet and 5 cholesterol-fed animals receiving a 33% protein diet. The figures in brackets represent the increase over the control animals due to cholesterol feeding.

	Diet			
	8% protein	33% protein	Cholesterol + 8% protein	Cholesterol + 33% protein
Liver weight (g/kg. final body weight)	34	37	35 (+ 6)	49 (+ 15)
Cholesterol (mg/kg. final body weight)	234	267	1160 (+ 926)	2230 (+ 1963)
Cholesterol (mg/100g. liver)	540	710	3260 (+2720)	4440 (+ 3730)

in both control and cholesterol-fed animals. Increased dietary protein did not however cause any change in the liver cholesterol of the control group of animals, whereas a marked increase in liver cholesterol was observed in the livers of animals fed a high protein diet in combination with cholesterol. Thus feeding rabbits cholesterol along with a higher level of dietary protein seems to cause increased deposition of cholesterol in both the adrenals and livers, compared to rabbits receiving a lower level of dietary protein.

The effect of the duration of cholesterol administration on adrenal size and composition

Throughout these experiments in which rabbits were fed with cholesterol the duration of feeding has been varied from 10 weeks to 16 weeks. Thus the data obtained from all the experiments has been computed together to determine whether the duration of cholesterol feeding has any influence on the effects produced on the adrenal gland. These data are given in tables 36 and 37. From these it is apparent that, although the changes caused by cholesterol administration varied considerably in degree between the different durations of time of cholesterol feeding, there was no consistent increase in the intensity of the adrenal effects as time of administration progressed. Consequently, the changes in adrenal size and structure are well established in less than 10 weeks of feeding cholesterol.

The effect of feeding rats cholesterol at different levels of dietary protein

An experiment was carried out in which female rats were fed

TABLE 36(a)

The effect of variations in time of cholesterol administration on adrenal weight and cholesterol content.

The results below were obtained from the control and cholesterol-fed rabbits in different experiments of varying durations. The numbers of control and cholesterol-fed rabbits in each experiment are indicated in the table. All results are expressed relative to per Kg. final body weight.

Duration of feeding	Gland weight (mg.)			Cholesterol (mg.)			Number of rabbits	
	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed
10 weeks	129	325	152	8	47	488	7	6
10 weeks	88	146	66	6	9	50	2	4
12 weeks	133	155	17	7	8	14	2	3
14 weeks	87	192	121	8	30	275	4	4
15 weeks	90	192	113	6	37	516	5	6
16 weeks	186	229	23	14	34	143	3	7

The effect of variations in time of cholesterol administration on adrenal RNAP, DNAP, protein nitrogen and lipid phosphorus

The results below were obtained from the control and cholesterol-fed rabbits in different experiments of varying durations. The numbers of control and cholesterol-fed rabbits in each group are those indicated in table 36(a). All results are expressed relative to per kg. final body weight.

Duration of feeding	RNAP (µg.)			DNAP (µg.)			Protein N (mg.)			Lipid P (µg.)		
	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase
10 weeks	57	78	37	34	43	26	1515	2123	40	214	262	22
11 weeks	41	60	46	25	31	24	1133	1541	36	133	177	33
12 weeks	55	66	20	24	25	4	1630	1577	-3	116	125	8
14 weeks	36	55	53	23	24	5	795	1092	37	143	168	9
15 weeks	47	66	40	27	30	11	1168	1808	55	140	185	32
16 weeks	87	83	-5	56	53	-4	2426	2134	-11	317	276	-13

TABLE 37

The effect of variations in time of cholesterol administration on adrenal size and composition expressed relative to the DNAP content of the gland

The results below are computed from the data of tables 36 (a) and 36 (b).

Duration of feeding	Adrenal weight (mg.)			Cholesterol (µg.)			RNAP (µg.)			Protein N (µg.)			Lipid P (µg.)		
	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase	Control	Cholesterol-fed	% increase
10 weeks	3.9	7.7	97	237	1135	378	1.7	1.9	14	46	50	9	6.5	6.4	- 1
12 weeks	3.6	4.8	33	227	359	58	1.7	2.1	23	48	54	13	5.7	7.0	23
13 weeks	5.5	6.4	16	305	306	0.5	2.3	2.9	26	67	68	1	4.6	5.3	11
14 weeks	4.3	8.7	112	333	1478	343	1.6	2.3	44	35	46	31	6.7	6.9	3
15 weeks	3.5	6.2	77	234	1143	387	1.8	2.2	22	44	57	29	5.4	6.0	11
16 weeks	3.4	4.3	27	266	616	131	1.8	1.8	-6	44	42	-4	5.8	5.3	-9

cholesterol in diets with varying levels of protein. In the morning each rat received 1 g VMR and half the rats (the control group) received 2.8 g. glucose while the other half (the cholesterol-fed group) were given 2.8 g. glucose containing 3% cholesterol. The control and cholesterol-fed rats were each subdivided into 3 groups each receiving 4.2 g. of either the protein-free diet, the protein diet, or the high protein diet in the evening meal. This feeding was continued for 14 days after which the animals were killed and the adrenals removed for analysis. The results of this experiment are given in table 38.

Feeding cholesterol to rats caused no marked changes in the weight of the adrenal glands. There appears to be an increase with feeding the high protein diet and cholesterol but this is due to the fact that one control animal had a particularly low adrenal weight. The increases in gland constituents after feeding cholesterol which were observed in the rabbit particularly RNAP, protein N and lipid P are not evident in the case of the rat. The ratio of these constituents to the DNAP content of the gland also show no change after cholesterol feeding (table 39). Thus cholesterol administration to the rat does not cause the hypertrophy which is observed in the case of the rabbit.

TABLE 38

The effect of feeding rats cholesterol at different levels of dietary protein on the chemical composition of the adrenal gland

The results given below are the mean of 2 rats and are expressed per 100g. initial body weight.

Protein intake	Gland weight (mg.)		RNP (µg.)		DNP (µg.)		Protein N (µg.)		Lipid P (µg.)	
	Con-trol	Choles-terol-fed	Con-trol	Choles-terol-fed	Con-trol	Choles-terol-fed	Con-trol	Choles-terol-fed	Con-trol	Choles-terol-fed
None	22.5	22.1	10.3	8.9	8.5	7.6	273	180	28.8	18.0
Protein	24.7	25.5	11.7	11.4	7.9	8.7	353	304	44.7	41.1
High Protein	27.2	31.1	15.5	14.8	12.0	10.5	445	396	53.7	35.1

TABLE 39

The effect of feeding rats cholesterol at different levels of dietary protein on the chemical composition of the adrenal gland relative to the DNAP content of the gland

Protein Intake	Gland weight (mg.)		DNAP (mg.)		Protein N (mg.)		Lipid P (mg.)	
	Control	Cholesterol -fed	Control	Cholesterol -fed	Control	Cholesterol -fed	Control	Cholesterol -fed
None	2.7	3.4	1.2	1.4	32	28	3.3	2.7
Protein	3.1	2.9	1.5	1.3	45	35	5.6	4.7
High Protein	2.7	2.9	1.3	1.4	30	38	4.5	3.4

D I S C U S S I O N

I. The effect of feeding individual amino acids on the activity of the adrenal gland

The present work was undertaken to investigate further the conclusions of Munro and Mukerji (1958; 1962) that amino acids can cause changes in adrenocortical function. Eighteen hours after feeding certain amino acids, notably glycine, methionine and leucine, to rats, they observed the following - (a) increased uptake of ^{32}P by liver RNAP, (b) an increase in liver RNAP, (c) increased deposition of liver glycogen. However, when these amino acids were fed to adrenalectomized rats, these effects were abolished indicating that intact adrenal glands are required for the mediation of a response on the liver by these amino acids.

There is evidence that the protein content of the diet effects adrenocortical function through ACTH secretory rate (Tepperman et al, 1943; Ingle, 1945; Leathem, 1945, 1947, 1951; Tuchman - Duplessis et al, 1948; Kaunitz et al, 1956; Munro et al, 1962.). Thus the weight of the gland and its chemical composition vary with the dietary protein level (Munro et al, 1962). This variation in the protein content of the diet appears to alter the activity of the adrenal cortex. This sensitivity of the adrenal to dietary protein seems to be at the pituitary level because the gland responds equally well to exogenous ACTH at low and higher levels of dietary protein and dietary protein does not alter the sensitivity of the gland to ACTH as may be expected if it did not act directly on the pituitary (Munro et al, 1962). The

question thus arises, do the amino acids cause the observed effects on the liver by increasing the secretion of ACTH from the anterior pituitary and thus acting in the same way as protein? If they act on the anterior pituitary it would be expected that feeding these amino acids will, like protein, cause an increase in adrenal weight and adrenal constituents. However, prolonged feeding of rats with these single amino acids produced no increase in adrenal weight nor in adrenal RNAP, DNAP, protein or phospholipid (tables 8, 9 and 10). In the same experiments, these adrenal constituents were observed to increase when an adequate protein diet was fed compared to a protein-free diet and thus the mechanism of action of protein and of the single amino acids on the adrenal cortex must differ. Furthermore, since single amino acids caused no increase in either adrenal size or constituents it may be concluded that they do not cause increased secretion of ACTH from the anterior pituitary. Thus the adrenal-dependent effects observed on the liver after feeding these single amino acids cannot be due to increased secretion of ACTH from the anterior pituitary resulting in increased production of adrenocortical hormones. Therefore if the increased uptake of ^{32}P by liver RNAP and the increased deposition of liver glycogen are in fact due to adrenocortical secretion, as would appear by the fact that the effects are abolished by adrenalectomy, the amino acids must stimulate the adrenal secretion by some means other than by increasing the secretion of ACTH. Can the action of these amino acids be a direct one on the adrenal cortex itself causing

increased secretion of adrenocortical steroids? Alternatively, the adrenals may play a "permissive" role (Ingle, 1951) in the action of amino acids on liver metabolism, that is, the presence of circulating adrenocortical hormones is necessary for the effects on the liver to take place. This type of permissive action has, for example, been demonstrated in the case of the metabolic response to injury, which occurs in adrenalectomized animals, provided that exogenous adrenocortical hormones are administered (Ingle, Ward, and Kuizenga, 1947).

Since this leaves us in a dilemma regarding the interpretation of the role of the adrenal cortex in the action of these amino acids on protein metabolism, it was necessary to establish whether or not the giving of these amino acids stimulates production of adrenal steroids even if they have no influence on adrenal size and composition.

II. Effect of feeding individual amino acids on the concentration of blood and adrenal corticosterone

If the single amino acids, glycine, methionine and leucine do in fact stimulate adrenal activity, an increased secretion of adrenal steroids may be expected. The main adrenal steroid in the blood of the rat is corticosterone and thus we would expect an increase in the concentration of this particular steroid. Thus the blood and adrenal concentrations of corticosterone were examined in rats that had been fed diets containing single amino acids, and considerable changes were observed. Methionine and leucine were found to cause rapid increases in blood corticosterone after being fed to rats and these increases

were significant after 24 hours, while the other amino acids fed, namely alanine, aspartic acid and glutamic acid did not produce these sustained effects (Tables 14 and 15). In addition, the giving of glycine, methionine and leucine caused a considerable accumulation after 24 hours of adrenal corticosterone, although these increases were not significant (Tables 16 and 17). Thus although methionine and leucine had no effect on adrenal size and gross composition, they appear to cause a rapid increase in adrenocortical activity as shown by increased production of corticosterone within 24 hours.

After prolonged administration of these three amino acids fed singly to rats, the levels of blood and adrenal corticosterone were found to be increased above the levels in rats fed a protein-free diet. After 11 days feeding, considerable elevations in blood corticosterone levels were observed in rats which had been fed casein, glycine, methionine, leucine or zein plus tryptophan and lysine, compared to animals receiving a protein-free diet (Table 18). However, none of these increases attained statistical significance due to individual variation within animals. Nevertheless, significant increases were observed in the adrenal corticosterone content of rats fed casein, methionine or leucine compared to those of rats receiving a protein-free diet, while the levels observed in rats fed a diet of zein plus tryptophan and lysine just failed to attain statistical significance (Table 18). Thus the stimulation of the adrenal cortex, as shown by increased production of corticosterone, by the single amino

acids methionine and leucine, is not limited to the rapid stimulation which was detected 24 hours after a single dose. Continual administration of smaller amounts of these amino acids over a longer period of time stimulated the production of corticosterone and led to considerable elevations in adrenal levels. After prolonged feeding, the adequate protein diets also showed large increases in adrenal corticosterone levels which were not evident after only a single meal of casein had been fed. Thus prolonged feeding of an adequate protein diet appears to be required to cause increased corticosterone production whereas methionine and leucine can stimulate the adrenal more rapidly. This emphasizes further that the mechanisms of action of whole protein and of amino acids is different.

A diet containing methionine or leucine can stimulate the adrenal to produce more corticosterone, although the finding that no change occurs in adrenal size or composition, indicates that they probably do not act through ACTH secretion. The possibility therefore exists that these amino acids influence the production of adrenal steroids by acting directly on the adrenal cortex itself. This would explain the observed increases in blood and adrenal corticosterone without any changes in adrenal size or composition. On the other hand, diets adequate in protein cause changes in adrenal size and composition as well as increasing blood and adrenal corticosterone levels, and thus the changes must be caused by a different mechanism to that of the single amino acids. Since changes in adrenal size and composition occur as well as changes in

corticosterone levels after feeding whole protein, it may be assumed that there is increased secretion of ACTH from the anterior pituitary. Thus the action of protein in causing adrenal stimulation appears to be mediated via the anterior pituitary while the single amino acids, methionine and leucine, may stimulate the adrenal cortex directly.

The present work showing that methionine and leucine can cause increased blood and adrenal corticosterone levels substantiates the work of Goth et al (1954) who showed that these amino acids caused a significant eosinopenia, indicative of increased adrenocortical stimulation, and also the work of Munro and Mukerji (1958; 1962), who concluded from the fact that these amino acids caused increased uptake of ^{32}P by liver RNAP and deposition of liver glycogen, effects which were abolished on adrenalectomy, that methionine and leucine caused increased adrenocortical secretion.

We have thus found that steroid production can take place without any change in adrenal cell size or constituents (Tables 8 and 16; Figure 4). It is well known that ACTH stimulates the adrenal cortex causing an increase in the output of adrenocortical steroids, and the work of Munro and Mukerji (1958; 1962) and the present work have shown that certain amino acids also exert a stimulating effect on this gland. However, amino acids do not cause the same effects on the adrenal as are observed after injections of ACTH. ACTH causes a large increase in gland size and in its content of RNAP, protein and phospholipid while feeding methionine and leucine produces none of these effects. Both

ACTH and single amino acids will, however, increase the level of blood corticosterone, and of the total amount of adrenal corticosterone (tables 18 and 19). One possibility which must be considered for the mechanism of action of these amino acids is that they may cause the release of small quantities of ACTH from the pituitary which are sufficient to stimulate the adrenal causing increased corticosterone production but insufficient to cause any changes in adrenal weight or composition. One point in which amino acids and ACTH differ in their stimulation of corticosterone production is that the concentration of corticosterone in the gland is increased after administration of amino acids whereas ACTH causes a decrease in the concentration (table 19); this decrease is however due to the fact that the adrenal weight has increased proportionately more than has the corticosterone. Also the doses of ACTH used in the present investigations and in those of Munro et al (1962) are quite large and thus it remains possible that the effect produced by amino acids may be equivalent to that of a very small dose of ACTH. Thus to investigate further the mechanism by which amino acids stimulate the adrenal glands it is necessary to feed these single amino acids to hypophysectomized rats. If the effect is a direct one on the adrenal cortex we would still expect to find elevated corticosterone levels. From the results obtained so far it appears more likely that methionine and leucine act directly on the adrenal gland, as if any ACTH stimulation was involved one would expect at least a small increase in gland weight which was not observed (figure 8).

Figure 8 summarises the current hypothesis on the effect of hormones and diet on protein metabolism. It shows how dietary protein and single amino acids may each exert their action on the adrenal cortex causing increased production of adrenal corticoids. Protein may act on the anterior pituitary causing increased production of ACTH and so promoting increased adrenal activity, while single amino acids may possibly exert their action directly on the adrenal gland causing increased adrenocortical secretion.

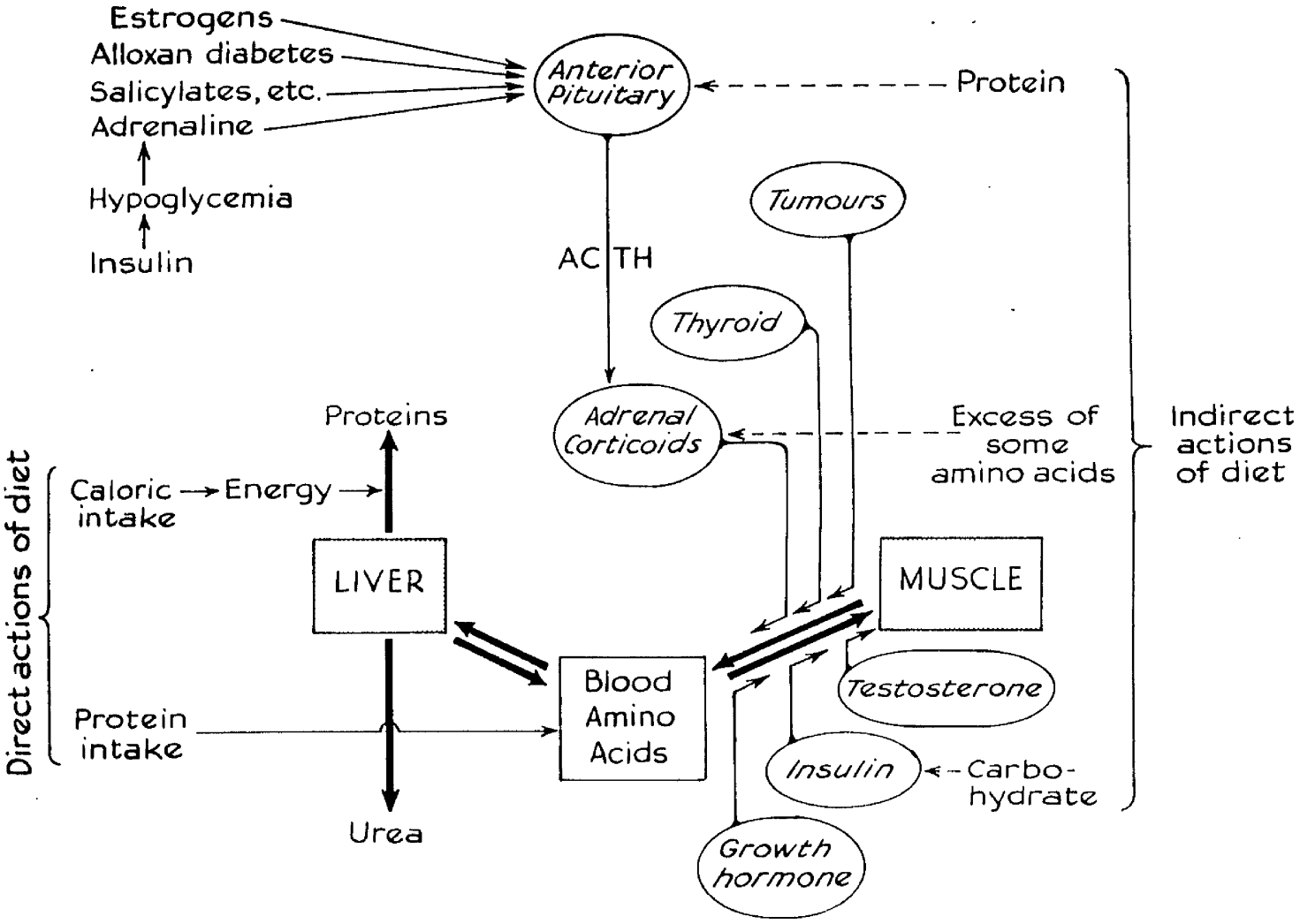

Estrogens
Alloxan diabetes
Salicylates, etc.
Adrenaline
Hypoglycemia
Insulin
Anterior Pituitary
Protein
Tumours
ACTH
Thyroid
Adrenal Corticoids
Excess of some amino acids
Indirect actions of diet
Direct actions of diet
Proteins
Caloric intake
Energy
LIVER
MUSCLE
Blood Amino Acids
Testosterone
Protein intake
Insulin
Carbo-hydrate
Urea
Growth hormone

Figure 8.

Another possibility exists for the mechanism of action of these amino acids on the adrenal cortex. Dixon, Moore, Stacke-Dunne and Young (1951) believe that there are two hormones, secreted by the anterior pituitary, acting on the adrenal cortex, one causing depletion of adrenal ascorbic acid and the other causing an increase in adrenal weight. If this is so, the amino acids may possibly cause the release of only one form of pituitary factor causing an increase in steroid production. Complete protein, on the other hand, would cause stimulation of both forms of factor, resulting in increased adrenal weight and adrenal constituents, and also increased steroid production.

III. The effect on the adrenal produced by cholesterol feeding.

The present studies substantiate the occurrence of adrenal enlargement in animals fed cholesterol which has been reported by previous investigators (Krylow, 1914; Sternberg, 1915; Reineck, 1929; Kay and Whitehead, 1935; McMillan et al, 1954; Bernick and Patek, 1961). These earlier studies demonstrated that hypertrophy of the adrenal glands had occurred but the types of cell involved were not specified and the fact that the number of cells may also have increased was not considered. In the present work these aspects have been investigated more fully and changes in other adrenal constituents have also been explored.

A large increase in gland size was observed but proportionately much smaller increases in the adrenal RNAP, protein and phospholipid, with gross deposition of cholesterol (Table 20). However, none of these increases were sufficient to account for the increase in gland size.

No significant change occurred in the DNAP content of the glands indicating that no new cells had been formed and that the increase in gland size was therefore due almost entirely to hypertrophy of the existing cells, which by histological examination was found to be confined to the zona fasciculata.

When the increase in adrenal size caused by feeding cholesterol was compared with the changes occurring after ACTH administration certain differences were observed. ACTH caused an increase in adrenal size which was of the same order of magnitude as caused by feeding cholesterol but the adrenal constituents were differently affected by the two agents. Unlike cholesterol, ACTH caused increases in the protein, RNAP and phospholipid of the adrenal which were proportional to the increase in gland weight (Table 26). The DNAP content of the gland was also considerably increased after ACTH administration although this just failed to attain statistical significance. However, the fact that the increase in DNA was nearly significant suggests that some new cells have probably been formed. The ratio of adrenal weight to DNAP was also significantly increased indicating that hypertrophy of the cells had occurred as well as hyperplasia (Table 27). Histological and histochemical examination also confirmed that the changes in cell morphology and composition after ACTH administration were quite different from those induced by cholesterol feeding. Since the type of adrenal enlargement caused by cholesterol feeding appears to differ from the enlargement due to ACTH administration,

this would suggest that the stimulation of the adrenal by cholesterol is of a different nature to the ACTH stimulation, and therefore that the action of cholesterol on the adrenal may not be directly mediated via the anterior pituitary.

It is, however, possible that cholesterol feeding may cause adrenal enlargement by sensitising the adrenal to the action of ACTH secreted at a normal rate from the pituitary. The results obtained regarding the changes in adrenal weight and constituents after ACTH was administered to cholesterol-fed rabbits indicated that cholesterol did not in fact alter the response of the adrenal gland to ACTH. The adrenal weight and constituents showed a further increase after treatment of cholesterol-fed rabbits with ACTH compared to the increase produced by cholesterol feeding alone (Table 29), and the increases produced by ACTH on the size and constituents of the adrenal glands of cholesterol-fed rabbits was of the same order as those produced on the adrenals of control rabbits not receiving cholesterol. Thus these results would indicate that the mechanism of action of dietary cholesterol in increasing adrenal size and constituents is not by sensitization of the adrenal gland to the ACTH secreted by the pituitary.

It would therefore appear that as the effect produced by cholesterol on the adrenal is not completely similar to an ACTH-type stimulation nor does it sensitise the gland to endogenous ACTH, that its action may possibly be a direct one on the adrenal gland itself. Cholesterol may, of course both act directly on the adrenal and also cause some pituitary

stimulation resulting in increased secretion of ACTH. The possibility exists that any action on the pituitary which does occur may be a general stress action caused by feeding cholesterol or as a result of the atherosclerosis due to the high dietary intake of cholesterol.

Many workers (Oppenheim and Brugger, 1952; Cooke et al, 1952; Adlersberg et al, 1953; Adlersberg et al, 1954; Gordon et al, 1954; Wang et al, 1955; Adlersberg, 1956) have found that the degree of atherosclerosis is diminished in cholesterol-fed rabbits when treated with cortisone. All of these workers except Cooke et al (1952) and Gordon et al (1954) found the level of blood cholesterol was further increased by the cortisone treatment. Wang et al (1955) suggested that the decrease in atherosclerosis after cortisone treatment is due to decreased tissue permeability as a result of the hormone administration. Dury and Ewell (1956) showed that the concentration of cholesterol in the livers of cortisone treated cholesterol-fed rabbits was lower than in cholesterol-fed rabbits not treated with cortisone. However due to the large increase in liver weight the total amount of cholesterol in the liver had increased. These workers also found a decrease in plasma cholesterol of cholesterol-fed rabbits after cortisone treatment which does not agree with the majority of other investigators. The present work showed that ACTH administration to cholesterol-fed rabbits reduced the cholesterol content of the adrenals, this however, is most likely to be due to the direct action of ACTH on the adrenals and whether this has any bearing on the atherosclerosis remains obscure at present.

Nevertheless Dury and Swell (1950) observed that in cholesterol-fed cortisone injected rabbits 2 days after feeding a labelled cholesterol meal, the specific activities of free and esterified cholesterol fractions in ileum region, liver and plasma, and the specific activity of aorta free cholesterol were lower than the specific activities of the respective cholesterol fractions in untreated animals. They concluded that cortisone injection of cholesterol-fed rabbits caused a change in metabolic behaviour of ingested cholesterol.

Thus the mechanism of action of adrenocortical hormones in the amelioration of atherosclerosis remains obscure. In the present investigation the results obtained on adrenal analysis after ACTH administration do not suggest any changes in the adrenal itself other than those expected and observed when ACTH is administered to rabbits not fed on cholesterol. However effects produced on other tissues have not been examined. To assess further the mechanism of action of cholesterol on the adrenal cortex it would be necessary to investigate this in hypophysectomized animals to give an indication of whether the pituitary is involved. Wells and Irshoff (1952) have fed cholesterol to hypophysectomized rats and they found that the elevation in plasma cholesterol levels induced by feeding 1% cholesterol was significantly greater in hypophysectomized than in intact rats. These findings are similar to those in the hypothyroid rat (Horlick and Havel, 1948; Page and Brown, 1952). In these experiments the giving of desiccated thyroid although reducing plasma cholesterol levels, did not reduce it to the

level of intact rats. They therefore concluded that the absence of pituitary hormones other than thyrotropin may have contributed to the observed results. The fact that hypophysectomy increases plasma cholesterol levels of cholesterol-fed rats, which is not entirely reduced by thyroid extract, may suggest that if the adrenals are involved the dietary cholesterol is acting directly on the adrenals and not on the pituitary. However, considerably more work is required to clarify this picture.

The present experiments showed that the sex of the animal had no influence on the effect produced by cholesterol feeding on adrenal size or adrenal RNAP, DNAP, protein, phospholipid or cholesterol (table 31). It has been observed in the United States that woman are more resistant to atherosclerosis than are men (White, Edwards and Dry, 1950; Stamler, 1958), and using cockerels as the experimental animal it has been shown that oestrogen administration induced significant inhibition of the coronary atherosclerosis caused by cholesterol feeding (Pick, Stamler and Katz, 1950). These workers also showed that oestrogen treatment of rats on potentially atherogenic diets also diminished atherosclerosis, but experiments carried out on rabbits showed that oestrogens apparently had no influence on the atherosclerosis. Thus in the present investigation in which no difference was observed in the adrenal response according to the sex of the rabbit, there are two possible conclusions to be drawn from this; either (a) cholesterol feeding has the same effect on the adrenal gland irrespective of the sex of the animal, or (b) the rabbit may possibly differ from other animals in so far as the sex of the animal

has no influence on the atherosclerosis produced by cholesterol feeding.

Another factor which is considered to have a possible influence on atherosclerosis is diet. In the present experiments an increased deposition of cholesterol in the adrenal and liver was observed when the cholesterol-fed rabbits received a high protein diet compared to those receiving a low protein diet (Tables 33 and 35). McMillan, Whiteside and Duff (1954) showed that in cholesterol-fed rabbits a state of undernutrition increased the hypercholesterolemia observed, although no difference was found in the extent of the aortic atherosclerosis. Various workers (Meyer, Kritchevsky, Logan and Cox, 1956; de Groot, 1958; 1959; Nath, Harper and Elvehjem, 1959; Nath, Seidel and Harper, 1961) have shown that the hypercholesterolemia observed in cholesterol-fed rats can be reduced by increasing the protein content of the diet, by the addition of casein, wheat glutin or other proteins to the diet. Stamler, Rich and Katz (1958) also observed that the serum cholesterol levels were lower in chicks fed an atherogenic diet when the diet was high in protein and that the high protein content protected them against atherosclerosis promoted by cholesterol feeding. De Groot (1959) showed that if combinations of different amino acids, of which methionine was one, were added to the diet instead of protein, a decrease in the serum cholesterol level of rats was again observed. Thus it would appear that, when a larger amount of protein is fed to animals along with a cholesterol diet there is an increased deposition of cholesterol in the adrenals (Table 33) and liver (Table 35), and possibly in the tissues

generally, while the blood cholesterol level is decreased compared to animals fed a diet with a lower protein content as seen from the survey of the literature. Various workers (Barnes, Primrose and Burr, 1944; Savage, 1951) have shown that fats are digested to a lesser extent on a low protein diet than on a diet containing a higher amount of protein. However, there is some controversy on this question as Jagerroos (1902), Chittenden (1905) and Coffey, Mann and Bollman (1943) reported that the protein level had little effect on digestibility. The present results would perhaps indicate that the protein content of the diet has some influence on the absorption or digestibility of the cholesterol in the diet resulting in increased deposition in the tissues. It may alternatively represent an increased capacity of the tissues to accumulate cholesterol, when a high intake of protein is provided.

The effect of feeding rats a cholesterol containing diet

In the experiments in the present investigation, in which rats received a cholesterol-containing diet, no changes were observed in the composition of the adrenal gland (Table 38). However other workers (Horlick and Havel, 1948) have found that hypercholesterolemia and atherosclerosis are not readily achieved in the rat and it appears to be particularly resistant to this disease. Page and Brown (1952) showed that hypercholesterolemia can be produced in the rat if a high fat diet and bile salts are given along with the dietary cholesterol, particularly if the animals are first rendered hypothyroid either with radioiodine or thiouracil but the rats remain resistant to atherosclerosis.

Nath, Wiener, Harper and Elvehjem (1950) proposed that a diet promoting both good growth and a significantly high serum cholesterol level should contain 25% hydrogenated coconut oil, 1% cholesterol and 0.5% cholic acid. The cholic acid is thought to promote cholesterol absorption, resulting in higher levels of serum cholesterol as well as deposition of cholesterol in the liver. These observations would explain why no changes occurred in the rat adrenal in our experiments after feeding a diet containing cholesterol. The rat is thus not a good experimental animal for these purposes, as it does not develop a significant spontaneous atherosclerosis and it is necessary to employ relatively severe dietary conditions. Bernick and Patek (1951), in their work on rats in which changes in the adrenals were found, fed a diet containing 1% cholesterol and 5% cottonseed oil. Cook and Thomson (1940) showed that the rabbit can absorb cholesterol more readily than the rat. This may help to explain the greater susceptibility of the rabbit to hypercholesterolemia and atherosclerosis.

IV. Some general comments on changes in adrenal composition under various conditions

In the course of these present investigations, examinations have been carried out on the adrenal glands of rats and rabbits which have undergone different kinds of treatment. One might anticipate that the effects of these stimuli would result in a similar change in cell composition due to their all acting through ACTH secretion. In fact, a variety of responses in adrenal composition has been observed according

to the nature of the treatment. Considerable differences occurred in adrenal size and composition under different dietary conditions; the type and extent of these changes varied according to whether a protein-containing diet, an amino acid-containing diet or a cholesterol-containing diet was fed. The changes in the adrenals bore both similarities and differences to the changes which occurred after treatment of the animals with ACTH. The changes in size, composition and corticosterone production under the different conditions are summarized in figure 9, which shows that various chemical patterns are produced.

It is generally accepted, that if the adrenal cortex is in a state of increased activity, there is an increase in the output of adrenal steroids. It is well known that ACTH causes an increase in the synthesis of adrenal steroids and this has also been found in the present investigation. This hormone does, however, also cause an increase in the adrenal RNA, protein and phospholipid content, but the mechanism of its action in causing increased cell metabolism and increased steroid production remains obscure. It is as yet unknown if this increase in cell metabolism produced by ACTH is directly connected with steroid synthesis. RNA in tissues is very closely associated with protein biosynthesis and it may be postulated that an increase in RNA in the adrenal may be associated with increased synthesis of the enzymes involved in steroid production. This would explain the increased cell metabolism associated with ACTH administration. Grant et al (1957) showed there is an increase in the 11-hydroxylating enzyme on stimulation of the human adrenal with ACTH which would give rise

Figure 9 shows how different types of stimuli cause varying changes in adrenal size, chemical composition and corticosterone content. It demonstrates that methionine and leucine can cause an increase in adrenal corticosterone with no marked changes occurring in cell size or in other cell constituents. Nutritionally complete protein and ACTH each cause increases in corticosterone content as well as in cell size, RNA, protein nitrogen and lipid phosphorus. The effects of cholesterol feeding and ACTH administration in the rabbit may also be compared where differences in the magnitude of the changes are observed. The results shown on adrenal size, RNA, DNA, protein nitrogen and lipid phosphorus in the rat after ACTH treatment are taken from Munro et al. (1962).

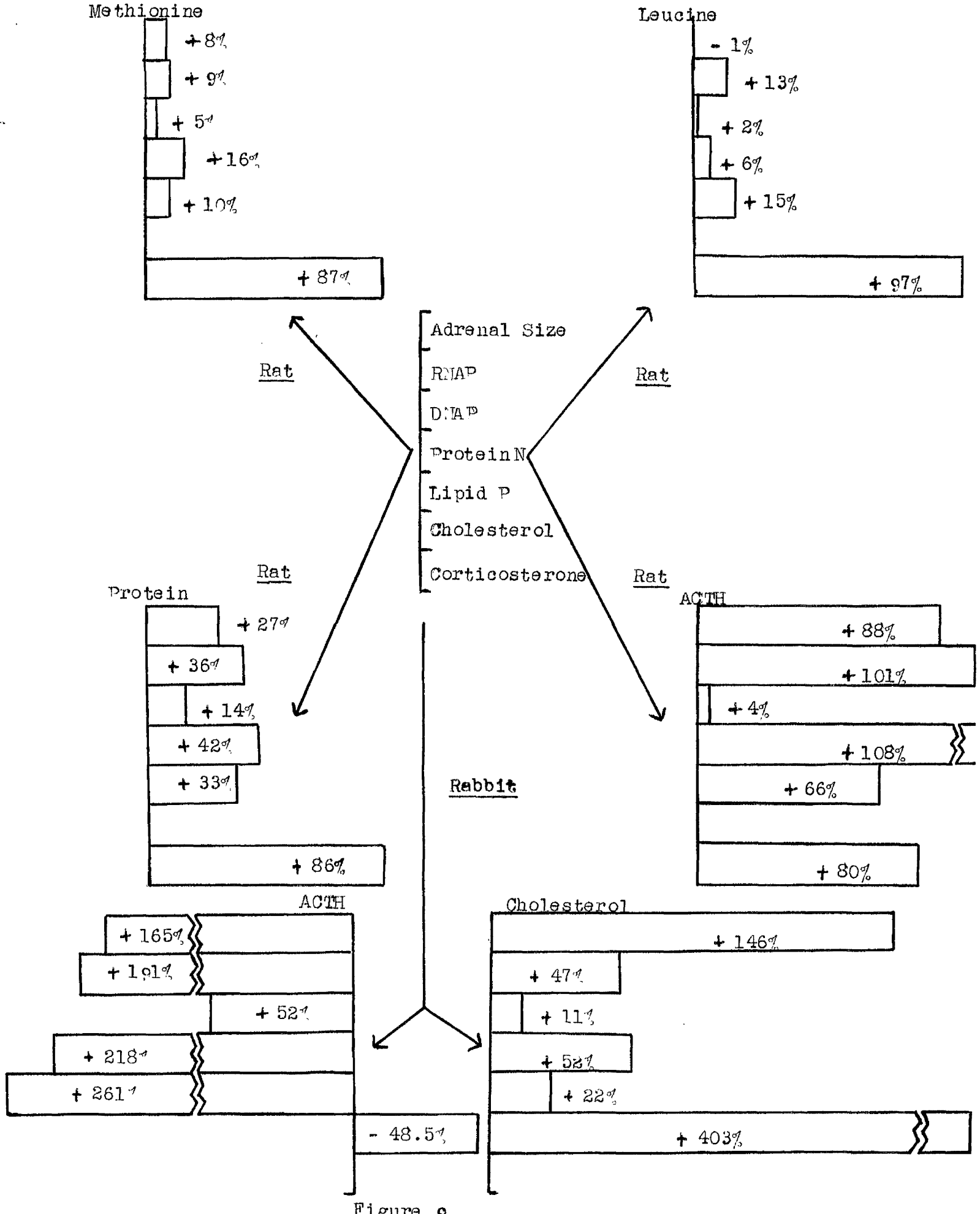

Methionine
+ 8%
+ 9%
+ 5%
+ 16%
+ 10%
+ 87%
Leucine
- 1%
+ 13%
+ 2%
+ 6%
+ 15%
+ 97%
Adrenal Size
RNA
DNA
Protein N
Lipid P
Cholesterol
Corticosterone
Rat
Rat
Rat
Rat
Protein
+ 27%
+ 36%
+ 14%
+ 42%
+ 33%
+ 86%
ACTH
+ 88%
+ 101%
+ 4%
+ 108%
+ 66%
+ 80%
Rabbit
ACTH
+ 165%
+ 191%
+ 52%
+ 218%
+ 261%
- 48.5%
Cholesterol
+ 146%
+ 47%
+ 11%
+ 52%
+ 22%
+ 403%

Figure 9

to increased production of cortisol. It would therefore seem plausible that, in the rat, the enzymes required for corticosterone synthesis may increase after ACTH administration, and that the increased RNA content represents the cell machinery for making more enzyme.

The amino acids, methionine and leucine, were found to cause increased production of corticosterone, but no change in cell composition was detected. Thus their mechanism of stimulation of steroid production must differ from that of ACTH. ACTH may possibly act in two ways in increasing steroid secretion. It has been found that it causes stimulation of steroid secretion very soon after administration (Hechter et al, 1951; Bush, 1953). This increase occurs before any change in adrenal size or composition can be detected. Thus its first action may be to cause a rapid increase in steroid output and its second to cause an increase in adrenal RNA resulting in an increase in enzyme protein which in turn gives rise to an increase in steroid production. The rise in blood corticosterone levels observed after feeding methionine or leucine was found to occur very soon after the rats received a first meal containing these single amino acids. Thus, on the basis of the above hypothesis, stimulation of the adrenal by amino acids would appear to act by the same mechanism by which ACTH produces the rapid stimulation of steroid secretion. As no changes in cell metabolism, in particular in RNA, in the adrenal were observed after feeding these amino acids the increase in steroid secretion is presumably not a result of increased enzyme synthesis. Koritz and Peron (1958) suggested that, as well as

the mechanism of action of ACTH proposed by Haynes and Berthet (1957), namely to stimulate adrenal phosphorylase resulting eventually in increased production of corticosteroids, that in addition it may also convert unavailable corticosteroid precursors into available ones. On the basis of this theory, the conversion of unavailable precursors into available ones may represent a rapid first action of ACTH and so possibly the mechanism by which the single amino acids stimulate corticosterone production. The amino acids would in this way stimulate the first step of corticosterone production and once the available precursors became available ones, the ACTH present in the blood would continue the process of production of corticosterone.

On the other hand, feeding complete protein caused increases in the adrenal content of RNA, protein and phospholipid and in corticosterone levels. This increase in corticosterone was, however, only observed after 11 days feeding and a rapid increase did not occur as in the case of feeding single amino acids and as does with ACTH. Thus, while the presence of complete protein in the diet stimulates the adrenal in a manner similar to that of ACTH, it differs from it in so far as it does not produce a rapid increase in steroid output. Thus it may possibly exert only part of the action of ACTH, namely to cause an increase in steroid synthesis as a result of increased RNA production. In this way its action would differ entirely from that of single amino acids.

The presence of cholesterol in the diet produced considerable increases in gland size and its constituents, although these differed in some ways from those produced by ACTH. It is not as yet possible, due to insuffi-

cient information, to suggest how and if these changes may be associated with steroid production and with the activity of the gland. There may also be changes in the secretion of corticosterone and cortisol, or cholester may just cause a general increase in cell metabolism with no change in gland activity.

Thus it is suggested that the means by which ACTH, single amino acids and proteins act on the adrenal gland differ. ACTH may have two mechanisms of actions - first to cause a rapid stimulation of steroid secretion, and second to increase the adrenal RNA and thus protein synthesis which in turn gives rise to steroid production. Amino acids cause only the first of these effects of ACTH while protein acts on the adrenal cortex by means of the prolonged action of ACTH - increasing steroid production as a result of increased protein synthesis.

V. Comments on liver changes after feeding amino acids or cholesterol

Although only the adequate protein diets fed to rats showed significant changes in adrenal size and composition, many of the diets fed showed considerable changes in liver composition. Both the adequate protein diets and the diets containing the single amino acids showed an increase in liver cell size as demonstrated by the increase in the liver weight to DNAP ratio (Table 11). Leucine appears to have a detrimental effect on the liver causing a significant decrease in liver DNAP content indicating a loss of cells. However, the decrease in DNAP content is greater than the smaller insignificant decrease in liver weight as is demonstrated by the significant increase in the ratio of liver weight to

DNAP. This thus indicates that as well as loss of liver cells in the leucine fed rats, hypertrophy of the remaining cells has occurred. The increase in cell size observed after feeding the casein, glycine, methionine or zein plus tryptophan and lysine diets without a change in liver weight may be attributed to the fact that insignificant increases in liver weight and, except in the case of glycine, insignificant decreases in liver DNAP content were observed, resulting in a significant increase in the ratio of liver weight to DNAP. This indicates that, although no significant changes occurred in overall liver size the size of the average individual liver cells had increased. It would thus appear that expressing the results obtained on liver composition in relation to the DNAP content of the liver and so in relation to the individual liver cells would be a more correct assessment of any changes occurring than by relating the results to the total liver.

The diets containing casein and zein plus tryptophan and lysine caused significant increases in liver RNAP content, while all the diets except methionine and leucine caused significant increases in the amount of RNAP per cell (Tables 11 and 13). Munro and Mukerji (1953) found the RNAP content of the livers of rats fed glycine, methionine or leucine to be significantly increased. However, their estimations of RNAP were carried out on rats which had been killed 19 hours after a single meal containing the amino acid was fed. In the present series of investigations the rats had been fed on the amino acid-containing diet for 11 days and thus had also been receiving a protein deficient diet for this

length of time which in itself leads to a decrease in the liver RNAP content. (Munro, Naismith and Wikramanayake, 1953; Clark, Naismith and Munro, 1957). Thus the two sets of results may not be validly compared.

An increase in protein nitrogen was observed after feeding each of the diets except methionine and leucine while each diet caused an increase in the amount of protein nitrogen per cell (Table 11). Munro and Mukerji (1958) also found an increase in liver protein content after feeding diets containing leucine. Trémolière, Derache and Lowy (1955) showed that one action of cortisone is to cause a deposition of protein in the liver. Thus the increase in liver protein observed when rats were fed the amino acid and protein diets considered in this present investigation may be attributed to increased secretion of hormones from the adrenal cortex. Goodlad and Munro (1959) showed that when cortisone is administered to rats, as well as the liver protein being increased, there is an overall loss of nitrogen, thus the carcass loses nitrogen while there is a gain in the liver (Figure 10). It may be significant that the animals which lost the most weight in the present investigation, namely those fed on methionine or leucine, and to a lesser extent glycine (Table 8), were fed the amino acid which Munro and Mukerji (1958; 1962) showed caused the largest incorporation of P^{32} into liver RNAP and the largest deposition of liver glycogen. Also the present investigation showed that these amino acids caused increased adrenal and blood corticosterone. All these facts would point to increased adreno-

Figure 10 shows diagrammatically the effect of adrenal corticoids in causing a decrease in the protein of the carcass with a concurrent gain in the liver protein.

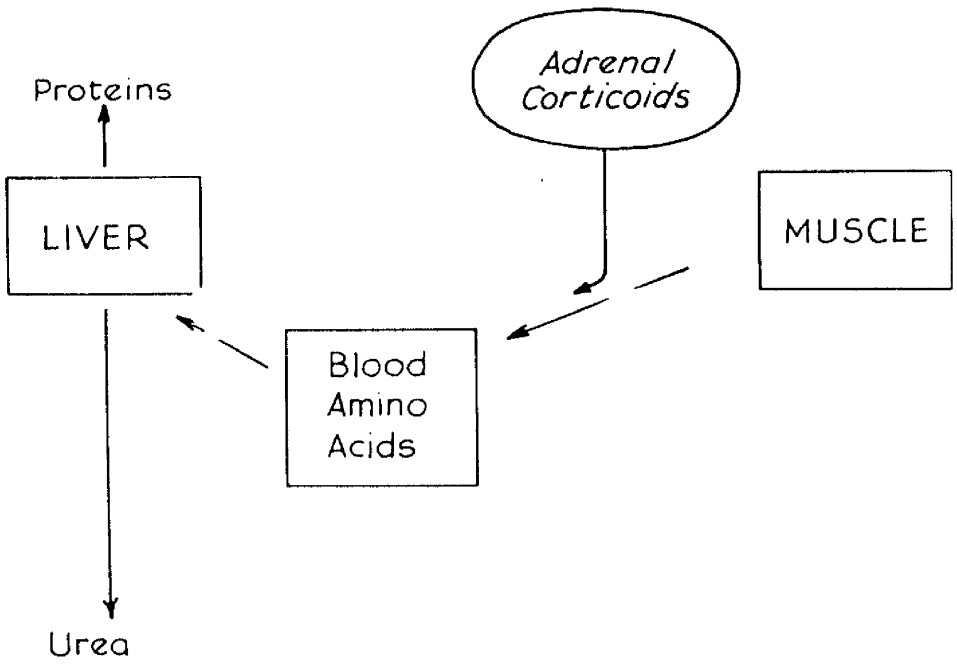

Proteins
Adrenal Corticoids
LIVER
MUSCLE
Blood Amino Acids
Urea

Figure 10.

cortical activity.

Feeding cholesterol to rabbits also caused considerable changes in liver size and composition. Significant increases in liver weight, RNAP and cholesterol were observed (tables 23 and 25). However, the increases in RNAP and cholesterol were not large enough to account for the increase observed in liver size. No increases were observed in liver protein which would be expected if there was increased secretion of adrenocortical hormones. This may thus possibly suggest that the increases observed in adrenal size and cholesterol are not indicative of increased adrenocortical secretion, and that cholesterol in some way acts directly on the adrenal gland and the liver, causing an increase in cell size and a deposition of cholesterol and also an increase in some other constituents.

SUMMARY

Effect of Amino Acid Administration on Adrenal and Liver Metabolism

Previous work showed that single amino acids in large doses can cause changes in liver protein and nucleic acid metabolism which are dependent on intact adrenal glands. The present work was undertaken to investigate further the effects produced by these amino acids, particularly on metabolism of the adrenal glands.

1. The effect of feeding various single amino acids, incomplete proteins and complete proteins on both adrenal and liver size and composition have been investigated in the rat. The effect of these diets on blood and adrenal corticosterone levels have also been examined.

2. The presence of a complete protein in the diet caused significant increases in adrenal weight, RNAP and protein nitrogen. The incomplete proteins and the single amino acids had no influence on either adrenal size or general chemical composition.

3. Feeding a single dose of methionine or leucine caused an increase in blood corticosterone 24 hours after feeding. After prolonged feeding of these amino acids for 11 days the levels of corticosterone in the adrenals were significantly increased while the blood levels although showing marked increases just failed to attain statistical significance. After prolonged feeding of casein, or zein plus tryptophan and lysine, similar increases in blood and adrenal corticosterone levels were observed, which were not apparent after a single dose of protein. The single amino acids, methionine and leucine, thus differ from protein in two respects in their stimulation of the adrenal cortex - (a) increased corticosterone levels

were observed 24 hours after a single dose of the amino acid, whereas increases after feeding protein are only observed after prolonged feeding, and (b) they cause no increase in adrenal size or constituents such as that observed after feeding a diet containing a complete protein.

4. The composition of the diet caused significant changes in liver composition. Complete protein and the single amino acids, glycine, methionine, and leucine caused increases in the size of the average liver cell. All the diets fed caused increases in the amount of protein per cell and each diet except methionine and leucine caused increases in the amount of RNAP per cell. The lipid phosphorus per cell was also raised after feeding the complete proteins or the incomplete protein zein.

5. It was concluded that the single amino acids, methionine and leucine, cause increased production of corticosterone by a direct stimulation of the adrenal gland and that their action is not mediated via the pituitary. Complete protein, on the other hand, causes an increase in adrenal size, constituents and corticosterone presumably by causing increased secretion of ACTH from the pituitary.

Effect of Cholesterol feeding on Adrenal and Liver Metabolism

1. Feeding rabbits a diet containing 1% cholesterol caused considerable increases in size and composition of the adrenals compared to control animals fed on stock diet. The adrenal weight increased with gross deposition of cholesterol. The RNAP and protein nitrogen also increased, but the increases observed in the adrenal constituents could not com-

pletely account for the enlarged size of the adrenals. This increase in adrenal size was due almost entirely to an increase in cell size as was shown by the fact that there was very little change in the DNAP content of these glands. Histological and histochemical studies showed that the main effect of cholesterol administration occurred in the zona fasciculata, where the cells underwent enlargement and showed gross changes histochemically.

2. The increase in size of the adrenals of cholesterol-fed rabbits was compared with the increase in size observed after treatment with ACTH. ACTH caused an increase in adrenal size of the same magnitude as did cholesterol feeding but the type of increase was different. In this case the DNAP also showed an increase, indicating that hyperplasia had occurred as well as hypertrophy. Also, the increase in adrenal size due to ACTH differed from that of cholesterol as ACTH caused an increase in RNAP and protein of a magnitude which corresponded with the increase in the size of the gland itself. A decrease in adrenal cholesterol was observed after ACTH as has been observed in other species.

Rabbits which had previously received a cholesterol-rich diet showed no change in their responsiveness to ACTH from animals on stock diet.

3. The sex of the rabbit had no significant influence on the effect produced on the adrenal gland by feeding cholesterol.

The effect of the presence of increased protein in the diet on adrenal composition after cholesterol feeding was examined. An increased deposition of cholesterol was observed but there was no change in the other

adrenal constituents compared to the rabbits receiving a lower amount of protein.

The effect of duration of time of cholesterol feeding was examined and there was no correlation between the duration of feeding and the effect produced on the adrenal gland.

4. The presence of cholesterol in the diet also caused considerable increases in the size of the rabbit liver with an increase in cholesterol and RNAP. An increased level of dietary protein caused increased deposition of cholesterol in the livers of rabbits fed cholesterol compared to those receiving a lower level of dietary protein.

5. Feeding rats a diet containing cholesterol had no influence on adrenal size or composition.

6. It has been concluded that the adrenal enlargement caused by cholesterol administration is not completely typical of increased secretion of ACTH from the pituitary gland, nor does cholesterol sensitise the adrenal to the action of ACTH. Its action may therefore possibly be a direct one on the gland itself.

General Conclusions. It is concluded that the adrenal cortex can show a variety of types of response to different stimuli.

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