STUDIES ON THE RIBONUCLEIC ACID METABOLISM OF THE RAT ADRENAL GLAND

by

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SECTION 1

I N T R O D U C T I O N

1.1. The Adrenal Gland.

(a) Historical.

The adrenal glands were first noticed by Eustachius in 1565 as a result of his studies on the kidney. He described them as "glandulae renibus incumbentes". There was, however, no significant advance in the knowledge of the function of the adrenal gland until 1855 when Addison published his observations on the effects of adrenal insufficiency in man. Brown-Séquard (1858), perhaps stimulated by this work, studied the effects of adrenalectomy on animals and found that such an operation invariably resulted in the death of an animal, thus proving that the adrenals were essential for life.

Although the idea of certain glands, without ducts, secreting directly into the blood stream was first suggested in 1766 (Haller), the concept of "internal secretion" was not fully appreciated till 1855 by Claude Bernard. The next half century saw a growing interest in these ductless glands and in 1905 Starling gave the name "hormones" to their secretory products. The findings of Smith (1927; 1930) that there is a fundamental relationship between the adenohypophysis and the adrenals marked the beginning of intensive research into the metabolism and function of the adrenals which has continued unabated to this day.

(b) Histology of the Adrenal Glands.

In the mid nineteenth century it was realised that the adrenal consisted of two parts, the "medulla" and the "cortex" (Huschke, 1845; Gray, 1852; Köllikor, 1854), the medulla being the inner part and the cortex the outer part of the gland. It was not until much later, however, that it was realised that the medulla and cortex are different with respect to function. The cortex was described by Arnold in 1866 as being divided into three zones, on the basis of work done with teased or macerated sections. He demonstrated that the outer layer of cells were in the form of little baskets lying directly underneath the capsule in which the gland is enclosed. He called this group the zona glomerulosa. Adjacent to this zone the cells were found to be in the form of bundles and the name given to them was the zona fasciculata. The innermost group of cells was named the zona reticularis due to the network of reticular fibres which were observed to run through this zone. The adrenal cortex has an extensive vascular blood supply (Flint, 1900; Bennett and Kilham, 1940; Grollman, 1941) and is lacking in nerve endings (Greep and Deane, 1949). Arnold's classification was given on gross morphological grounds and later the various zones were also found to be quite different when their

histology was examined more closely.

Zona glomerulosa.

This is a small zone, the width of which varies in different species. In human adrenals it is on occasion difficult to distinguish this zone from the zona fasciculata (Symington 1960) but it is generally easily recognisable in the rat adrenal. In the cells themselves there is found a high content of cholesterol and lipids and a large number of mitochondria (Hoerr, 1931; Deane and Greep , 1946; Miller, 1950). These cells are also rich in RNA (Symington and Davidson, 1956; Symington Duguid and Davidson, 1956).

Zona fasciculata.

This is generally the widest zone of the three, comprising, in the human adrenal, 78% of the cortex, while 16% is composed of glomerulosa cells and 6.4% of reticularis cells (Swinyard, 1940). The cells of this zone are larger than those of the other zones and are arranged in a columnar fashion. These cells are poor in RNA (Symington and Davidson, 1956) but are abundant in lipid except in those cells of a small sudanophobic zone where the zona fasciculata meets the zona glomerulosa. The cytoplasm of the zona fasciculata does not stain deeply with haematoxylin and eosin, suggesting that the amounts of cell constituents such

as protein are small.

Zona reticularis.

The cells of this zone form a network around sinuses. They are smaller in size than the cells of the zona fasciculata and the cytoplasm stains more deeply than fasciculata cells. In general there is an absence of lipid material but the amount of RNA is high and a large number of mitochondria are usually observed (Carr, 1959; 1962; Symington, 1960).

(c) The Nature of the Secretions of the Adrenal Cortex.

The secretory products of the adrenal cortex have been known to be necessary for life since 1855 (Brown-Séquard) but it has only been in the last thirty years that these secretions have been isolated and identified. The early isolations and chemical identifications which showed these secretions to be steroid in nature were carried out by three groups of workers, Kendall, Reichstein, and Wintersteiner and Pfiffner. By 1938 twenty one different steroids had been isolated from bovine adrenal, among them corticosterone and hydrocortisone (Reichstein, 1937; Mason, Hoehn, McKenzie and Kendall, 1937; Mason, Hoehn and Kendall, 1938). Cortisone (Wintersteiner and Pfiffner, 1955) and 11-dehydrocorticosterone (Mason, Myers and Kendall, 1936; Mason et al, 1937) 11-deoxy-corticosterone was isolated in 1939 by Reichstein and

von Euw. These five steroids show biological activity with effects on the metabolism of certain tissues and in particular the carbohydrate and protein metabolism. Such steroids are usually referred to as the glucocorticoids. Although they were first isolated from beef adrenals they are found in the adrenals of all mammals studied. By 1959, over 40 steroids has been isolated from adrenal extracts (Wettstein, 1959) although they are not all secreted by the glands and are not all biologically active.

The relative amounts of glucocorticoids secreted varies from one species to another (Bush, 1955a,b; Romanoff, Hudson and Pincus, 1953). For example, the human adrenal is known to secrete predominantly cortisol, the rat adrenal only corticosterone and the rabbit adrenal secretes a mixture of the two (Bush 1955b; Kass, Hechter Macchi and Mou, 1954). Bush (1955a) has suggested that the ratio of cortisol to corticosterone is genetically linked for each species, but Hechter, Macchi, Korman, Frank and Frank (1955) found that the ratio of cortisol to corticosterone in the blood of dogs selected at random varied from 1.2 : 1 to 20 : 1, and Grant, Forrest and Symington (1957) have shown that ACTH administration dramatically alters this ratio. A similar change in secretion after ACTH

treatment has been observed in rabbits (Kass, et al, 1954). The reason for the absence of cortisol in the blood of rats has been tentatively explained as due to the low amount of the 17α-hydroxylase compared to the 11β-hydroxylase (Hofman, 1957).

The adrenal steroids are all basically similar in structure, all consisting of a perhydrocyclopentenophenanthrone ring system. They all possess an α,β-unsaturated ketonic group in ring A and all have a 2-carbon side chain at carbon atom 17, $-\overset{O}{\overset{\|}{C}}-CH_2OH$, referred to as the α-ketol side chain. Both these groupings seem to be necessary for biological activity.

Although it was realised as early as 1898 (Soddu, 1898) that the adrenal was involved in some way with mineral metabolism, it was not until the 1950's that the discovery and identification of aldosterone was made (Grundy, Simpson and Tait, 1952; Simpson, Tait, Wettstein, Neher, von Euw and Reichstein, 1953). This steroid, the 18-aldehyde of corticosterone, was shown to have powerful effects on electrolyte balance, being many times more potent in causing renal retention of sodium in adrenalectomised rats than is deoxycorticosterone (Prunty, McSwiney, Mills and Smith, 1954; Mach and Fabre, 1955; Simpson and Tait, 1955). A steroid which can cause sodium retention in rats has been isolated

from pig adrenals by Neher, Desaulles Vischer, Wieland and Wettstein (1958) and identified as 3β, 16α dihydroxy-5α-pregnane-20-one, but the significance of this remains unknown.

Sex hormones are also found in the adrenal cortex. Progesterone was isolated from bovine adrenals by Beall and Reichstein (1938) and since then many other steroids which influence the sex accessory glands have been isolated (Short, 1960). The existence of these steroids in the adrenal cortex is, of course, not evidence for their secretion, but Short (1960) has analysed the androgen content of the adrenal venous blood of women stimulated by ACTH and has found that there are appreciable amounts of such compounds secreted. The androgens found were androstenedione, dehydroepi-androsterone, 11β hydroxyandrostenedione and 17α hydroxy-progesterone. Only the last two androgens are found in bovine adrenal venous blood. Progesterone has been found to be secreted by the human adrenal in appreciable quantities (Balfour, Comline and Short, 1957). It is well known that adrenal disorders in man can result in virilization or feminization due to secretion of the sex hormones in abnormal amounts into the blood stream (Short, 1960; Wilkins, 1962; Symington and Jeffries, 1962) and the fact that man is particularly susceptible to these disorders may be coupled with the

observed higher amounts of sex hormones secreted by the adrenals when compared to the amounts secreted by the adrenals of other species.

(d) Corticosteroid Biosynthesis in the Adrenal.

Most tissues appear to be capable of synthesising cholesterol, the liver being the most active in this respect (Gould, 1958), but only a few can convert the cholesterol to steroid hormones. Perhaps the most interesting feature of the biosynthesis of steroids is that the pathways are so similar in the different tissues (Samuels, 1956; Dorfman, 1957), that is in the adrenals, ovaries, testes and placenta. Indeed the differences probably are reflections of the control mechanisms operating on the biosynthetic pathways producing the steroids characteristic to each particular organ.

It is generally accepted that the adrenocortical steroids are synthesised from acetate (Dorfman, 1957). The sequence of reactions has recently been reviewed by Grant (1960; 1962). By a long series of reactions acetate is condensed to form cholesterol through intermediates such as Δ^3 isopentenyl pyrophosphate and squalene (Popjak, 1958; Goodwin, 1960). The side chain of cholesterol is degraded by a pathway involving at least five enzymes to yield progesterone which is

the key intermediate in the biosynthetic pathway of corticosteroids and the sex hormones. By a series of complex hydroxylases which appear to require $NADPH_2$ and molecular oxygen, progesterone is oxidised to the adrenocortical steroids (Dorfman, 1957; Grant 1962). Some aldosterone is derived from corticosterone but this mechanism is not yet fully understood (Ayres, Eichorn, Hechter, Saba, Tait and Tait, 1960; Stachenko and Giroud, 1962).

Some controversy has arisen as to whether cholesterol is an obligatory intermediate in adrenal steroid biosynthesis (Stone and Hechter, 1954; Hechter, 1958). Also, Heard, Bligh, Cann, Jellinck, O'Donnell, Rao and Webb (1956) have observed that cell-free homogenates of pig adrenals could incorporate 1-^{14}C-acetate into corticosterone and cortisol but the specific activity of the cholesterol isolated was lower than those of the corticosteroids. These results suggested that cholesterol was not an obligatory intermediate in cortisol and corticosterone synthesis. However, it is possible that newly formed labelled cholesterol is not extensively diluted into the metabolic pool but is transformed into corticosteroid without equilibrating with the unlabelled cholesterol. This would seem to be the case, as Werbin and Chaikoff (1961) found that

after feeding guinea pigs with ^{14}C-cholesterol until the cholesterol of the adrenal gland was in isotopic equilibrium, urinary cortisol had a specific activity similar to that of adrenal cholesterol. However, Caspi (1962) using degradation experiments has shown that the pattern of labelling of corticosteroids from ^{14}C-acetate was not entirely explained by a pathway via cholesterol. Thus the question still remains open.

(c) Adrenocorticotrophic Hormone (ACTH).

It has been known since the work of Smith (1927; 1930) that a relationship exists between the pituitary and the adrenal cortex. He showed that hypophysectomy was followed by an atrophy of the adrenal cortex and that this atrophy could be reversed by administration of pituitary extracts. These observations stimulated research into the nature of the factor in the pituitary which affected the adrenal cortex and after years of work by many workers, preparations were isolated from sheep pituitaries (Li, Simpson and Evans, 1948) and pig pituitaries (Sayers, White and Long, 1948) which were capable of increasing adrenal weight. The significant feature of these early preparations was that, although they were capable of increasing adrenal weight in hypophysectomized rats, they had no effect on the

weight of other tissues such as thyroid and gonads. Further purification of ACTH was achieved by Payne, Raben and Astwood (1950), Astwood, Raben, Payne and Grady (1951) and Dixon, Moore, Staeke-Dunne and Young (1951), and by 1954 Bell had elucidated the sequence of 39 amino acids of a preparation from pig pituitaries. Later work, notably that of Li (1956) has determined the sequence of the ACTH's of various species. It has also been established that the biological activity is not unduly affected by removal of the 11 amino acids from the carboxyl terminals of the molecule (Li, 1956) and indeed the variations in sequence of the amino acids in the ACTH of different species are to be found in these 11 amino acids (Li, 1956; Hofman, 1962). Recently, peptides with ACTH like biological activity have been chemically synthesised by Hofman, Gajima, Yanaihara, Liu, and Lande (1961) and Schwyzer and Sieber (1963).

ACTH appears to be stable in the isolated pituitary but is destroyed by cell-free preparations of pituitary (Barret and Sayers, 1958) and of liver, kidney and adrenal (Geschwind and Li, 1952).

ACTH is removed from the blood by essentially two processes; it is absorbed from the blood by the tissues and notably by the kidney but this uptake

seems to be nonspecific (Cats and Kassenaar, 1957). ACTH can also be destroyed by a system in the blood itself (Meakin, Tingey and Nelson, 1960). However, inactivation of injected ACTH can be diminished by suspending the ACTH in $Zn(OH)_2$ (van der Vies, 1960).

(f) The Effects of ACTH on Adrenal Cortex.

ACTH is known to have many effects on the adrenal gland. An early observed effect was that ACTH increased the weight of the gland (Simpson, Evans and Li, 1943). In 1946 Sayers, Sayers, Liang and Long noticed that ACTH administration caused a decrease in the cholesterol and ascorbic acid content of the adrenal and on the basis of this depletion of ascorbic acid a bioassay was proposed (Sayers, Sayers and Woodbury, 1948). ACTH has been found to cause a decrease in the glycogen content of the adrenal gland (Noble and Papageorge, 1955), but this finding has been questioned by Vance, Girard and Cahill (1962). ACTH also causes an increase in the permeability of the adrenal (Canis, Miller and Axelrod, 1955; Hechter and Lester, 1960) and increased uptake of inorganic phosphorus into the total acid soluble phosphorus of the adrenal (Gemzell and Samuels, 1950; Riedel, Logan, De Luca and Rossiter, 1954). Effects on the adrenal nucleic acid metabolism have also been observed but

these will be more fully described in a later section.

However, the most important effect of ACTH is that which causes an increase in the output of corticosteroids. It was shown that adding ACTH to the fluid perfusing a bovine adrenal caused an almost immediate increase in corticosteroids in the perfusate (Hechter, 1949; 1956; Pincus, Hechter and Zaffaroni, 1951). Increase of corticosteroid production after ACTH administration has also been observed in rat adrenal slices (Saffran, Grad and Bayliss, 1952). Increases in the corticosteroid output from the adrenal cortex *in vivo* after ACTH administration have been observed by Bush (1955a,b) using the technique of Vogt (1951).

As previously mentioned ACTH, besides stimulating the output of corticosteroids, has been shown to alter the relative amounts secreted (Kass *et al*, 1954; Grant *et al*, 1957). These observations would suggest that ACTH, besides stimulating the output of the adrenal steroids, also affects the level of hydroxylases.

The other important steroid secreted by the adrenal cortex is aldosterone and ACTH appears to be capable of stimulating an increase in aldosterone in the decerebrate dog (Farrell, Fleming, Rauschkolb, Yatsu, McCally and Anderson, 1958). ACTH is not the only factor controlling aldosterone secretion for in

hypophysectomized rats the width of the zona glomerulosa increases (Swann, 1940; Deane and Greep, 1946) and more recently Farrell (1959,a; 1959b) has described a factor which can be isolated from the diencephalon which will stimulate the secretion of aldosterone and not of cortisol or corticosterone.

(g) Zonation and Function of the Adrenal Cortex.

In 1883 a hypothesis was introduced by Gottschau suggesting there was a relationship between the three zones of the adrenal cortex. He believed that the adrenal cells originated at or near to the capsule, migrating inwards through the various zones and finally degenerating in the medulla. The 'escalator theory' as it was called was accepted in a modified form by many workers (Graham, 1916; Bennett, 1940; Salmon and Zwemer, 1941; Gruenwald and Konikov, 1944).

Swann in 1940, was the first to suggest that the three zones of the adrenal cortex, as well as being histologically different, also differed functionally. He also suggested that all the zones of the adrenal cortex are not under the control of ACTH. Deane and Greep, (1946) and Deane, Shaw and Greep, (1948) provided evidence that the zona glomerulosa and the zonae fasciculata and reticularis have different functions. Their conclusions were based on histological studies of rats which had been hypophysectomized or fed diets

containing varying amounts of salts. These findings were confirmed by Singer and Stacke-Dunne (1954) and Farrell, Rauschkolf and Royce (1955). The 'zonal theory' (Jones, 1946) has been confirmed by the work of Ayres, Gould, Simpson and Tait (1956) and Giroud, Stachenko and Venning (1956). These workers were able to demonstrate that aldosterone is only produced by the zona glomerulosa cells of the ox and rat adrenals respectively. Jones (1948; 1957) believed that the zona reticularis only produced sex hormones. It is now thought that the zona reticularis and the zona fasciculata represent different aspects of functional activity (Yoffey, 1955; 1956; Dawson, 1956; Symington and Davidson, 1956; Symington et al, 1956).

Thus the belief at present is that the zona glomerulosa is not normally controlled by ACTH, and produces aldosterone in response to changes in electrolyte balance. The zona reticularis produces the normal requirements for glucocorticoids and that ACTH stimulation above normal will cause the zona fasciculata to produce and secrete steroids and in doing so becomes histologically similar to the zona reticularis. The reason for sex hormones being secreted by the adrenal appears to be little understood.

(h) Mechanism of Action of ACTH.

Although much work has been done on the mechanism of action of ACTH and hypotheses have been advanced suggesting the metabolic loci of its action none of them has yet satisfactorily explained how ACTH stimulates the secretion of corticosterone and causes the other observed effects.

Stone and Hechter (1954) using ^{14}C labelled precursors observed that in the perfused bovine adrenal ACTH increased the synthesis of corticosterone and cortisol from acetate or cholesterol but that ACTH had no effect on the conversion of progesterone to corticosterone and cortisol. This suggested that ACTH influenced the conversion of cholesterol to progesterone.

ACTH has been demonstrated to have a stimulating effect on hydroxylation of the steroid nucleus (Kass, et al. 1954) and later work has shown that 11-β hydroxylation is dependent on the presence of $NADPH_2$ (Hayano, Wiener and Lindberg, 1953; Sweat and Lipscomb, 1955; Grant and Brownie, 1955). Furthermore, there is an active hexosemonophosphate shunt in the adrenal (Glock and Maclean, 1954; Kelly, Neilson, Johnson and Vesting, 1955), during the operation of which $NADPH_2$ is formed. The addition of liver phosphorylase and glycogen to adrenal homogenate was found to stimulate

steroidogenesis and also ACTH increased the phosphorylase activity of bovine adrenal slices (Haynes and Berthet, 1957). These results led Haynes and Berthet to postulate that ACTH caused adrenal phosphorylase to be actived so increasing the amount of glucose-1-phosphate which would be converted to glucose-6-phosphate. The glucose-6-phosphate would then be oxidised by the hexose monophosphate shunt with the concomitant formation of $NADPH_2$ which would thus be available for steroid hydroxylations. Later findings showed that the addition of 3',5' adenosine monophosphate (3',5' AMP) to adrenal tissue could activate adrenal phosphorylase (Haynes, 1958) and stimulate *in vitro* steroid production (Haynes, Koritz and Peron, 1959). It had already been established that 3',5' AMP activates liver phosphorylase (Sutherland and Rall, 1957) and so it was suggested that ACTH acted by increasing the amount of 3',5' AMP which would then activate phosphorylase so resulting in an increased amount of $NADPH_2$. There is other evidence supporting this hypothesis. An increase in glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in the border zone between the *zona reticularis* and *zona fasciculata* has been observed after ACTH stimulation (Greenberg and Glick, 1960). Also the glycogen content of the *zona fasciculata* is diminished

by exposing rats to stress (Noble and Papageorge, 1955). The stimulation of corticoid formation caused by 3',5' AMP cannot be additively increased by addition of ACTH. This would suggest that both substances act by a similar mechanism (Birmingham, Kurlents, Kane, Muhlstock and Traikov, 1960).

There is, however, data which suggests that this hypothesis must be applied with caution. Glucose does not stimulate the production of corticosteroids by adrenal tissue *in vitro* (Schönbaum, Birmingham and Saffran, 1956; Vance, Girard and Cahill, 1962). This is the converse of that predicted by the Haynes and Berthet hypothesis. Also ACTH has no effect on the glycogen content of the rat adrenal (Vance *et al.*, 1962). The phosphorylase activity of bovine adrenal slices has not been found to be significantly changed by ACTH (Williams, Johnson and Field, 1962). Observations on the oxidation of ^{14}C- labelled glucose by adrenal tissue has thrown doubt as to whether glucose metabolism via the hexose monophosphate shunt is stimulated by ACTH (Field, Pastan, Herring and Johnston, 1961; Williams *et al.*, 1962; Vance *et al.*, 1962; Weaver and Landau, 1965). The enhancement by glucose of the stimulatory effect of ACTH on steroid secretion (Schönbaum *et al.*, 1956) has been questioned by

Field et al., (1961), and by Vance et al., (1962).

There is also the possibility that ACTH may exert its effect by altering the permeability of the cellular membrane (Hechter, 1955), and it has been shown that ACTH accelerates the transport of amino acids into the cortical cell(Hechter and Lester, 1960). ACTH has also been noted to increase the entry of D-xylose into the rat adrenal glands in vivo (Eichhorn, Halkerston, Feinstein and Hechter, 1960).

(1) Control of Secretion of ACTH.

As mentioned earlier, it is known that ACTH secreted by the pituitary can stimulate the adrenal cortex in a variety of ways. The question arises as to how this secretion of ACTH is controlled.

Many stimuli such as heat, cold and fear cause an increase in blood corticosteroids which is itself produced by an increase in blood ACTH. It thus seems that changes in environment and emotion can affect the release of ACTH, supposedly by a neural mechanism. The anterior pituitary itself is relatively free of nerve fibres running to the rest of the organ (Harris, 1955), and cutting the pituitary stalk (Fortier, Harris and McDonald, 1957) did not permanently stop the release of ACTH by stressors. It therefore appeared that the release of ACTH is not under direct nervous control.

It has been shown, however, that after lesion of the hypothalamus especially at or near to the median eminence, ACTH is no longer released after stress of any kind. This and other evidence (Harris, 1955; Brodish and Long, 1962) suggested that the factor causing release of ACTH originated from the median eminence of the hypothalamus. At first it was believed that this factor, the ACTH releasing factor (CRF), was identical with vasopressin which is synthesised in the hypothalamus (Scharrer and Scharrer, 1954) and there was a great deal of circumstantial evidence suggesting this was so. For example stress was often associated with antidiuresis (Mirsky, Stein and Paulisch, 1954) and McCann (1957) obtained evidence which seemed to prove that the CRF was vasopressin. However, it is now generally accepted that CRF is not identical with vasopressin although they are thought to be similar in structure. Administration of large amounts of water which inhibit the release of vasopressin also cause ACTH to be secreted (Nagareda and Gaunt, 1951). Also, even when ACTH secretion is inhibited by injecting large doses of steroids, vasopressin can be shown to be released (De Wied and Mirsky, 1959). More convincing evidence has been supplied by Saffran (1962) who, using *in vitro* techniques

for assay has shown that ORF can be at least partially separated ~~isolated~~ from vasopressin.

1.2. The Nucleic Acids.

1. Historical.

The discovery of the nucleic acids was first made by Miescher in 1868 and preparations of nucleic acids from many sources were made during the next few decades (Allen, 1962). It was thought that there were two nucleic acids; one resembling that isolated from thymus tissue and which could be isolated from nuclei of animal tissues and the other found in the cells of plant tissues (Jones, 1921). This idea was never founded upon much experimental evidence and it was not long before many workers disproved this concept. By 1924 it was realised that both nucleic acids could be found in animal tissues (Jones and Perkins, 1924). Until the late 1930's it was assumed that the nucleic acids were nuclear in origin but due to the work of Behrens (1938), Caspersson and Schultz (1938; 1939) and of Brachet (1940; 1941) RNA was demonstrated to exist in the cytoplasm. The early work on the nucleic acids is reviewed by Chargaff and Davidson (1955).

2. Deoxyribonucleic Acid. (DNA)

DNA can be regarded as a polynucleotide chain with 5',3' phosphate ester linkages (Brown and Todd, 1955). It contains the nucleotides deoxyadenylic acid,

deoxyguanylic acid, deoxycytidylic acid and thymidylic acid. Some other nucleotides are also found to a lesser extent (Wyatt and Cohen, 1953; Dunn and Smith, 1955). The DNA molecule consists of two anti-parallel chains interwoven into a double helix winding round the same axis and held together by specific hydrogen bonds between the constituent bases (Wilkins, Stokes and Wilson, 1953; Watson and Crick, 1953). The possibility that some of the DNA in the cell is not double-stranded has been raised (Sondich and Rosenkranz, 1963). The molecular weight of native DNA is very high and indeed a recent report suggests that the DNA of Escherichia coli is a single molecule of molecular weight of about 10^9 (Cairns, 1962; 1963). Avery, MacLeod and McCarty (1944) were first to demonstrate that the DNA molecule was the carrier of genetic information.

Although DNA is primarily located in the nuclei, reports have been made suggesting that DNA can be found in the cytoplasm (Chèvremont, Chèvremont-Comhaire and Baeckland, 1959; Beck and Moses, 1963; Nass and Nass, 1963a,b).

8. Ribonucleic Acid (RNA).

(a) Structure of RNA

Hydrolysis of RNA yields D-ribose, phosphoric acid

and the bases adenine, guanine, cytosine and uracil. Some species of RNA also contain smaller amounts of other bases (Adler, Weiseman and Gutman, 1958; Smith and Dunn, 1959). RNA can be regarded as a polynucleotide chain linked by 3',5' phosphate ester linkages (Markham, 1957). The secondary structure of RNA is less well defined than that of DNA and it is thought to consist of comparatively short helices formed by hydrogen bonding between bases of the same chain (Doty, Boedtker, Fresco, Haselkorn and Litt, 1959a; Spirin, Gavrilova, Bresler and Mossevitsky, 1959). Brown and Zubay (1960) proposed a structure for sRNA in which a high proportion of the bases were hydrogen bonded and it is known that sRNA in the solid state possesses a perfect DNA-like helix (Spencer, Fuller, Wilkins and Brown, 1962). There is some controversy as to whether RNA molecules possess a tertiary structure. Doty and his colleagues believe that RNA has no tertiary structure (Doty et al., 1959; Doty, Boedtker, Fresco, Hall and Haselkorn, 1959b), but Dvorkin and Spirin (1960) have published evidence suggesting that RNA does have a tertiary structure.

(b) Heterogeneity of Cellular RNA

The highest proportion of the cellular RNA is found in the subcellular components known as the ribosomes

(Hoagland, 1960) which are found in the mammalian cell both free and attached to the endoplasmic reticulum (Palade and Siekevitz, 1956 a,b; Roberts, 1958). Isolated ribosomes, which have a sedimentation coefficient 70-80, dissociate on lowering the concentration of magnesium ions of the medium in which they are suspended into two sub-units, one of 50s and one of 30s (Bolton, Hoyer and Ritter, 1958; Tissières, Watson, Schlessinger and Hollingworth, 1959; Lamfrom and Glowacki, 1962). RNA has been isolated from these particles and it has been found that the 50s ribosomes contain an RNA molecule of s value between 23 and 30 and the 30s ribosomes contain an RNA molecule of s value between 16 and 19 (Timasheff, Brown, Colter and Davies, 1958; Hall and Doty, 1959; Tissières et al., 1959; Kurland, 1960). The absolute value depends upon the origin of the ribosomes. The subject has been reviewed by Spirin (1963).

SRNA on the other hand has a molecular weight of about 25,000 to 30,000 (Tissières, 1959; Cox and Littauer, 1960) and is quite distinct from ribosomal RNA in base composition and perhaps even in secondary structure.

The remaining RNA in the mammalian cell is found in the nucleus and in the mitochondria. The mitochondrial

RNA has not been studied as fully as that of the microsomes, but on the other hand, that of the nucleus has been shown to be heterogeneous with respect to metabolic activity and physical size (Logan and Davideon, 1957; Osawa, Takata and Hotta, 1957; 1958; Georgiev and Mantieva, 1960; 1962; Hiatt, 1962; Sibatani, de Kloet, Allfrey and Mirsky, 1962; Sporn and Dingman, 1962).

A cellular RNA constituent whose existence has only been recently discovered is messenger RNA or template RNA. The existence of such an RNA fraction was first suggested by Jacob and Monod (1961) and Brenner, Jacob and Meselson (1961) on the basis of experiments carried out on bacteriophage T2 infected cells of E. coli by Volkin and Astrachan (1956), Nomura, Hall and Spiegelman (1960), and Gros, Hiatt, Gilbert, Kurland, Risebrough and Watson (1961). RNA fractions from mammalian tissues have recently been reported which have the properties of messenger RNA similar to that found in bacteria, namely (i) has a DNA-like base composition (Sibatani, et al., 1962), (ii) is rapidly labelled and of unstable nature (Sibatani et al., 1962; Hiatt, 1962; Scholtissek, 1962) and (iii) is capable of stimulating protein synthesis in vitro (Barondes, Dingman and Sporn, 1962; Braverman, Gold

and Eisenstadt, 1965). It thus seems that a type of RNA exists in mammalian tissues analagous to the messenger RNA in bacteria.

(c) Function of RNA.

It is now firmly established that RNA is intimately involved in protein synthesis. The association was first suggested by Brachet (1950) and Caspersson (1942; 1950). The steps in the activation of the amino acids, their attachment to sRNA molecules and the transfer of the amino acids from the sRNA to the ribosomal RNA, have been reviewed by Hoagland (1960) and Berg (1961). Recently, the means by which the amino acid sequence of a protein is determined and the mechanism whereby protein biosynthesis is controlled have become clearer (Crick, Barnett, Brenner and Watts-Tobin, 1961). It is believed that the base sequence of an unstable RNA fraction (messenger RNA), of nuclear origin, provides the code for the amino acid sequence of a protein (Crick, 1963). Multi-ribosomal structures have been demonstrated in rabbit reticulocytes and in rat liver and it is believed that the ribosomes are held together by strands of messenger RNA (Warner, Knopf and Rich, 1963; Wettstein, Staehelin and Noll, 1963; Noll, Staehelin and Wettstein, 1963).

Models of protein synthesis compatible with the present available data have been proposed (Gierer, 1963; Gilbert 1963a,b).

(d) Biosynthesis of RNA.

(i) in vitro studies

RNA biosynthesis in vitro is studied by demonstrating the incorporation of radioactive RNA precursors into an acid insoluble product which can then be shown for example, by treatment with alkali or ribonuclease (RNase) to have the properties of a polyribonucleotide. Systems have been discovered and one is the system which catalyses the following reaction

$$x\ NRPP \xrightleftharpoons{Mg^{++}} (NRP)_x + P_i$$

where N is a purine or pyrimidine base, R is ribose, and P is phosphate. The enzyme, polynucleotide phosphorylase, was first isolated from Azotobacter vinelandii (Grunberg-Manago and Ochoa, 1955; Grunberg-Manago, Ortiz and Ochoa, 1956). It requires a primer polyribonucleotide for optimal activity, it incorporates all four nucleotide mono-phosphate residues from the diphosphates but they appear to be incorporated at the end of the chain and the sequence of the nucleotides in the formed product is more a reflection of the concentration of the nucleoside diphosphates than of the nature of the primer. (Ortiz and Ochoa, 1959; Ochoa

and Mii, 1961). Although it is found widely in bacteria it does not seem to be present in animal tissues to any appreciable extent (Grunberg-Manago, 1962), and its function is still doubtful.

Another well studied incorporation of nucleotides into RNA has been that which catalyses the addition of two molecules of cytidylic acid and one of adenylic acid from their respective triphosphates to the terminal end of a sRNA molecule (Heidelberger, Harbers, Leibman, Takagi and Potter, 1956; Canellakis, 1957; 1959; Zamecnik, Stephenson and Hecht, 1958; Hecht, Zamecnik, Stephenson and Scott, 1958a; Harbers and Heidelberger, 1959).

Perhaps the most extensively studied system of RNA biosynthesis is that which incorporates ribonucleoside triphosphates into polyribonucleotide. The first example of such a system was reported by Weiss (Weiss and Gladstone, 1959; Weiss, 1960) and similar systems have been found in animal tissues, plants and bacteria (Smellie, 1962; 1963; Furth, Hurwitz and Anders, 1962; Chamberlin and Berg, 1962; Hurwitz and August, 1963). The enzymes catalysing the incorporation of these triphosphates requires a primer molecule of either RNA or DNA.

The enzymes of bacterial origin, specifically requiring DNA, have been well studied, and it seems that the product has a base sequence which is complementary to that of the DNA primer employed. Mammalian systems of similar properties have been less well characterised (Abrams, Edmonds and Biswas, 1962; Burdon, 1962; Smellie, 1962).

Enzymes are also known which catalyse the formation of polyribonucleotides and are dependent on a RNA primer, some of these enzymes will only catalyse the synthesis of homopolymers (Abrams et al., 1962; Burdon and Smellie, 1961; Edmonds and Abrams, 1962; Klemperer and Kammen, 1962), whilst others incorporate all four nucleotides (Reddi, 1961; Smellie, 1962).

(ii) in vivo studies.

The original observation of Bergstrand, et al., (1948) that radioactive precursors are incorporated more rapidly into nuclear than into cytoplasmic RNA have been confirmed by many workers (Smellie, 1955; Plaut, 1962; Graham and Rake, 1963). However, the interpretation of kinetic data has proved to be more difficult than was first believed.

Evidence is accumulating that cytoplasmic RNA is of nuclear origin (Reich, Franklin, Shatkin and Tatum 1961; 1962; Shatkin, 1962; Perry, 1962). It was

found that actinomycin D almost completely blocked the synthesis of RNA in cultivated mammalian cells. Actinomycin D is also known to block *in vitro* DNA-primed RNA biosynthesis (Goldberg and Rabinowitz, 1962; Hurwitz, Furth, Malamy and Alexander, 1962). It thus seems that cytoplasmic RNA originates from the nucleus. The possibility of RNA being synthesised in the cytoplasm and dependent on a RNA of nuclear origin cannot be entirely ruled out. Moreover, recent kinetic studies on the incorporation of radioactive precursors into RNA of the nucleus and cytoplasm of mammalian cells cultured *in vitro* were interpreted as showing that RNA was synthesised and degraded in the nucleus and that insignificant amounts of undegraded RNA migrated from the nucleus to the cytoplasm. It was thus concluded that RNA synthesis can take place in the cytoplasm. (Harris and Watts, 1962; Harris, 1963a).

4. The Nucleases.

(a) General.

Nucleases have been found in mammalian tissues (Jones, 1920; McCarty, 1946; Khorana, 1961), in plants (de la Blanchardiere, 1915; Greenstein, 1948; Holden and Pirie, 1955; Frisch-Niggemeyer and Reddi, 1957; Shuster, 1957), and in bacteria (Plenge, 1903; Sherry,

Tillett and Christensen, 1948; Sato and Egami, 1957; Nishimura and Nomura, 1958; 1959), and it may then be assumed that they exist in all living cells. It has been suggested that the nucleases be classified on the basis of 4 criteria (Laskowski, 1959) -

(1) type of substrate.

(2) mode of action (endonuclease or exonuclease).

(3) products formed.

(4) adjacent bases or sensitive terminal.

Relatively few of the nucleases have been completely classified according to this scheme but a growing interest is being taken in these enzymes with respect to their mode of action and their biological function.

(b) Enzymes hydrolysing RNA and DNA.

These enzymes are generally known as phosphodiesterases and have been detected in a wide variety of biological material. They can be divided into two main groups, one group which forms nucleoside 5' phosphates and the other which forms nucleoside 5' phosphates.

Venom phosphodiesterase is probably the best characterised enzyme of the first group. It was first discovered by Ugawa (1932) and initially studies were carried out by Takahashi (1952), Gulland and Jackson, (1938a; 1938b). Using synthetic substrates, the substrate specificity and the effect of various inhibitors

on a partially purified enzyme preparation have been examined (Singer, Hilmoe and Heppel, 1958; Razzell and Khorana, 1959a; 1959b). It is an exonuclease (Laskowski, Hagerty and Laurila, 1957) and has little action on polynucleotides with a 3'phosphomonoester group (Volkin and Cohn, 1953; Koerner and Sinsheimer, 1957; Privat de Garilhe, Cunningham, Laurila and Laskowski, 1957).

Other enzymes with similar properties have been found in mung bean sprouts (Sung and Laskowski, 1962) in Azotobacter Agile (Stevens and Hilmoe, 1960) and in intestinal mucosal cells (Carter, 1951).

An enzyme which is found in the spleen is the best example of the second group (Heppel and Hilmoe, 1955). The action of spleen phosphodiesterase is in many ways analagous to that of venom phosphodiesterase, although of course these enzymes hydrolyse different bonds. 5'phosphomonoester end groups are not attacked by the spleen enzyme (Heppel and Hilmoe, 1955; Heppel, Ortiz and Ochoa, 1956). Enzymes have been found in fish (Tomlinson, 1959) and in micrococcus pyogenes (Alexander, Heppel and Hurwitz, 1961; Williams, Sung and Laskowski, 1962) which have actions similar to those of the spleen enzyme. The micrococcus pyogenes enzyme, however, appears to be an endonuclease.

(c) Enzymes hydrolysing DNA.

The deoxyribonucleases (DNase's) can be characterised on the basis of their reaction products. One group, of which pancreatic DNase I is best understood (Laskowski, 1961), hydrolyses DNA to oligonucleotides carrying a 5'phosphodiester group. Enzymes of the DNase I type have also been reported in the kidney (Cunningham and Laskowski, 1953), urine (Kowlessar, Altman and Hempelman, 1954), in plasma (Gavosto, Buffa and Mariani, 1959) and in streptococci (Plenge, 1963; McCarty, 1948; Potter and Laskowski, 1959).

Another group of the DNase's (DNase II) have been demonstrated in spleen (Catchside and Holmes, 1947), in thymus (Mayer and Greco, 1949a,b). These enzymes produce oligonucleotides or nucleotides with a terminal 3'phosphomonoester group.

(d) Enzymes hydrolysing RNA.

Undoubtedly the nuclease which is most understood is bovine pancreatic ribonuclease. It has been purified and its amino acid sequence has been determined (Hirs, Moore and Stein, 1960; Spackman, Stein and Moore, 1960). The reactions catalysed by pancreatic RNase have been elucidated (Brown and Todd, 1955; Schmidt, 1955; Markham, 1957); RNase hydrolyses the phosphodiester between the 5' and 5' position producing oligonucleotides

with pyrimidine 2'3' cyclic phosphate end groups. The cyclic phosphate is slowly hydrolysed to a 3'phosphate. Thus RNase produces a mixture of oligonucleotides and a purine rich "core".

Bovine pancreatic RNase is remarkably resistant to acid pH's, but its activity is destroyed by a pH greater than 12.7, although not by treatment with liquid ammonia (Sela and Anfinsen, 1957). RNase of pancreatic origin has been demonstrated in other animals (Dickman and Morrill, 1959; Aqvist and Anfinsen, 1959) and calf spleen has been shown to contain an enzyme with specificity similar to that of bovine pancreatic RNase (Kaplan and Heppel, 1956).

RNase's have been found in plants which degrade RNA to 3'oligonucleotides or cyclic nucleotides. They do not leave a resistant "core", and have a pH optimum in the region of pH 5 (Holden and Pirie, 1955; Frisch-Niggemeyer and Reddi, 1957; Shuster, 1957; Reddi, 1958; 1959; Shuster, Khorana and Heppel, 1959).

Other less well characterised RNase's have been detected in many animal tissues. Mayer and Greco (1949a,b) showed nuclease activity in spleen and thymus extracts. In rat liver there has been shown to be at least two RNase's, one with a pH optimum around 7.8 and the other with a pH optimum of around 5.8 (de Lamirande, Allard,

Da Costa and Cantero, 1954; Roth, 1954; 1959; Reid and Nodes, 1959). Both enzymes were originally believed to be associated with the mitochondria, but an acid RNase has been found in the cell sap of rat liver (Reid and Nodes, 1963).

The ratio of the activities of the two enzymes (alkaline RNase and acid RNase) in rat liver is in much dispute, but the lack of agreement is probably due to some workers being unaware of the presence of impurities in commercially prepared RNA which interferes with RNase assays. These impurities, which are probably certain metal ions, were shown to affect RNase assays by three groups of workers (Roth and Wojnar, 1961; Shortman, 1961; Eichel, 1962).

The levels of alkaline RNase in various mouse tissues have been determined (Ellem, Colter and Kuhn, 1959) and the RNases in rat adipose tissue have been studied (Eichel, Figueroa, and Goldenberg, 1961).

(e) Nuclease Inhibitors.

Inhibitors of DNase I have been found in various tissues, notably in the kidney, (Dabrowska, Cooper and Laskowski, 1949; Cooper, Trautman and Laskowski, 1950) and inhibitors of DNase II have also been detected (Siebert et al., 1953; Kowlessar et al., 1954). No physiological role appears to have been demonstrated

as yet for these inhibitors.

The experiments of Pirotte and Desreux (1952) gave the first indication of the existence of a naturally occurring RNase inhibitor and, later, Roth (1956) showed it to be widespread in the tissues of the rat. More recently, an RNase inhibitor has been found in rat adipose tissue (Eichel et al., 1961). However, the inhibitor in rat liver is the one which has most thoroughly been investigated. Roth (1958a) partially purified this inhibitor and found it was heat-labile, non dialysable and sensitive to certain sulphydryl reactants. More recently the rat liver inhibitor has been purified six thousand fold (Shortman, 1961). Later studies of the purified product showed that although it was an effective inhibitor of bovine pancreatic RNase and of liver alkaline RNase, it was ineffective towards a number of plant acid RNases. It was also found that it was dissimilar in many ways to heparin which is also an inhibitor of bovine pancreatic RNase. The effects of proteolytic enzymes upon the purified inhibitor suggested that it was a protein (Shortman, 1962a).

1.3 Hormones and Nucleic Acids.

Hormones have been known to affect the nucleic acid metabolism of their respective target organs for a number of years (Leslie, 1955). As the relationship between RNA and protein biosynthesis became established, it became clear that the effect of hormones on nucleic acid metabolism was more than merely casual or indirect. Likewise, as the knowledge of RNA metabolism has increased it has become possible to study the effects of hormones at lower levels of cell organisation.

Perhaps the first biochemical study of the action of cortisone on nucleic acids was that of Lowe, Williams and Thomas (1951). They showed that administration of cortisone to rabbits resulted in a decrease in the concentration of RNA in the liver and an increase in the $\frac{RNA}{DNA}$ ratio. These results are indicative of hypertrophy. Similar effects have been noted by other workers after prolonged cortisone treatment (Silber and Porter, 1953; Trémolières, Derache and Griffaton, 1954; Goodlad and Munro, 1959). The current work, however, which is being conducted in many laboratories is the investigation of the early action of cortisone on liver metabolism. Cortisone or hydrocortisone has been shown to induce an increase in the amount of certain liver enzymes. These enzymes include tryptophan pyrrolase (Thomson and Mikuta, 1954;

Knox and Auerbach, 1955), tyrosine-α-oxoglutarate transaminase (Lin and Knox, 1957; Kenney and Flora, 1961; Kenney, 1962a,b), tryptophan transaminase (Civen and Knox, 1959), glutamic-alanine transaminase (Segal and Hopper, 1965) and phosphoglyceraldehyde dehydrogenase (Matzelt, Oriol-Bosch and Voigt, 1962).

Jervell (1965) has shown that hydrocortisone induces a rapid increase in the amount and uptake of certain radioactive precursors into liver RNA of adrenalectomized rats. These changes occur concomitantly with increases in tryptophan pyrrolase. Kenney and Kull (1963) have examined the metabolism of nuclear and cytoplasmic RNA of the liver of adrenalectomized rats treated with hydrocortisone and find that nuclear RNA synthesis appears to increase 2-3 fold after hydrocortisone treatment. Under similar conditions of labelling, the cytoplasmic RNA is unchanged. The increase in nuclear RNA synthesis occurs <u>before</u> an increase in tyrosine transaminase is detected.

Increases in the incorporation of ^{32}P into RNA after cortisone administration have been noted by Feigelson, Feigelson and Greengard (1960) and by Munro and Mukerji (1962).

Another class of steroids whose effects have been extensively studied are the oestrogens. Much of the

knowledge of the mechanism of action of oestrogens on the uterus has come from the work of Mueller and his colleagues. Oestrogen treatment for 6 hours *in vitro* was shown to increase the ability of surviving uterine segments to incorporate [3-^{14}C]-serine into the nucleic acids (Heyraneu and Mueller, 1956). Later experiments showed that oestrogen treatment *in vivo* caused the RNA content of the uterus to increase (Aisawa and Mueller, 1961).

Studies using puromycin, which blocks protein synthesis in cell free systems (Yarmolinsky and de la Haba, 1959) indicated that the uterine response to oestrogens *in vivo* was abolished by injection of puromycin *in vivo* (Mueller, Gorski and Aisawa, 1961; Hamilton, 1963). More recently, Ui and Mueller (1963) have demonstrated that actinomycin D injection *in vivo* depressed the oestrogen effect on the rat uterus. This finding was suggestive that new messenger RNA production was necessary for the oestrogen response. Similar results have been obtained by other workers (Wilson, 1962; 1963; Hamilton, 1964). In confirmation of this work Noteboom and Gorski (1963a,b) have demonstrated an increase in RNA nucleotidyl transferase in uterine nuclei 1 hour after oestrogenic stimulation. Oestradiol has also been shown to increase the RNA nucleotidyl

transferase in chicken liver, but the increase was not observed until 24 hours after the initial injection of oestradiol (Weill, Busch, Champon and Mandel, 1963).

The effects of testosterone on their respective target organs have been less extensively studied. The lowering of the nucleic acid content of the seminal vesicles and ventral prostrate by castration has been shown to be reversed by injection of testosterone (Rabinovitch, Junqueira and Rothschild, 1951; Lostroh, 1961; Kassenaar, Kouvenhoven and Querido, 1962; Kochakian and Harrison, 1962). More recently Shutsung Liao and Williams-Ashman (1962) have demonstrated that an action of testosterone on the prostrate gland is to cause the production of messenger RNA.

The effects of the pituitary hormones on the nucleic acid metabolism of their respective target organs has also been studied, but investigations have been less intensive than that of the steroid hormones mentioned.

Prolactin administration increases the nucleic acid content of the pigeon crop gland (McShan, Davis, Soukup and Meyer, 1950), the RNA content of the rat mammary gland (Ota, Yokoyama and Shinde, 1962; Cole and Hopkins, 1962) and of the rabbit mammary gland (Densmur, 1963). It may be noted that prolactin is not the only hormone affecting nucleic acid levels in

the mammary glands as oxytocin and progesterone also have been shown to affect the RNA content. The effect appears to be different for each hormone (Griffith and Turner, 1962; Denamur, 1963; Ota et al., 1962).

Fiala, Sproul and Fiala (1957) have noted a stimulating effect of thyrotrophin on the rat thyroid gland and Mantovinovic and Vickery (1959) have observed hypertrophy of the guinea pig thyroid, i.e. increase in RNA without concomitant increase in DNA after thyrotrophin administration. Hall (1963) has demonstrated a stimulation by thyrotrophin of purine synthesis in calf thyroid slices incubated in vitro. More recently Seed and Goldberg (1963) have presented evidence that thyrotrophin is required to stabilise the template RNA necessary for thyroglobin synthesis.

Hypophysectomy of rats has been found to be followed by decreases in liver RNA and protein. Injection of growth hormone restores the RNA or protein to their normal level (Di Stefano, Bass, Diermeier and Tepperman, 1953). Administration of growth hormone was found to increase the uptake of ^{32}P into the subcellular fraction of rat liver, but it was found that the RNA concentration decreased (Talwar, Panda, Sarin and Toland, 1962). However, it is likely that the RNA content increased on treatment with the hormone.

Recently Korner (1962; 1963) has claimed that growth hormone may regulate the rate of protein synthesis by altering the ability of the ribosomes to assemble activated amino acids into protein and that somehow growth hormone alters the rate of messenger RNA synthesis.

From this survey of the literature, it will be seen that hormones may affect cellular metabolism by influencing nucleic acid and protein metabolism, but it still remains to be shown at what level they act.

1.4 The Nucleic Acids of the Adrenal.

The presence of basophilic granules in the cytoplasm of the cells of the human adrenal cortex was first observed by Bantee (1936). Rich and Berthong (1949) using the ribonuclease-cytochemical method of Brachet (1940; 1941), identified these granules as being largely composed of RNA; they also found that these granules were particularly abundant in the adrenal cells of patients who had died after severe infection. A more intensive biochemical and histological investigation was carried out by Symington and Davidson (1956). These authors examined the adrenals removed from patients either at operation or post-mortem and showed that a period of stress prior to removal of the adrenals led to an increase in the concentration of RNA and in the ratio of RNA to DNA, but it led to a decrease in the DNA concentration. The results were interpreted as demonstrating that ACTH causes hypertrophy of the adrenal cell. Histochemical evidence has shown that the RNA increase, although seen in all the zones, is greatest in the zona fasciculata in the human adrenal (Symington and Davidson, 1956; Symington, Duguid and Davidson, 1956) and in the guinea pig (Burns and Hale, 1959). Increases in the DNA content of human adrenals of patients suffering from various dysfunctions

have been observed by Bransome and Reddy (1962). However, these results cannot be directly compared with those of Symington and Davidson as the studies of Bransome and Reddy were on malfunctioning or diseased adrenals whereas the studies of Symington and Davidson were on adrenals from patients with relatively normally functioning adrenals. Bransome and Reddy (1961) have also observed the effects of ACTH on the nucleic acids of dog adrenals andfound that even after 6 days treatment with ACTH, which increased the RNA content about 400%, the DNA content was not significantly altered.

The content of RNA in various subcellular fractions of the rat adrenal gland at various time intervals after ACTH administration has been investigated by Fiala, Sproul and Fiala (1956). They found that the RNA content was increased in all the subcellular fractions but the greatest increase was in a fraction which they called the "large chromidia". An increase in DNA content was also noted by these authors but this is not in agreement with the findings of Ramaiah (1959). The findings of Fiala *et al.* that ACTH induces hyperplasia may be due to the fact that rats were administered relatively high amounts of ACTH which may have contained contaminating growth hormone. In the dog adrenal at least, growth hormone can result in the DNA content increasing (Bransome and Reddy, 1961).

It is well established that hypophysectomy can cause a decrease in the incorporation of inorganic ^{32}P into total acid soluble phosphorus and a decrease in the rate of transfer of inorganic phosphorus into the cell (Reiss and Halkerston, 1950; Gemzell and Samuels, 1950; Riedel, Logan, de Luca and Rossiter, 1954) and a decrease in the incorporation of inorganic ^{32}P into phospholipid has also been noted (Riedel, Logan and Rossiter, 1954). These changes are reversed by injection of ACTH.

The incorporation of inorganic ^{32}P into RNA under various experimental conditions has also been studied by Rossiter and his co-workers. Administration of ACTH was found to have no significant effect on the incorporation after 20 hours but it was found that the incorporation by adrenal glands of hypophysectomized rats treated with ACTH was significantly increased compared with hypophysectomized rats which were not treated (Logan, Heagy and Rossiter, 1955). Later, it was found that the adrenals of rats subjected to a cold stress of 16 hours duration showed significantly increased incorporation of ^{32}P orthophosphate into adrenal RNA nucleotides by about 30% (Nicholls, Heagy and Rossiter 1956a); however, if the duration of cold stress was increased to 8 days it was found that the incorporation

into the ribonucleotides, guanylic and cytidylic acids, obtained by alkali hydrolysis of adrenal RNA, was significantly lowered.

Ramaiah (1959) conducted a study on the effect of ACTH at different dose levels, acting for different time intervals, on the content of protein, phospholipid, RNA and DNA of the rat and guinea pig adrenal. He also examined the effect of ACTH on the uptake of ^{32}P orthophosphate into the nucleic acids. Since most of this work has not yet been published the parts relevant to this thesis will be considered below.

Figure 1 summarises part of his work. Several interesting features can be seen. In rat, the increase in RNA content is not observed until 24 hours after ACTH administration; this increase is maximal at the end of the second day and prolonged administration of ACTH does not result in any further increase in RNA. The change in gland weight runs parallel with the changes in its content of RNA. Surprisingly, the specific activity of the RNA was found to fall as a result of ACTH. The content of DNA is not altered by ACTH in agreement with the findings of others investigating human, calf (Symington and Davidson, 1956) and dog adrenals (Bransome and Reddy, 1961). While chemical analysis did thus not demonstrate any increased

Figure 1.

Comparison of the action of ACTH on guinea pig and rat adrenal glands.

Animals were given ACTH (Acthar-gel, Armour Laboratories) or the carrier for varying intervals of time.

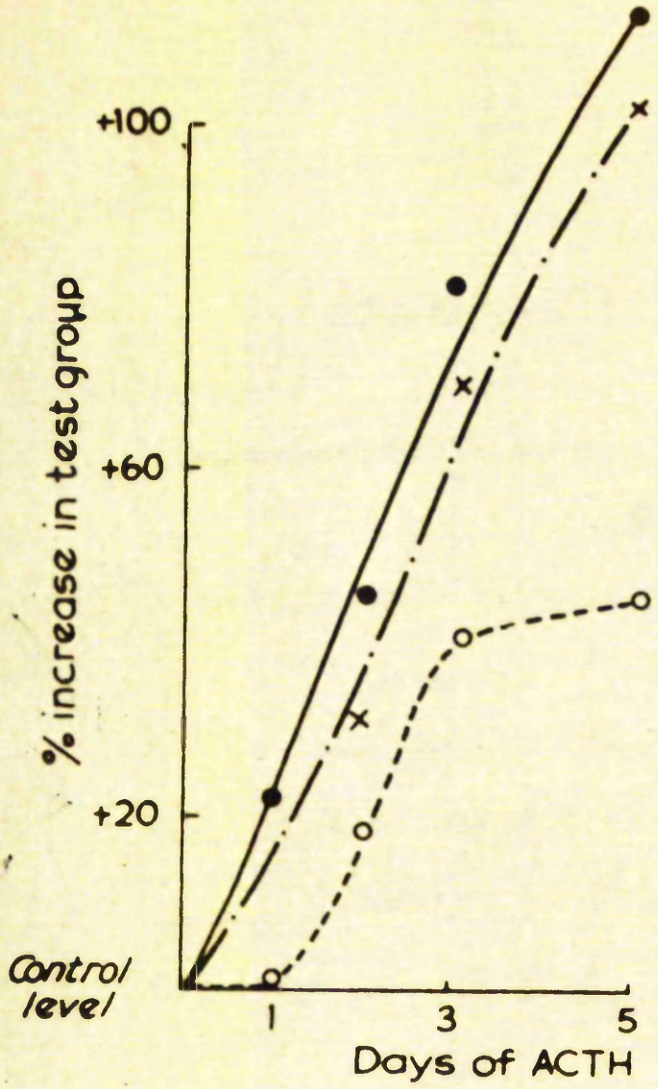
^{32}P as inorganic phosphate was administered 2hr. before killing.

Top: changes in gland weight, amount and specific activity (relative to adrenal inorganic phosphate) of RNAP.

Bottom: increases in amount and specific activity (relative to adrenal inorganic phosphate) of DNAP.

(from Ramaiah, 1959).

Figure 1.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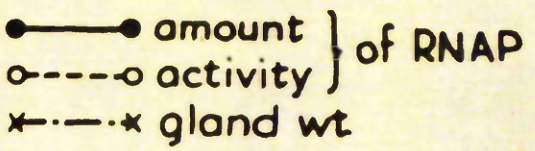
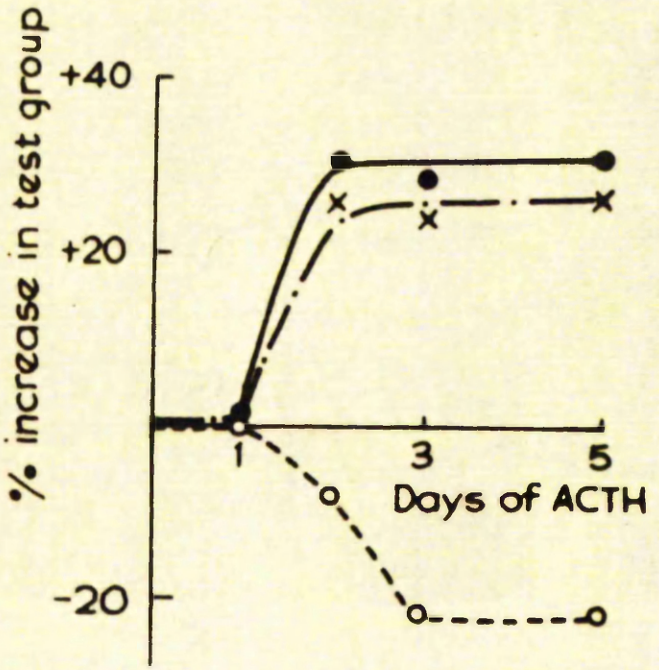
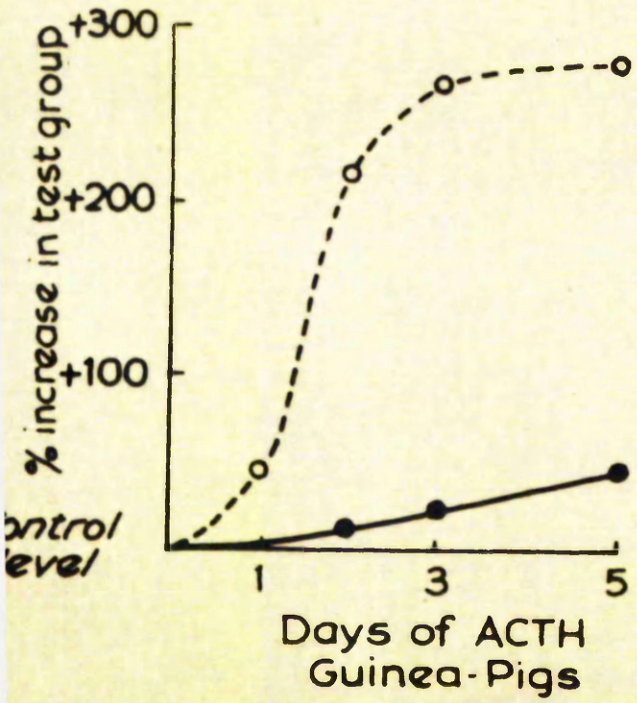
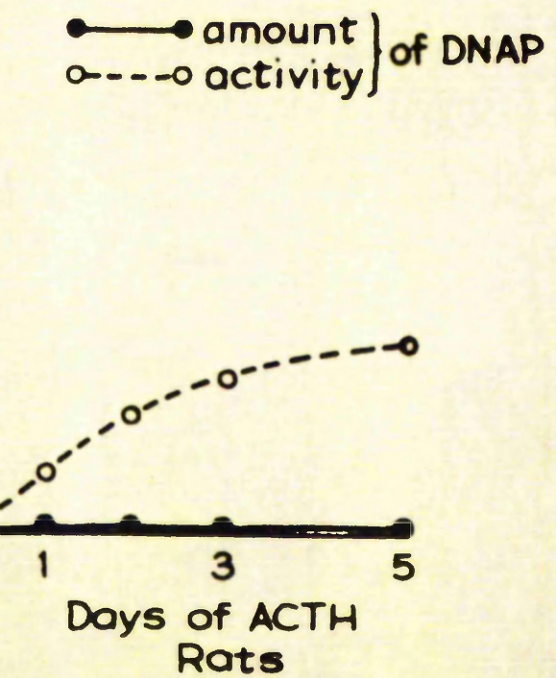
+60
+20
Control level
% increase in test group
1
3
5
Days of ACTH


amount
activity
of RNAP
gland wt


+40
+20
-20
% increase in test group
1
3
5
Days of ACTH


+300
+200
+100
% increase in test group
1
3
5
Days of ACTH
Guinea-Pigs


amount
activity
of DNAP
+200
+100
% increase in test group
1
3
5
Days of ACTH
Rats

cell division, the specific activity of DNA rose indicating that there is in fact increased DNA synthesis.

It will be noticed that the action of ACTH on the metabolism of the nucleic acids of the guinea pig adrenal is different. The incorporation of inorganic ^{32}P into RNA is increased, the increase in RNA is seen immediately after ACTH and the DNA content is also increased by ACTH.

Ramaiah suggested that ACTH caused the cessation of RNA breakdown in the adrenal. To test this hypothesis Antoni (1961) attempted to fractionate the adrenal RNA and to examine the metabolic activities of each species. He isolated the subcellular fractions of the adrenal gland and analysed them for RNA, DNA, protein and phospholipid. He found increases in RNA content in all cell fractions and the increase, after three days treatment with ACTH, was especially marked in the cell sap. This increase was 145%. Unfortunately the fraction which he called the cell sap also contained free ribosomes; thus it is not known whether the increase is due to the amount of mRNA altering or to the amount of ribosomal RNA changing. Antoni also separated RNA on Ecteola-cellulose and found the adrenal RNA to be metabolically heterogeneous.

1.5. Purpose of Present Investigations.

The results of Ramaiah suggested that the RNA metabolism of the rat adrenal is altered by ACTH, in rather an unusual fashion. Whereas, studies on the effects of other hormones on the nucleic acids of their target organs indicate that hormones cause an increase in the uptake of isotope into RNA, it was found that ACTH treatment lowered the *in vivo* incorporation of ^{32}P into the RNA of the rat adrenal.

Ramaiah examined the incorporation into total RNA of the adrenal gland. In this investigation, the effects of ACTH upon the metabolism of the RNA of the subcellular components have been examined.

The effect of ACTH treatment on the metabolism of RNA of adrenal glands incubated *in vitro* has been studied and it was hoped to ascertain whether there was a rapidly labelled RNA fraction in the adrenal gland analogous to the rapidly labelled fraction found in other biological systems, and if there was, whether ACTH influenced this fraction in any way.

Some of the enzyme systems involved in the breakdown of RNA have also been investigated to determine whether ACTH treatment *in vivo* exerts any effect on these mechanisms.

SECTION 2

METHODS

2.1. General.

1. Animals.

Female albino rats from the departmental colony, housed under thermostatic conditions, were used in all experiments. The body weights were in the range 150-165 g., but were matched to within 5g. for each experiment.

2. Isotopes.

Carrier free, inorganic ortho-phosphate solution (PBS1, Radiochemical Centre, Amersham) was used for the in vivo studies. The solution was diluted with water, such that the volume injected into each rat was 0.2ml. Amounts varying between 500-600μc were injected intramuscularly.

Phosphate buffered saline (PBS2, Radiochemical Centre, Amersham) was used for the in vitro experiments.

Tritiated uridine, designated as "nominally labelled 5-6 ^{3}H uridine", (TRA27, Radiochemical Centre, Amersham) was dissolved in water and stored at -100° until required for use.

3. Adrenocorticotrophic Hormone (ACTH).

Zinc stabilised ACTH (Cortrophin ZN, Organon Laboratories, Ltd), injected intramuscularly, was used for in vivo studies and lyophilised ACTH (Cortrophin, Organon Laboratories, Ltd.) used for in vitro experiments.

4. Removal of Adrenals.

(a) In vivo experiments.

After killing the rats, by decapitation, the adrenals were quickly removed, chilled to 0°, trimmed free from extraneous fat and connective tissue and weighed. In some experiments, the adrenals were stored at -10° before further experimentation.

(b) In vitro experiments.

Rats were killed by cervical dislocation followed by decapitation, carried out as quickly as possible after removal from their cages; the adrenals were excised and placed on filter paper in a petri dish. The filter paper was moistened with Krebs-Ringer 0.025M-sodium bicarbonate buffer (Umbreit, Burris and Stauffer, 1949), preparation of which is given below. With practice it was possible to remove the adrenals within 25 sec. of taking the rat from its cage.

2.2. Incorporation Experiments.

1. In vivo

The rats were given 5i.u. ACTH twice daily, except when the effect of the duration of ACTH treatment being studies was 15hr.; in this case, the rats received a single injection of 5i.u. ACTH 15hr. before killing. ^{32}P was administered 2hr. before killing in all experiments. From each rat, immediately after

killing, a sample of approximately 1ml. blood was collected, from the neck vessels, in heparinised tubes and samples pooled in groups of three. The inorganic phosphate was isolated from the plasma as described in 2.9.3 and the specific activity determined.

2. In vitro.

(a) Preparation of incubation medium.

All solutions were made up in glass distilled water.

The medium was Krebs-Ringer 0.024 M-bicarbonate (Umbreit et al., 1949) containing glucose and was prepared as follows. 200ml. of 4.5%(w/v) NaCl, 8.0ml. of 5.75%(w/v) KCl, 8.0ml. of 10.55%(w/v) KH_2PO_4 and 8.0ml. of 19.1%(w/v) $MgSO_4.7H_2O$ were added together, diluted with 800ml. of water, 6.0ml. of 6.1%(w/v) $CaCl_2$ added and the mixture diluted finally to 548ml. with water. This was known as "double Ringer" and was stored at 2°. It was freshly prepared every two weeks. The incubation medium was prepared by adding together the following, 54.0ml. of "double Ringer", 27.0ml. of 1%(w/v) glucose, 29.0ml. of water and 21.0ml. of 1.8%(w/v) $NaHCO_3$ which had been previously gassed with CO_2 (from Drikold, I.C.I.) for 60min. at room temperature. The medium was gassed with 95% O_2/ 5% CO_2 for 10 to 20min. before use.

(b) Incubation.

The adrenals were removed from eight rats as in 2.1.4(b) and each adrenal was quartered using a pair of 3" dissecting scissors. The quarters were divided into eight groups such that each group contained a quarter from one adrenal pair. The adrenals and quarters were kept moist (with medium) during this procedure. Each group was placed in a 2"x1" cork-stoppered flask, and 1.5ml. of incubation medium added. The flasks were gassed with 95% O_2/5% CO_2 for 20 to 30 sec., stoppered and incubated at 37° in a shaking water bath for 30 to 45min. After this pre-incubation period, the medium was discarded and a further 1.5ml. of medium added to control groups and 1.5ml. of medium containing 100-500m.i.u. ACTH added to test flasks. The flasks were gassed and incubated as above. The medium was changed at approximately 30min. intervals, ACTH being added where appropriate. Radioactive isotope was added at varying times after treatment of adrenal quarters with ACTH *in vitro*. Occasionally, aliquots of medium were assayed for corticosterone content.

2.3. Tissue Fractionation.

1. Separation into sub-cellular fractions.

All centrifugations mentioned in this section were carried out at 0°.

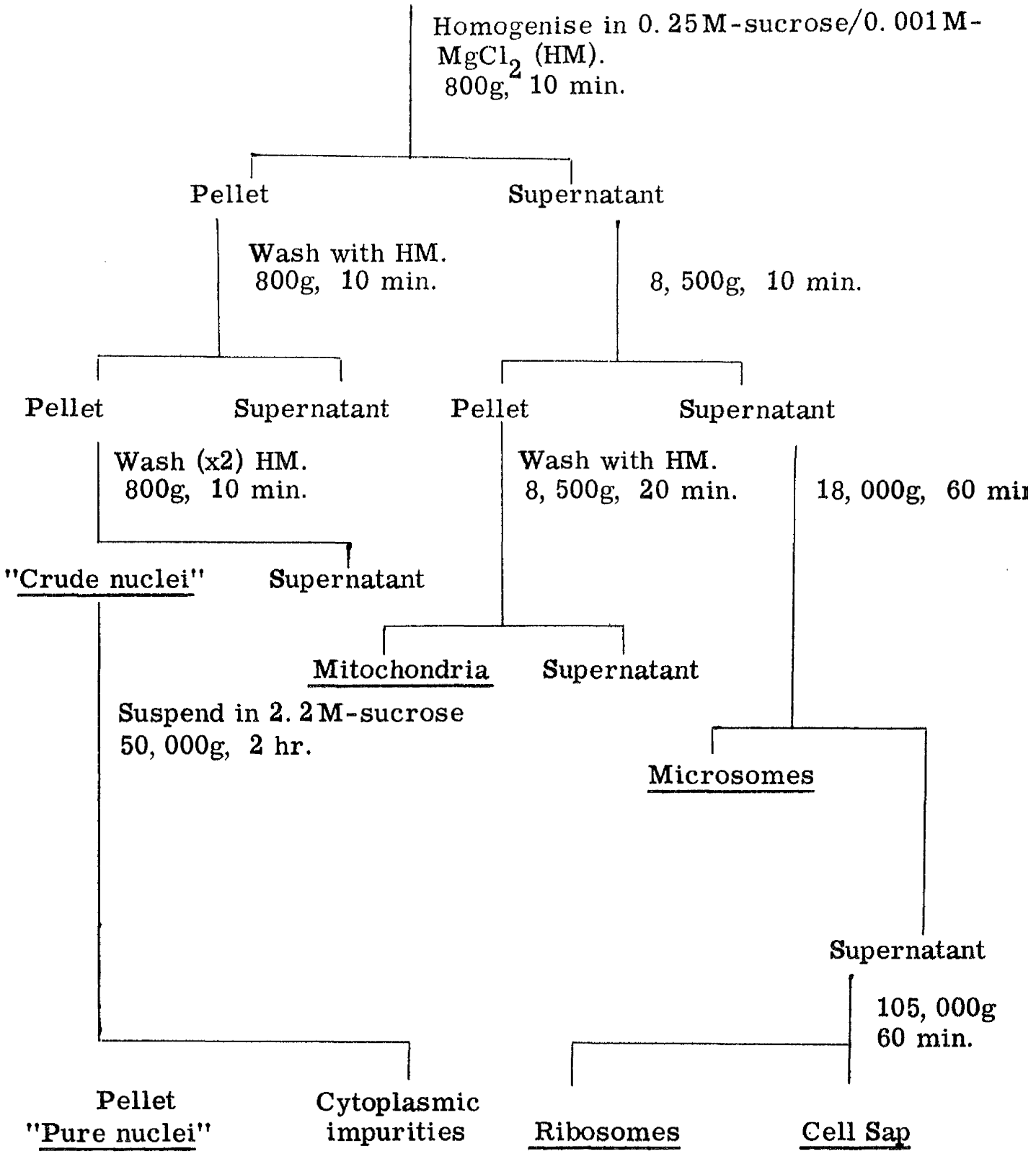
The adrenals were homogenised at 0° in 4-5ml. of 0.25 M-sucrose containing 1μmole $MgCl_2$ per ml., hereafter referred to as the homogenising medium, in a Potter-Elvejhem (1956) type of homogeniser fitted with a perspex pestle. The adrenal homogenate was spun at 800g for 10min. and the pellet, which consisted of nuclei contaminated with red blood cells, whole cells, and cytoplasmic debris, was washed three or four times with homogenising medium. The first wash was combined with the supernatant and subsequent washes discarded. The combined supernatants were centrifuged at 8,500g for 10min. using the super-speed attachment of a M.S.E. medium centrifuge to give a pellet referred to later as the mitochondria. After washing the mitochondria with 1.0ml. of homogenising medium and combining the wash after spinning to the 8,500g supernatant, the combined supernatants were centrifuged at 18,000g for 60min. to spin down the fraction later referred to as microsomes, and leaving a supernatant which was then spun in a rotor 40 or rotor 50 in a Spinco model L Ultracentrifuge at 105,000g for 1hr. The pellet so obtained was named the ribosomes and the supernatant is hereafter referred to as the cell sap.

A flow sheet summarising the fractionation procedure is given in Figure 2.

Figure 2

SCHEME USED FOR CELL FRACTIONATION OF ADRENAL GLANDS

All operations are carried out at 0°



In some experiments, aliquots of the cell sap were taken for inorganic phosphate specific activity determinations.

2. Purification of Nuclei.

The impure nuclei, as obtained by the above method, were first washed with homogenising medium until the supernatant was only faintly opalescent and were then thoroughly mixed in 2.22 M-sucrose which contained 1 μmole $MgCl_2$ per ml. The final concentration of the sucrose was between 2.20M and 2.21M. The suspension was centrifuged in a rotor 40 in a Spinco model L Ultracentrifuge at 50,000g for 2hr. Ideally this centrifugation should have been carried out using a swinging-bucket rotor but in these experiments an angle head was used, the only disadvantage being that the nuclei collected as a white gelatinous smear down the centrifugal side of the tube, making collection of the nuclei more difficult. It was found that the best way of recovering the nuclei was to decant the supernatant and cut the tube in half down the longitudinal axis, one half containing the nuclei, and the other the cytoplasmic impurities.

2.4. Isolation of RNA.

RNA was isolated by methods which were modified from that of Kirby(1956). The modifications employed depended on the nature of the experiment.

1. Extraction of RNA prior to chromatography on DEAE Sephadex.

Each subcellular fraction was suspended at 0° in 4.0ml. of 0.2M-sodium phosphate buffer, pH 6.85. 4.0ml. of 90%(w/v) phenol in water (90% phenol) was quickly added to the suspension and the resulting mixture shaken mechanically in 10ml. glass-stoppered test tubes at 2° for 40 to 60min. The 90% phenol was freshly made up each day and was discarded if at all pink in colour. This criterion was used whenever phenol was used in RNA isolation procedures. The phases were separated at 4° by centrifugation at 1,000g for 40min. The top (aqueous) layer was carefully removed with the aid of a pasteur pipette and the phenol phase and interphase re-extracted with a further 4.0ml. of buffer. After spinning, the two top layers were combined and extracted with five 5ml. portions of ether. The last traces of ether were removed by aspirating nitrogen through the solution, which was then dialysed at 4° against four changes of water over a period of 36 to 48hr. A spectrum from 220mμ-320mμ of the dialysis residue was taken as a check of purity. Lyophilisation was employed to concentrate the solution prior to application on to a DEAE-Sephadex column.

2. Isolation of RX fraction.

In some experiments, the phenol phase and interphase, after washing with a further 5ml. aliquot of buffer, were added to 5ml. of ethanol:ether (3:1 v/v), and left at -10° either overnight or for a period of 1 to 2hr. The precipitate was successively extracted with 5ml. portions of ethanol:chloroform (3:1 v/v) ethanol:ether, (3:1 v/v) and finally ether. The extracts were discarded and the lipid free precipitate, which was called the R fraction, was extracted with 2-4ml. of 10%(w/v) NaCl at 100° for 20min. The suspension was spun, the supernatant removed and the precipitate re-extracted. The combined supernatants were dialysed against three changes of 1mM-$MgCl_2$ or water to remove NaCl prior to subsequent examination. The dialysed supernatants were called the RX fraction.

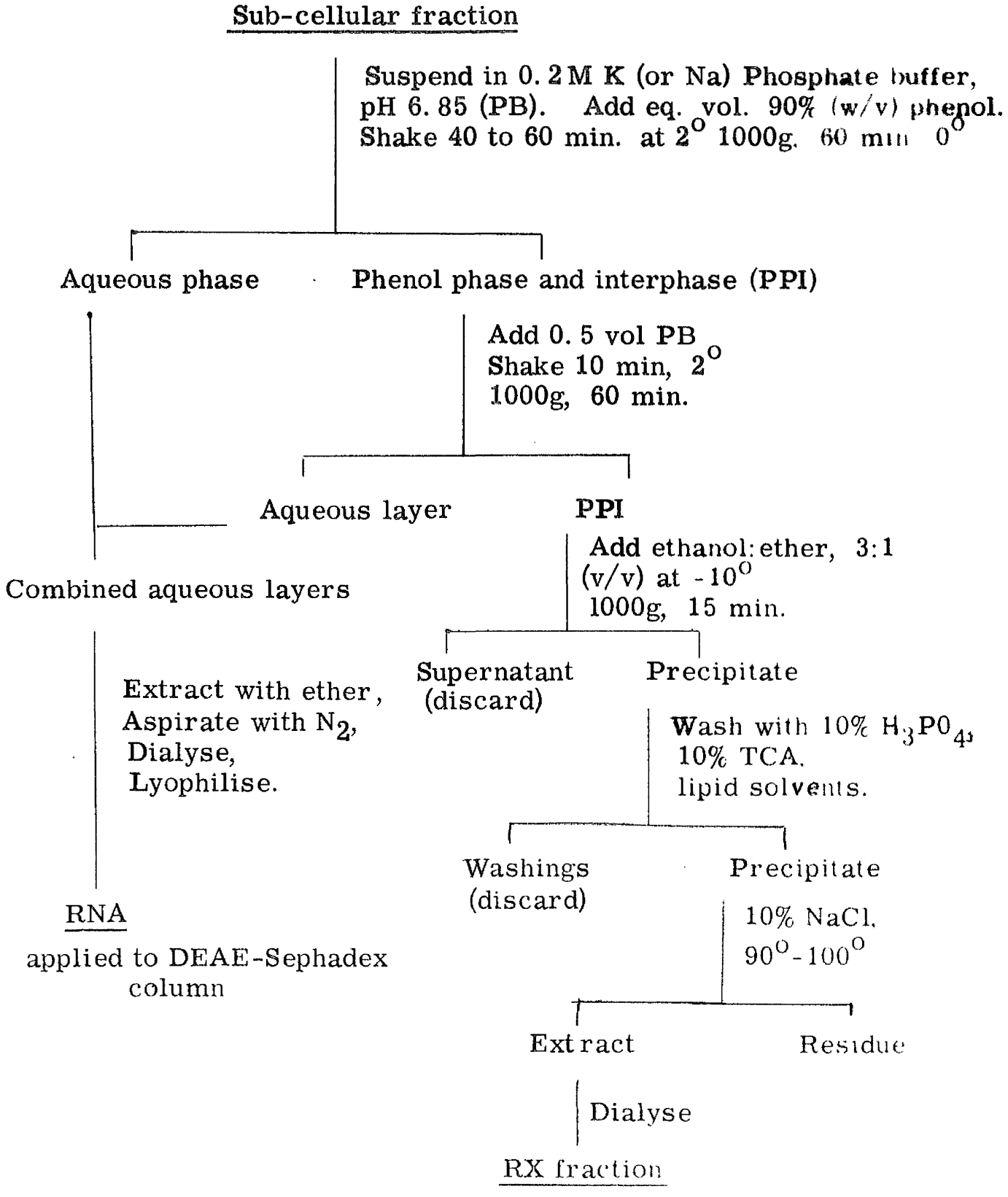
A summary of the above procedure is given in Figure 3a.

3. Fractionation of RNA by a modification of the phenol extraction procedure.

In later experiments, the above method was modified to yield a separation of the RNA's from a subcellular fraction into a further three classes as described below. The subcellular fraction was suspended, at 0°, in 3.5ml. of 0.2M-sodium phosphate buffer pH 6.85, an equal

Figure 3a

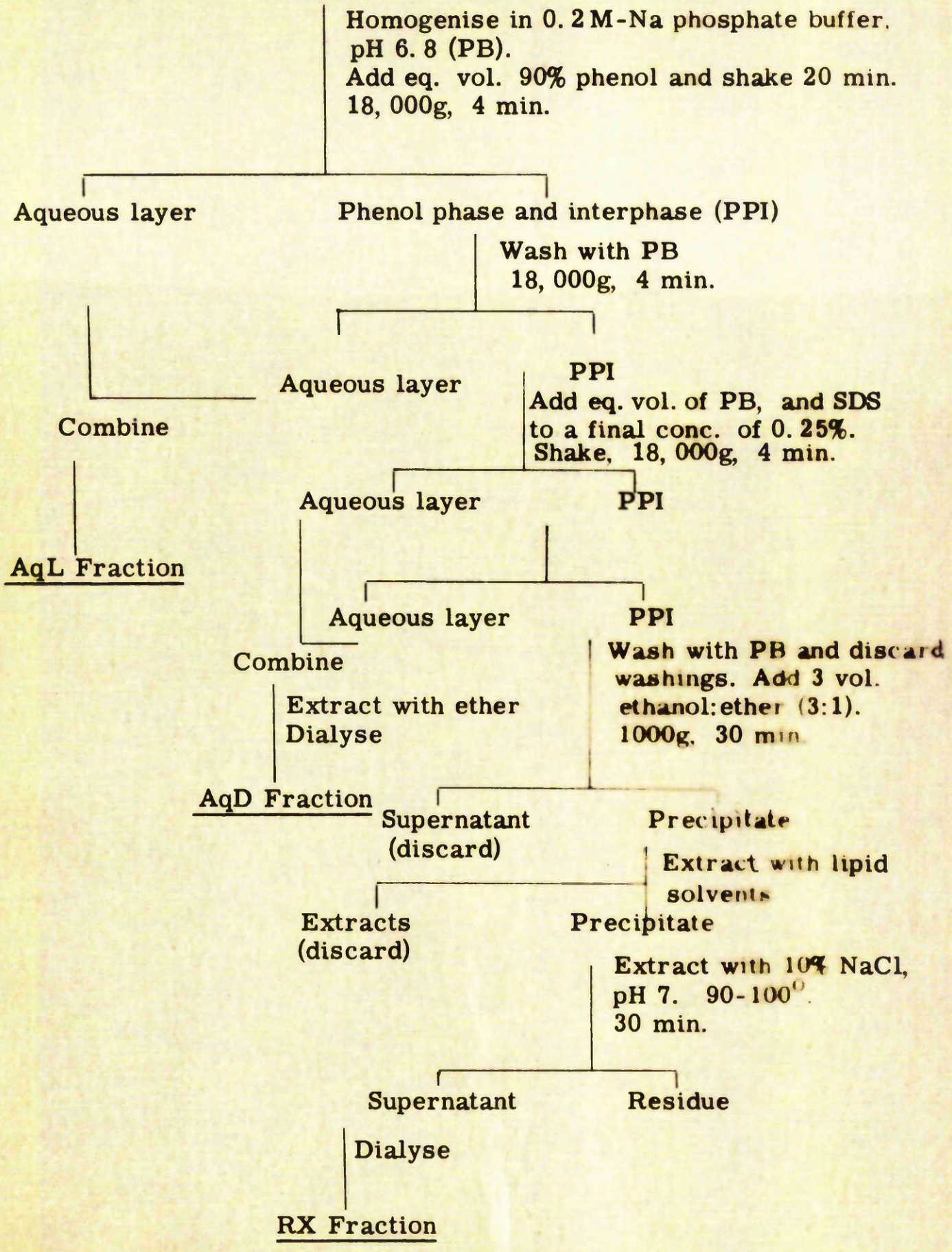
OUTLINE OF METHOD USED FOR ISOLATION OF RNA PRIOR TO CHROMATOGRAPHY ON DEAE-SEPHADEX AND FOR ISOLATION OF RX FRACTION



volume of 90% phenol added and the mixture shaken in a 8ml. stoppered polythene centrifuge tube for 20 to 30min. After separating the two phases by centrifugation at 18,000g for 4min. at 4^{o}, the top layer was removed and the lower phase washed with 1-2ml. of 0.2M-sodium phosphate buffer, pH 6.85, and re-centrifuged. The combined top layers were re-extracted with 2.0ml. of 90% phenol to lessen the possibility of contamination by protein and the excess phenol removed by ether as above. The aqueous extract was dialysed at 4^{o} for 24 to 36hr. against three changes of 1mM-$MgCl_2$. The dialysed extract was called the AgL fraction.

The phenol phase and interphase were washed with a further 5.0ml. of 0.2M-sodium phosphate buffer pH 6.85, centrifuged as before and the aqueous layer discarded. 3.6ml. of buffer and 0.4ml. of 5%(w/v) sodium dodecyl sulphate (SDS) in water were added to the phenol phase and interphase. After shaking for 20min. at room temperature, the phases were separated by centrifugation, the top layer removed and kept, and the lower layer washed with a further 1.0ml. of 0.2M-sodium phosphate buffer pH 6.85. The combined top layers were extracted free of phenol as above and dialysed as above to yield a fraction which was referred to as the AgD fraction.

OUTLINE OF RNA FRACTIONATION PROCEDURE



The remaining phenol phase and interphase was extracted with 5.0ml. of 0.2M-sodium phosphate buffer, pH 6.85, and then treated as in 2.4.2. to obtain the RX fraction.

4. Purification of RNA used as substrate for ribonuclease assays.

The method employed was that of Shortman (1961). 5g. of yeast RNA, Na salt (BDH) which was of a light brown colour, was dissolved in 100ml. of water and 100ml. of 90% phenol quickly added. The resulting mixture was shaken continuously at room temperature for 30 to 40min., centrifuged at 5,000g for 20min. at 4°, and the top layer removed. The phenol phase and interphase was re-extracted with a further 50ml. of distilled water, re-centrifuged and the top layers combined. Two vols. of 2%(w/v) potassium acetate in ethanol were added to the top layers and allowed to stand at -10° for 30min. before collecting the precipitated RNA by centrifugation. The RNA was washed with ethanol:ether (3:1y/v) and dissolved in approximately 100ml. of water. The residual ethanol in the RNA solution was removed by extraction with ether and the traces of ether removed by aspiration with nitrogen. An equal vol. of 2-methoxyethanol ("special for chromatography" grade, B.D.H) was added to the RNA solution

followed by 1 vol. of 2.5M-K_2HPO_4 and 0.05vol. of 33%(v/v) H_3PO_4. After shaking the mixture the top layer was dialysed firstly against distilled water then against two changes of 0.01M-EDTA, pH 7.3 and then against three changes of 0.15M-NaCl and finally against five changes of distilled water. The non-diffusible material was lyophilised and dissolved in distilled water such that $E_{260m\mu}$, read in 1cm. cells, of the final solution was 200. The solution was stored at -10^{0}. The overall yield was usually approximately 50%.

5. Isolation of RNA prior to sedimentation analysis.

The preparation of bentonite used in this method was as below.

2g. of bentonite (powder form, Technical grade, B.D.H) were suspended in 40ml. of water and centrifuged at 1,000g for 15min.; the supernatant was re-centrifuged at 8,000g for 20min. and the resulting sediment of this centrifugation was suspended in 0.01M-EDTA pH 7.0 for 48hr. at room temperature and then re-centrifuged as above. The sediment after centrifuging at 8,000g was taken up in 0.01M-sodium acetate, pH 6.0, recentrifuged differentially and the sediment suspended in 0.01M-sodium acetate pH 6.0 to a conc. of 50mg. per ml.

Figure 3c

OUTLINE OF METHOD USED FOR ISOLATION OF RNA FOR SEDIMENTATION ANALYSIS

Adrenal quarters

Add bentonite.
Homogenise in 0.01 M Na acetate, pH 5.2/ 0.5% SDS.
Add eq. vol 90% phenol, shake 20 min.
18, 000g, 4 min.

- Phenol phase and interphase (PPI) (discard)
- Aqueous layer

  Add 90% phenol.
  Shake
  18, 000g, 4 min.

  - Aqueous layer

    Add 2 vol 2% KAC in ethanol.
    Cool to -10^{o}. 1000g, 30 min.

    - Supernatant (discard)
    - Precipitate

      Digest in 0.01 M tris, pH 7.4/ 0.001 M-$MgCl_2$ in presence of DNase and bentonite. Precipitate with 2% KAC in ethanol.
      Dissolve ppte. in SM buffer (see text). Dialyse against SM buffer. Precipitate with 2% KAC in ethanol.

      - Supernatant (discard)
      - Precipitate

        Dissolve in SM buffer
        Extract with ether. N_2
        1000g, 30 min.

        - Precipitate (if any) (discard)
        - Supernatant (RNA)

          Apply to 5-20% sucrose gradient (for sedimentation analysis)
  - PPI (discard)

The tissue was homogenised in 4.0ml. of 0.01M-sodium acetate buffer pH 5.2 which contained 1μmole of $MgCl_2$ per ml. and 5mg. of SDS per ml. About 1 to 2mg. of bentonite in 0.01M-sodium acetate pH 6.0 were added to the tissue before homogenising. 4.0ml. of 90% phenol were added to the homogenate and the mixture shaken vigorously for 20 to 25min. at room temperature. The phenol and water phases were separated by centrifugation at 18,000g for 4min. at 4°, and the top (aqueous) layer transferred by pasteur pipette to another tube to which 0.5mg. of bentonite, and 1.6ml. of 90% phenol had been added. This mixture was briefly shaken, re-centrifuged, the top layer removed and two vols. of 2%(w/v) potassium acetate in ethanol (previously chilled to -10°) added to the top layer. The nucleic acids were allowed to precipitate by standing this mixture at -10° for 60min., before collecting them by centrifugation at 1,000g for 25min. The pellet was dissolved in 1.8ml. of 0.01M-tris buffer pH 7.8 containing 1μmole $MgCl_2$ per ml.; 0.5mg of bentonite and 0.2ml. of 0.01M-tris pH 7.4 containing 40μg. of RNase (Bovine pancreatic, Sigma Chem. Co.) were added and the solution incubated at 37° for 15 to 30min. The reaction was stopped by placing the tubes in ice and adding a further two vols. of 2%(w/v)

potassium acetate in ethanol. The tubes were allowed to stand at -10°, then the RNA was collected by centrifugation, dissolved in 2ml. of 0.01M-sodium acetate buffer pH 5.2 containing 1μmole $MgCl_2$ per ml. and 50μmoles of NaCl per ml. This buffer is referred to hereafter as SM buffer. After dialysing the RNA solution against 20vols. of SM buffer at 2° for 12 to 18hr., the RNA was precipitated and collected as above and dissolved in 0.5-1.0ml. of SM buffer. Residual ethanol was removed by ether extraction and the last traces of ether were removed from the solution by aspiration with nitrogen. The RNA solution was centrifuged at 1,000g for 30min. before it was applied to the top of a sucrose density gradient.

2.5. Fractionation of ribonucleic acids on anion exchangers.

1. Ecteola-cellulose.

(a) Preparation

Ectoola-cellulose (Serva Entwicklungslabor, Heidelberg) with exchange capacity 0.24m. equiv. per g. was used. Before use it was shaken in water, then washed successively, with N-HCl, water until it was free of HCl, N-NaOH, water until the Ecteola-cellulose was free of alkali; this washing procedure was repeated once and the Ecteola-cellulose was finally suspended in water. A column 7-8cm. x1cm. (diam.) was prepared

and water followed by 0.01M-potassium (or sodium) phosphate buffer, pH 6.85, passed through the column.

(b) Elution of RNA.

The level of buffer solution above the surface of the Ecteola-cellulose was allowed to fall until the level of buffer above the column was about 2mm.; the RNA solution (0.2 to 0.3ml.) was applied to the top of the column, allowed to enter the column and washed into the column with small aliquots of 0.01M potassium (or sodium) phosphate buffer, pH 6.85. A volume of this buffer was allowed to pass through the column followed by an NaCl concentration gradient.

In the early experiments, the NaCl gradient used was exponential, and was obtained by passing 2M-NaCl, in 0.01M-sodium phosphate buffer, pH 6.85, into a constant volume mixing flask containing 0.01M-sodium phosphate buffer, pH 6.85, which was connected to the top of the column. In later experiments a linear NaCl gradient was used. This was achieved by connecting a mixing flask (flask I) to the column and connecting a second flask (flask II) to flask I. Both flasks were identical in size and were placed at the same level above the top of the column. Flask I was filled with 250ml. of 0.01M-sodium phosphate buffer, pH 6.85, with the connection between the flasks closed, and flask II filled with

230ml. of 2M-NaCl in 0.01M-sodium phosphate buffer, pH 6.85. After applying RNA to the column about 20-30ml. of buffer from flask I was allowed to pass through the column and the connection between flask I and II was opened, so commencing the NaCl gradient. When the NaCl conc. passing through the column had risen to 1.2-1.4M, the contents of the flasks were changed such that flask I contained either M-NaCl or 2M-NaCl in 0.01M-sodium phosphate buffer, pH 6.85, and flask II contained M-NH_4OH in either M-NaCl or 2M-NaCl. A NH_4OH gradient was now passed through the column. The final eluting solution was N-NaOH.

The eluate was collected in 5ml. fractions and the E at 260mμ determined, using either a Unicam SP 500 Spectrophotometer or a Beckman DB Spectrophotometer.

2. DEAE-Sephadex

(a) Preparation.

10g. of DEAE-Sephadex (A50 Medium grade, exchange capacity 3.9m.equiv. per g., Sephadex Uppsala) were allowed to swell in water over a period of 3 to 4hr. The supernatant was carefully decanted and a further 500ml. of water added and after allowing the particles to settle the supernatant was again decanted; this was repeated twice more. This procedure eliminated the "fines". The DEAE-Sephadex was transferred to a Buchner filter

and washed successively with 500ml. of 0.5 N-HCl, water until the DEAE-Sephadex was free of acid, 500ml. of 0.5N-NaOH, water until the DEAE-Sephadex was free of alkali, 250ml. of 5%(v/v) H_3PO_4 and finally 0.01M-potassium phosphate buffer, pH 6.85, until the buffer passing through the DEAE-Sephadex was also pH 6.85. The DEAE-Sephadex was stored as a suspension in 0.01M-potassium phosphate buffer, pH 6.85. A fresh column (9cm. x1cm. diameter) was prepared for each fractionation. Before applying the RNA solution to the column, 0.01M-potassium phosphate buffer, pH 6.85 was passed through the column until E 260mμ of effluent was equal to that of the influent.

The RNA was applied as above and a linear NaCl conc. gradient, followed by a linear NH_4OH gradient used to elute the RNA from the column. Details of the gradients are given above. The eluate was collected in 5ml. fractions by means of a Locarte fraction collector and the $E_{260m\mu}$ determined.

2.6. Sedimentation Analysis of RNA.

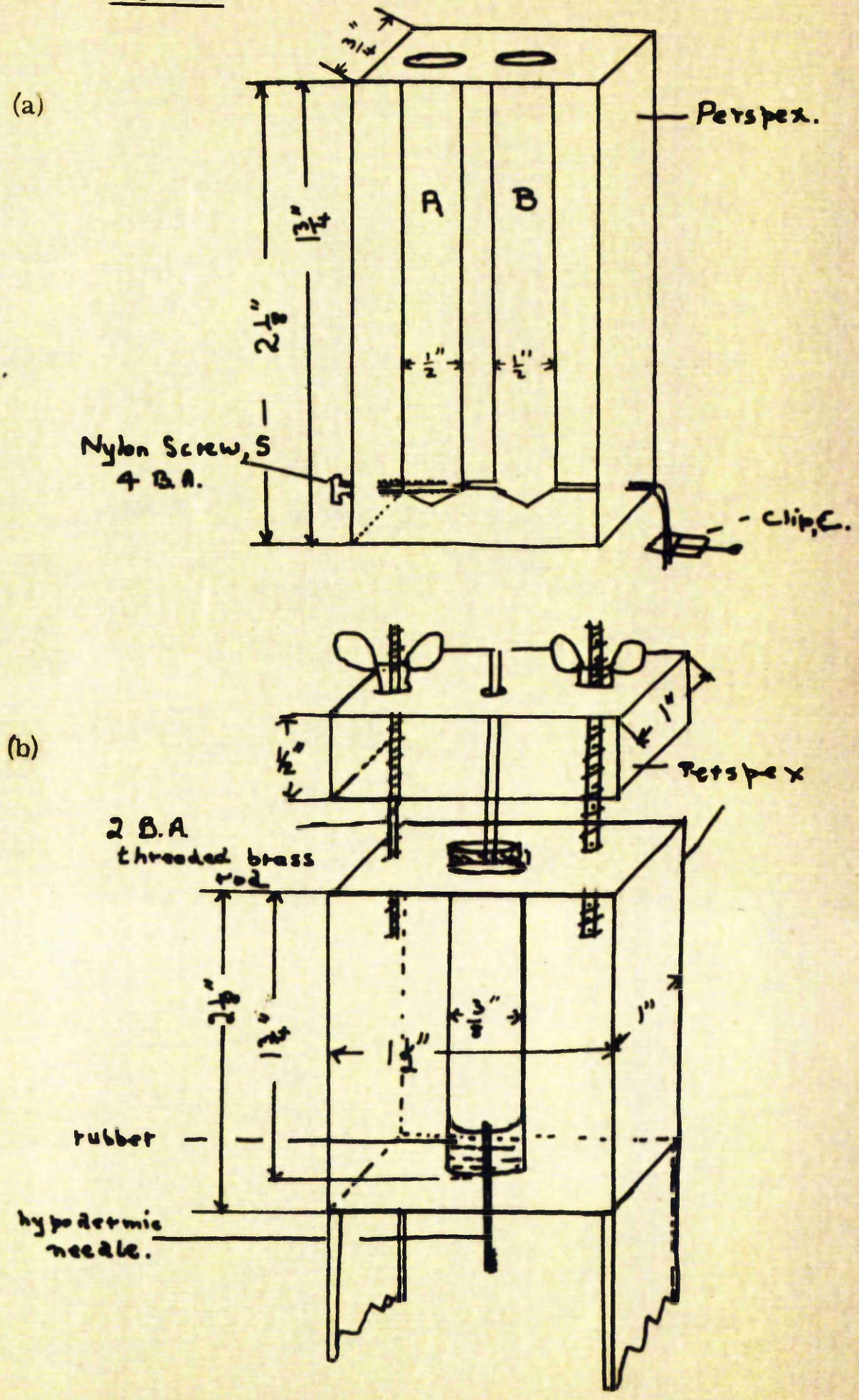
Sucrose density gradients were prepared using the device shown in Figure 4a. Screw, S, and clip, C, were closed, 2.4ml. of 5%(w/v) sucrose in S.M. buffer pipetted into chamber A and 2.4ml. of 20%(w/v) sucrose in S.M. buffer pipetted into chamber B. Screw S was

Figure 4.

(a) Device used to prepare linear sucrose concentration gradients.

(b) Apparatus used to puncture the bottom of a centrifuge tube (Spinco Rotor 39L) prior to collection of the fractions.

Figure 4


(a)
Perspex.
A
B
Nylon Screw, S
4 B.A.
Clip, C.
(b)
Perspex
2 B.A
threaded brass
rod
rubber
hypodermic
needle.

opened, a gentle stream of air bubbled through the 20% sucrose in chamber B, clip C opened and the solution collected in an appropriate centrifuge tube. This procedure produced a linear gradient of sucrose concentration down the centrifuge tube.

The RNA solution was carefully layered on top of the sucrose gradient and the tubes centrifuged in a swinging-bucket rotor (SW 39L head) using a Spinco model L preparative ultracentrifuge. Time of centrifuging varied between 10.5hr. and 12hr. and centrifugal force employed varied between 30,000g and 42,000g. After centrifugation, the bottom of each tubes was punctured with a 14 or 16 gauge hypodermic needle and the sucrose solution collected in 13 drop fractions using the device shown in Figure 4.b. 0.8ml. of water was added to each fraction and the $E_{260m\mu}$ read in 1cm. microcells in a SP 500 Unicam spectrophotometer.

2.7. Radioactive Assay.

1. ^{32}P assay.

In some experiments samples were assayed in the liquid state using a M6 Geiger-Müller tube (20th Century Electronics) coupled to a conventional scaler. The tube was operated in the geiger region. In other experiments, liquid samples were pipetted on to a stainless steel planchets and evaporated to dryness

with the aid of an infra-red lamp. The samples were counted by means of a Nuclear-Chicago gas flow counter fitted with a "Micromil" window, operating in the geiger region.

The counts were corrected for background and decay.

2. ^{3}H assay.

1ml. of the aquoous sample being assayed was added to 9.0ml. of NE 572 "Scinstant" (Nuclear Enterprises Ltd., Edinburgh) in dioxane (scintillation grade, Nuclear Enterprises Ltd., Edinburgh) and the samples assayed for ^{3}H using a Nuclear-Chicago (Series 720) liquid scintillation spectrometer.

2.8. Assay of RNase, pCMB released RNase and RNase Inhibitor.

All solutions were made up in glass distilled water.

1. Special Reagents.

(a) Chemicals.

p-chloromercuribenzoate (pCMB) (Light and Co. Ltd.) was dissolved in water, using 2N-NaOH to assist solution, to give a final concentration of 0.0102M. The pH was adjusted as close to pH 8 as solubility of the pCMB would allow. The final pH was usually about 9.0-9.2.

The tris used for the buffer solution was "specially purified grade" (B.D.H.)

(b) Gelatin and RNase.

Gelatin was purified as follows.

1g. of gelatin was added to 100ml. of 0.01M-EDTA pH 7.8 and dialysed successively against 0.01M-EDTA pH 7.8, five changes of 0.15M-NaCl, and finally against five changes of water. All dialysis was done at 4°. The dialysis residue was lyophilised and a 1% solution in water was made and stored at 2°.

Crystalline pancreatic RNase (Bovine pancreatic, crystallised x5, Sigma Chem. Co.) was dissolved in 0.1% gelatine to a concentration of 500µg./ml. This constituted the stock RNase solution and was stored at -10°. A sub-stock of 5µg./ml. also in 0.1% gelatin, was prepared twice monthly. Each day, when an assay was being carried out, a standard was prepared by diluting the sub-stock of RNase 1 part in 100 with 0.1% gelatin.

2. RNase Assay.

This method was modified from that of Shortman (1961). The assays were carried out in 15ml. conical centrifuge tubes which had been previously cleaned in chromic acid. All assays were done in triplicate. The reaction mixture was set up as follows; to 0.2ml. of 0.06M-tris buffer pH 7.8 containing 450µmoles NaCl/ml. (Hereafter referred to as tris/NaCl buffer pH 7.8),

0.1ml. of homogenate of suitable dilution in water, 0.1ml. of water, 0.2ml. of a 1 % solution of RNA, purified by method given above, were added at 0° in the above order, mixing after each addition. A reaction mixture set up as above, but omitting the homogenate, served as a control. RNase standards were also assayed out concurrently, details of which are given below. The mixtures were incubated in a shaking water bath at 37° for 20min. The reaction was stopped by transferring the tubes to a water bath held at 0°. 0.1ml. homogenate was added to the control tubes and 0.9ml. of precipitating agent added to each tube with thorough mixing. The precipitating agent was a mixture of 1vol. 0.75% uranyl acetate in 2.5N-PCA in 10vol. N-HCl in 76% ethanol. The tubes were kept in ice for 25 to 45min. after precipitation before spinning at ~5° at 1,000g for 35min. 0.5ml. of the supernatant was diluted to 5ml. with water and $E_{260m\mu}$ determined. The conditions of precipitation had to be held to if reproducible results were to be obtained.

3. Latent RNase assay (or pCMB-released RNase assay).

This assay was identical to RNase except that 0.1ml. of 10.2mM-pCMB was substituted for the 0.1ml. of water.

4. Assay of RNase Inhibitor.

To 0.2ml. of tris/NaCl buffer, pH 7.8, at 0°, 0.1ml. of RNase (0.05µg/ml.) in 0.1% gelatin, 0.1ml. of homogenate, or cell fraction, diluted with 75mM-EDTA, pH 7.8, and 0.2ml. of 1% yeast RNA solution was added. The tubes were thoroughly mixed after each addition. The dilution of the homogenate with EDTA is chosen so that it inhibits the action of 0.005µg. RNA by about 50%. The control consisted of 0.2ml. tris/NaCl buffer, pH 7.8, and 0.2ml. of 1% yeast RNA solution. The tubes were then incubated at 37° for 20min., cooled in ice for 2 to 3min., 0.1ml. of RNase solution and 0.1ml. of EDTA diluted homogenate added to the blank reaction mixture, and 0.9ml. of precipitating agent added and preparation of diluted supernatant carried as above, and its absorbance at 260mµ determined.

5. Assay of RNase Standard.

When any of these assays were carried out, the activities were compared to those of a standard amount of pancreatic RNase assayed under the same conditions. The standards were set up, similar to the assays above, as follows - to 0.2ml. of tris/NaCl buffer, pH 7.8, 0.1ml. of RNase solution containing 0.005µg. RNase in 0.1% gelatin, 0.1ml. of water and 0.2ml. of 1% yeast RNA solution were added with mixing. A "half standard"

was also set up with 0.05ml. of RNase solution containing 0.0025µg. RNA and 0.15ml. of water. Blank tubes with RNase omitted were set up. The tubes were incubated, the reaction mixtures precipitated and the supernatants diluted and read as above.

6. Expression of results.

The definition of units are those of Shortman (1961).

(a) Free RNase.

When a given amount of tissue gave the same increase in $E_{260m\mu}$ as did 0.0025µg. of pancreatic RNase acting at pH 7.8 and carried out simultaneously and at the same ionic strength it was said to possess 1 unit of "free" RNase activity.

(b) Latent RNase.

When a given amount of tissue after treatment with pCMB gave the same increase in $E_{260m\mu}$ as did 0.0025µg. pancreatic RNase acting at pH 7.8 and carried out simultaneously at the same ionic strength, it was said to possess 1 unit of pCMB released RNase. ("latent" RNase).

(c) RNase Inhibitor.

Adrenal tissue samples were diluted in 75mM-EDTA pH 7.8 to a concentration which gave 50% inhibition of the added pancreatic RNase under assay conditions already stated. A dilution of $\frac{1}{100}$ of the adrenal was usually employed, i.e. about 30-40µg. of tissue were added to the reaction mixture.

Figure 5

PROCEDURE FOR SEPARATION OF RNA AND DNA

All operations are carried out at 0^{o}, unless otherwise stated.

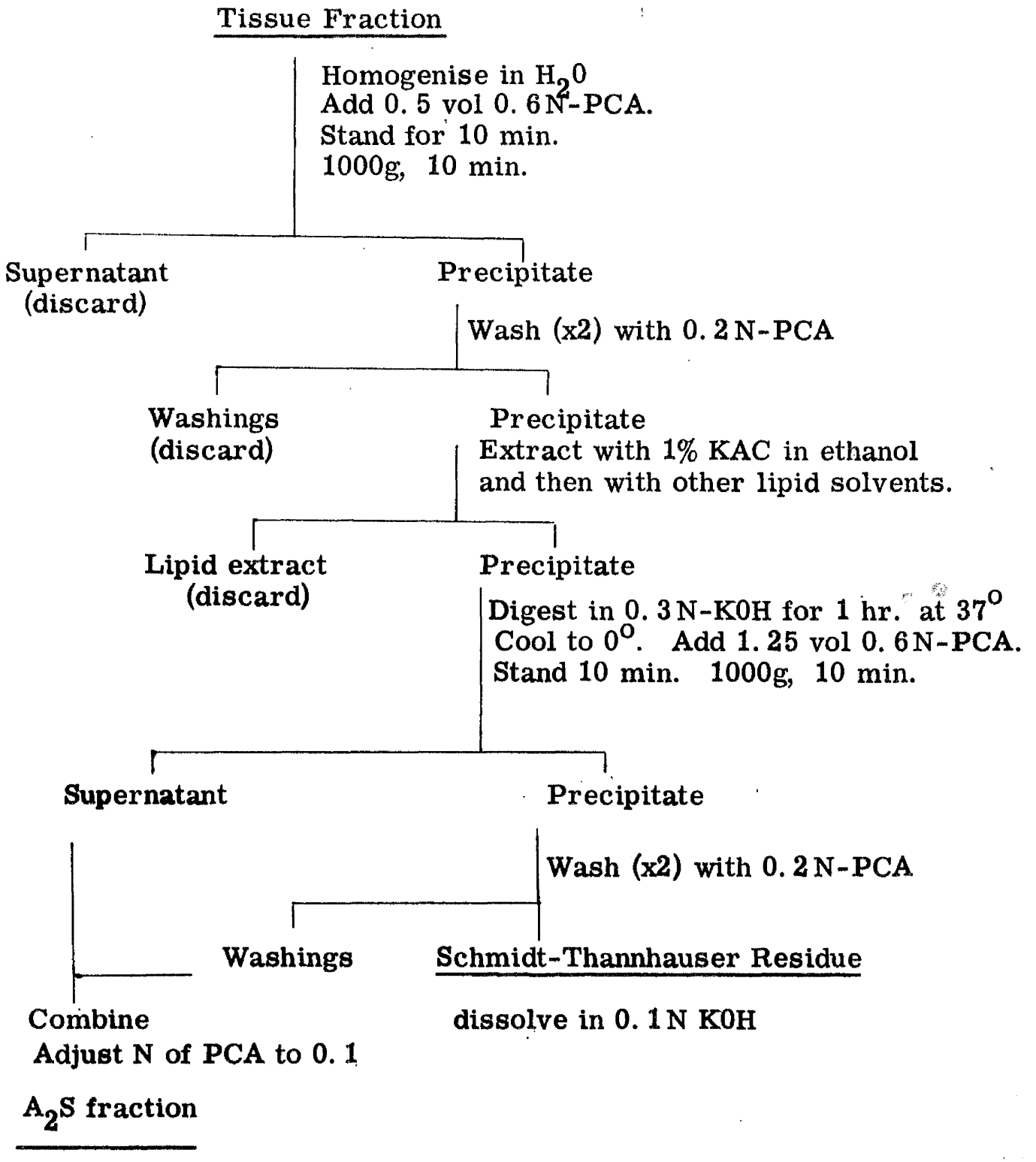
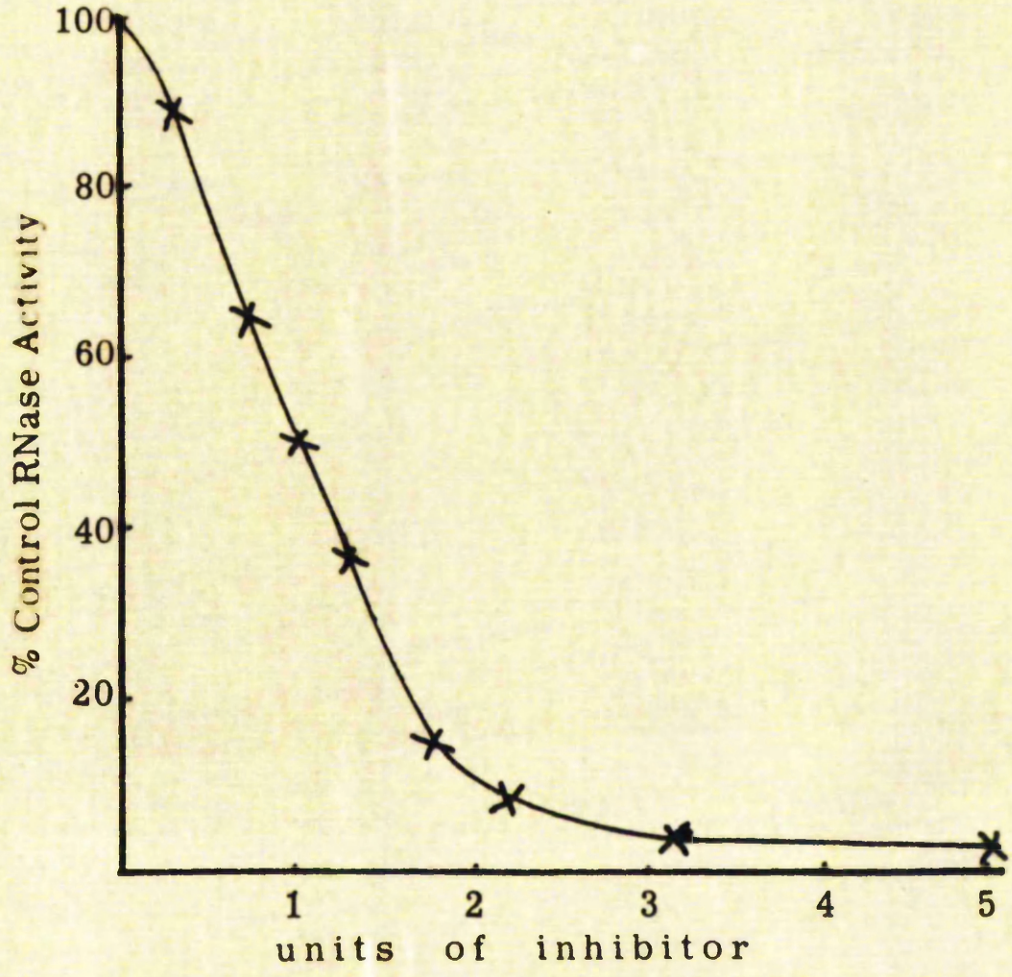


Figure 6.

The relationship between percent control bovine pancreatic RNase activity and units of RNase inhibitor. (from Shortman, 1961).

Figure 6


100
80
60
40
20
% Control RNase Activity
1
2
3
4
5
units of inhibitor

One unit of inhibitor activity was defined as that which gave 50% inhibition of 0.005µg. of bovine pancreatic RNase under the stated conditions of assay. The inhibitor concentration was read from the graph in Figure 6.

2.9. Chemical Estimations.

1. Analysis of RNA and DNA.

The method was modified from that of Schmidt and Thannhauser (1945).

The sample being analysed was suspended or homogenised in a suitable vol. of water at 0°, 0.5vol. of 0.6N-perchloric acid (PCA) added and mixed thoroughly. After allowing the mixture to stand at 0° for 10min., it was spun at 1,000g for 10min. at 0°. The precipitate was washed twice with 5ml. aliquots of 0.2N-PCA and the supernatant and washings were discarded. The precipitate was then successively extracted with 5ml. portions of 1%(w/v) potassium acetate in ethanol, ethanol:chloroform (3:1, v/v), ethanol:ether (3:1, v/v) and finally ether. These extracts were discarded. The air-dried lipid-extracted precipitate was incubated in 4.0ml. of 0.3N-KOH at 37° for 60min. This alkaline digest was acidified at 0° by addition of either 5ml. of 0.6N-PCA or 0.2ml. of 10N-PCA. After allowing 10min. for complete precipitation of DNA and protein

the precipitate was collected by centrifugation, and washed twice with 0.2N-PCA. The supernatant and washings were combined, diluted and normality of PCA adjusted to 0.1. This constituted the A$_2$S fraction. The RNA content of A$_2$S fraction was calculated on the basis that E$_{260m\mu}$ = 1.000, read in 1cm. cells, was given by a solution of hydrolysed RNA of concentration 35.18μg. The precipitate (Schmidt-Thannhauser residue) was dissolved in 0.1N-NaOH for DNA estimation: DNA was estimated by method of Ceriotti (1952). To 2.0ml. of solution containing DNA, 1.0ml. of 0.04%(w/v) indole in water and 1ml. of concentrated HCl was added in 10ml. glass stoppered tubes. The tubes were heated in a boiling water bath for 10min. After rapid cooling, the solutions were extracted three times with 4ml. portions of chloroform, shaking for 30sec. after each chloroform addition. After the third extraction, the tubes were spun at 200g for 5min. to aid separation of the aqueous and chloroform layers. The E of the aqueous layer at 490mμ was read in a Unicam SP500 spectrophotometer. Blanks and standards were also carried through this procedure. For the blanks 2ml. of water and for the standard 2ml. of a standard solution of DNA were used. The DNA standard was prepared by dissolving an accurately weighed amount (about 20mg.)

of pure, dry DNA in distilled water with a drop of N-NaOH to aid solution, the final volume being 50ml. The DNA used was purified from calf thymus by the method of Kay, Simmons and Dounce (1952). 1ml. of the DNA solution was diluted with 0.5N-PCA and heated to 70° for 20min. and made up to 50ml. This was used as standard DNA solution. The amount of DNAP in this standard was estimated by the method of Griswold, Hunoller and McIntyre (1951).

2. Estimation of RNA by orcinol method.

A modification of the method of Mejbaum (1939) was used.

The orcinol reagent was 0.0%(w/v) orcinol (BDH) in 0.08%(w/v) $FeCl_3.6H_2O$ (Analar., BDH) in conc. HCl (Analar., BDH) and was freshly prepared for each assay.

To 1ml. of a solution (containing not more than 10µg. RNAP) were pipetted into a 6"x$\frac{5}{8}$" test tube previously cleaned in chromic acid. The standards consisted of 2ml. of solution containing 10µg. of D-ribose, and the blanks consisted of 2ml. of water. 2ml. of orcinol reagent were added to each tube, the reagents mixed, the tubes capped (aluminium caps, Oxoid) and placed in a vigourously boiling water bath for 30min. After rapid cooling, the colour intensity was measured against

the reagent blank at 660mμ using a Unicam SP600 spectrophotometer.

10μg. of ribose was assumed to be equivalent to 4.15μg. RNAP.

5. Estimation of Inorganic Phosphorus.

The estimation used was based on the method of Gpiswold et al., (1951.)

(a) Isolation of Inorganic P from Blood.

A 0.5vol. of 30%(w/v) trichloroacetic acid (TCA) was added to the blood at 0° and then thoroughly mixed. After allowing the tubes to stand in ice for 10min., they were centrifuged at 1,000g for 20min. The supernatants were transferred to another centrifuge tube, 1ml. of Mathleson's Reagent (1909) added, and the pH adjusted to 10 with conc. NH_4OH solution using phenolphthalein as indicator. The tubes were stored at 0°, then centrifuged at 1,000g for 10min. at 0°. The supernatant was discarded, the precipitate washed twice with 1.6N-NH_4OH, dissolved in 5ml. of 2N-HCl, and diluted with water such that the final solution contained approximately 2μg. of inorganic P. This solution contains the inorganic P of the blood.

(b) Isolation of Inorganic P from cell sap.

The aliquot of cell sap, which was usually of vol. 0.5ml. was first diluted to 4ml. before addition of

(2ml.) 50% TCA. Thereafter the isolation procedure was as above.

(c) Estimation of P

This reducing agent used was made up as follows. 15.6g. of sodium metabisulphite, 4.0g. of $Na_2SO_3.7H_2O$ and 1g. 2-napthol-1-amine-4-sulphonic acid were dissolved in 250ml. water and stored in a brown bottle.

1ml. of test solution (containing not more than 4µg. P), 3ml. of 1.66N-H_2SO_4, 0.5ml. of reducing agent and 0.5ml. of 2.5%(w/v) ammonium molybdate were added together with thorough mixing after each addition. 1.ml. of water and 1ml. of a solution containing 0.772µg. KH_2PO_4 served as a blank and a standard. The tubes were incubated in a boiling water bath for 10min. and rapidly cooled after this incubation. The intensity of the blue colour was read at 820mµ against the blank solution.

4 Estimation of Corticosterone.

The fluorometric method of Silber, Busch and Oclapas (1956) was used. This method was used to estimate the amount of corticosterone produced by quartered adrenals *in vitro* and thus served as a criterion that the quarters were responding to ACTH added to the incubation medium. The incubation medium (1.5ml.) was diluted to 5.0ml. with water and centrifuged at 1,000g for 10min. 1ml. samples of supernatant were

pipetted into a glass stoppered centrifuge tube and 5ml. of distilled 2,2,4-trimethylpentane added and the tube shaken for 30sec. The top layer was removed, and 4ml. of water and 15ml. redistilled CH_2Cl_2 added to the aqueous layer. The tubes were shaken for 30sec., and the top (aqueous) layer removed. 1ml. 0.1N-NaOH was added and the tubes again shaken for 10 to 15sec. After centrifugation the top layer was discarded. A 10ml. aliquot of the lower layer was pipetted into a 35ml. centrifuge tube containing 2ml. 30N-H_2SO_4. The tube was shaken for 30sec., centrifuged and the top layer removed. 30 to 90min. after addition of the CH_2Cl_2 layer to 30N-H_2SO_4 the fluorescence of the 30N-H_2SO_4 estimated using an Aminco-Bowman Spectrophotofluorometer. The wavelength of exciting light was 465mμ and the fluorescent light was 530mμ. An 109 Ilford filter was fitted in front of the photo-multiplier tube. The slit arrangement was No. 5.

Standard solutions containing 0.5μg., 0.3μg. and 0.1μg. of corticosterone were assayed simultaneously as the unknowns.

2.10. Buffers.

Tris buffers were adjusted to the desired pH using 2N-HCl. EDTA solutions were adjusted to the

required pH by addition of 2M-NaOH.

Phosphate buffers: 0.2M-K_2HPO_4 (or Na_2HPO_4) was added to 0.2M KH_2PO_4 (or $NaH_2PO_4.2H_2O$) until the required pH was obtained. This was diluted $\frac{1}{20}$ to give 0.01M phosphate buffer.

2.11. Determination of sedimentation coefficients.

Ultracentrifugal analyses were carried out on a Spinco model E ultracentrifuge using U.V. optics. Runs were done in either water or 5M buffer at 20° at 47,770 rev./min. Photographs were taken at 4 or 8min. intervals. The sedimentation coefficient was calculated using the following equation,

$$w^2 s = \frac{d \log_e r}{dt}$$

Where w = angular velocity (rad./sec.)

r = distance of boundary from centre of rotation (cm./sec.)

t = time (sec.)

s = sedimentation constant.

A value of $s = 10^{-13}$ was defined as 1 Svedberg unit. ( ).

2.12. Statistical Analysis.

Student's t-test (1946) was used for the analysis of significant differences between means. 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.

SECTION 3

R E S U L T S

3.1. Estimation of RNA and DNA.

1. General.

The original method of Schmidt and Thannhauser (1945) has been modified by many workers (Hutchison and Munro, 1961) and as the result of an intensive investigation, Fleck and Munro (1961) recommended that digestion of an acid precipitated liver sample for 1hr. in 0.3N-KOH at 37° was sufficient to hydrolyse RNA such that it was no longer acid precipitable. More recently, Hallinan, Fleck and Munro (1962) showed that treatment of an acid-precipitated homogenate with lipid solvents extracted appreciable amounts of RNA and protein. Since the estimation of RNA and these observations were made on liver tissue samples it was thought desirable to appraise the methods used for their validity and to establish whether these findings also apply to adrenal tissue.

2. Comparison of 1hr. and 17hr. as digestion times for hydrolysis of RNA in 0.3N-KOH to acid-soluble products.

Replicate samples of liver and adrenal homogenates were precipitated by addition of 0.5vol. of 0.6N-PCA at 0°, allowed to stand 10min. at 0° and then centrifuged. The acid-insoluble residues were washed twice with 0.2N-PCA and then successively extracted with ethanol, ethanol:chloroform (3:1v/v), ethanol:ether

(3:1, v/v) and ether. The acid-extracted lipid-free powder was incubated in 0.3N-KOH for either 1 hr. or 17hr. at 37°. The alkaline digest was then cooled to 0° and 1.25vol. of 0.6N-PCA added to precipitate DNA and protein. The DNA and protein (Schmidt-Thannhauser residue) was washed twice with 0.2N-PCA, the supernatant and washings combined and made up to a suitable volume and the normality adjusted to 0.1N with respect to PCA. The RNA in this fraction, the A_2S fraction, was estimated by the orcinol method. The Schmidt-Thannhauser residue was dissolved in 0.1N-KOH and the amount of DNA estimated by the method of Ceriotti. The results are given in Table 1. These results show that a 1hr. digestion period is sufficient to hydrolyse adrenal and liver RNA to products soluble in 0.2N-PCA. There is good agreement between the values of RNAP/ml. of A_2S fraction as obtained by the two methods of estimation only when a 1hr. incubation time interval is used. The increase in $E_{260m\mu}$ after 17hr. compared to that after 1hr. is due to release of acid-soluble products from protein (Fleck and Munro, 1962).

3. <u>The effect of extraction of RNA by lipid solvents after acid precipitation.</u>

PCA was added to replicate samples of liver and adrenal homogenates to a final conc. of 0.2N. After

Table 1.

Comparison of 1hr. and 17hr. digestion times for hydrolysis of RNA to acid-soluble products.

Experimental details are given in text. Hydrolysis was carried out in 0.3N-KOH at 37°. Amount of RNA in A_2S fraction measured by orcinol method and on basis that $E_{260m\mu} \equiv 1.000$ is given by a solution of hydrolysed RNA in 0.1N-PCA at a concentration of 5.412 μg./ml. Values are averages of three determinations.

Tissue	Digestion Time (hr.)	$E_{260m\mu}$ of A_2S Fraction	Amount of RNAP/ml. A_2S fraction (a) by orcinol	(b) by U.V.
Liver	1	0.520	1.69	1.75
Liver	18	0.570	1.75	1.94
Adrenal	1	0.640	2.37	2.26
Adrenal	18	0.740	2.31	2.53

standing, at 0°, for 10min., the acid-precipitated material was collected by centrifugation, washed twice with 0.2N-PCA and either

(i) digested immediately in 0.3N-KOH for 1hr. at 37°

or (ii) extracted with the lipid solvents used in 3.1.2 before digestion in 0.3N-KOH for 1hr. at 37°,

or (iii) as (ii) except that the first lipid extraction was carried out using 1%(w/v) potassium acetate in ethanol instead of ethanol.

or (iv) extracted with ether to which sufficient ethanol was added to prevent the precipitate appearing as a gel. The precipitation was then successively extracted with ethanol:chloroform (5:1, v/v), ethanol:ether (3:1, v/v), and ether, and then hydrolysed in 0.3N-KOH for 1hr. at 37°.

The separation of the alkaline digest into the A_gS fraction and the Schmidt-Thannhauser residue was carried out as above and the amounts of RNA and DNA estimated (Table 2).

The highest amounts of RNA are found in the A_gS fraction of liver and adrenal homogenates if treatments (iii) or (iv) are carried out after acid precipitation. It seems that ethanol extracts about 5% of the RNA from an acid-precipitated liver homogenate and about 25% of the RNA from an acid-precipitated adrenal homogenate.

Table 2.

Effect of various lipid solvent extraction procedures on the estimation of nucleic acids.

Experimental details are given in text. RNA was measured by ultraviolet absorbance determinations as in Table 1. Values are the average of four determinations.

Tissue	Lipid Extraction Procedure	Amount of RNAP in A_2 fraction	Amount of DNAP in Schmidt-Thannhauser residue	RNAP / DNAP
Liver	(i)(none)	89.4	20.6	4.3
	(ii)(1st solvent ethanol)	85.0	21.8	3.9
	(iii)(1st solvent KAc/ethanol)	93.2	21.2	4.41
	(iv)(ether and ethanol)	92.8	21.2	4.34
Adrenal	(i)	26.1	16.2	1.61
	(ii)	19.8	19.1	1.03
	(iii)	26.6	18.4	1.45
	(iv)	26.5	20.0	1.33

KAc/ethanol refers to 1%(w/v) potassium acetate in ethanol.

This is in agreement with the findings of Venkataraman and Lowe (1959), Venkataraman (1960) and Hallinan, Fleck and Munro (1963) but not of Hutchison, Downie and Munro (1963).

The amount of DNA estimated in the Schmidt-Thannhauser residue seems to depend on the lipid extraction procedure used. The value of DNAP obtained is lower if the lipids are not extracted than if the lipids are extracted. Use of any of the lipid extraction procedures each results in the higher value of DNAP being obtained for liver but different values are obtained for adrenal DNAP. Similar observations have been made by Hutchison et al., (1962). No reason can be given for these findings except that it may be that there is a factor soluble in lipid solvents and insoluble in acid which is present in these tissues, and which can inhibit the reaction of DNA with acidified indole.

The varying RNAP and DNAP values obtained are reflected in the $\frac{RNAP}{DNAP}$ ratios.

4. Conclusion.

On the basis of these experiments, it was concluded that the most satisfactory method for the estimation of RNA and DNA in adrenal tissue was that of Schmidt and Thannhauser (1945) with the modifications as given in 2.8.1.

3.2. The Effect of ACTH on the Metabolism of the RNA's of the Adrenal as separated by Column Chromatography.

1. General.

As already mentioned, treatment of rats with ACTH has been found to cause a reduction in the uptake of ^{32}P into adrenal whole cell RNA (Ramaiah, 1959). In this series of experiments, it was intended to investigate further the effect of administration of ACTH, *in vivo*, upon the metabolism of RNA of the various subcellular fractions. It was hoped that this approach would establish whether this effect of ACTH is upon all the species of RNA in the adrenal cell or whether the effect is upon only certain species.

Rats were injected with ACTH for lengths of time varying between 24 hr. and 72hr. The RNA was isolated from each subcellular component of the adrenals and chromatographed on columns of DEAE-Sephadex.

2. Validation of Methods.

(a) Phenol Extraction.

The RNA isolation method in this series of experiments was essentially that of Goldthwait (1961), who stated that a higher proportion of liver cell sap RNA was released into the aqueous phase on addition of phenol if the cell sap were first made 0.2M with respect to sodium phosphate, pH 6.85, before addition of the phenol.

To test whether this observation also applied to extraction of RNA from adrenal tissue, aliquots of adrenal tissue homogenate were extracted with 90% phenol and the amount of RNA in the aqueous phase estimated. The tissue was homogenised in either water or 0.2M-sodium phosphate buffer, pH 6.85. It was found that extraction with phenol released 40% of the total RNA of the "water homogenate" into the aqueous phase and 80% of the total RNA of the "buffer homogenate" into the aqueous phase. These figures are in broad agreement with those of Goldthwait (1961).

(b) Choice of Anion Exchanger for Chromatography of RNA.

Antoni (1961) separated the RNA isolated from the various subcellular components of rat adrenal glands into various fractions using Ecteola-cellulose. However, attempts to repeat this separation were met by a series of difficulties and Ecteola-cellulose was found to possess a number of disadvantages as an anion-exchanger suitable for chromatography of RNA. These are given below.

An exponential concentration gradient of NaCl was passed through a column (1cm. diam.) of Ecteola-cellulose (0.62mg.) washed as described in the methods section. This was followed by a concentration gradient of NH_4OH and finally N-NaOH was passed through the column.

Five ml. fractions were collected and the $E_{260m\mu}$ determined (Figure 7a). It can be seen that there are varying amounts of ultraviolet absorbing material in each fraction and that the amounts vary from fraction to fraction. This fact means that the determination of RNA being eluted from Ecteola-cellulose would be almost impossible, and may perhaps explain why the $\frac{E_{260m\mu}}{E_{280m\mu}}$ ratios of the RNA fractions eluted from Ecteola cellulose were found to be low, 1.1-1.6, (Antoni, 1961).

This type of experiment was repeated using DEAE-Sephadex instead of Ecteola and it was found that DEAE-Sephadex was more satisfactory as the ultraviolet absorbance of the "blank" eluate was less and there was virtually no variation in the absorbance at 260mμ of the eluate fraction (Figure 7b).

Figure 8 shows the fractionation of whole adrenal RNA (isolated by method 2.4.1) achieved by column chromatography on DEAE-Sephadex. It can be seen that the RNA is separated into three main types. Two of these types are eluted by NaCl and the other is eluted by NH_4OH. It proved to be impossible to use N-NaOH as eluant, for, when the eluant was changed to M-NaOH the flow-rate fell to almost zero. However, in later experiments, in which labelled RNA was

Figure 7.

(a) Blank elution profile ($E_{260m\mu}$) from an Ecteola-cellulose column.

An exponential gradient of NaCl (0 to 1.0M) was passed through a column of Ecteola-cellulose (3.5cm. x 1.0cm.). This was followed by an exponential gradient of NH_4OH (0 to approx. 1.0M). 5ml. fractions were collected.

(b) Blank elution profile ($E_{260m\mu}$) from a DEAE-Sephadex column.

A linear gradient of NaCl (0 to 1.0M) was passed through a column of DEAE-Sephadex (7.5cm. x 1.0cm.). This was followed by a linear gradient of NH_4OH (0 to approx. 0.05M).

Figure 7

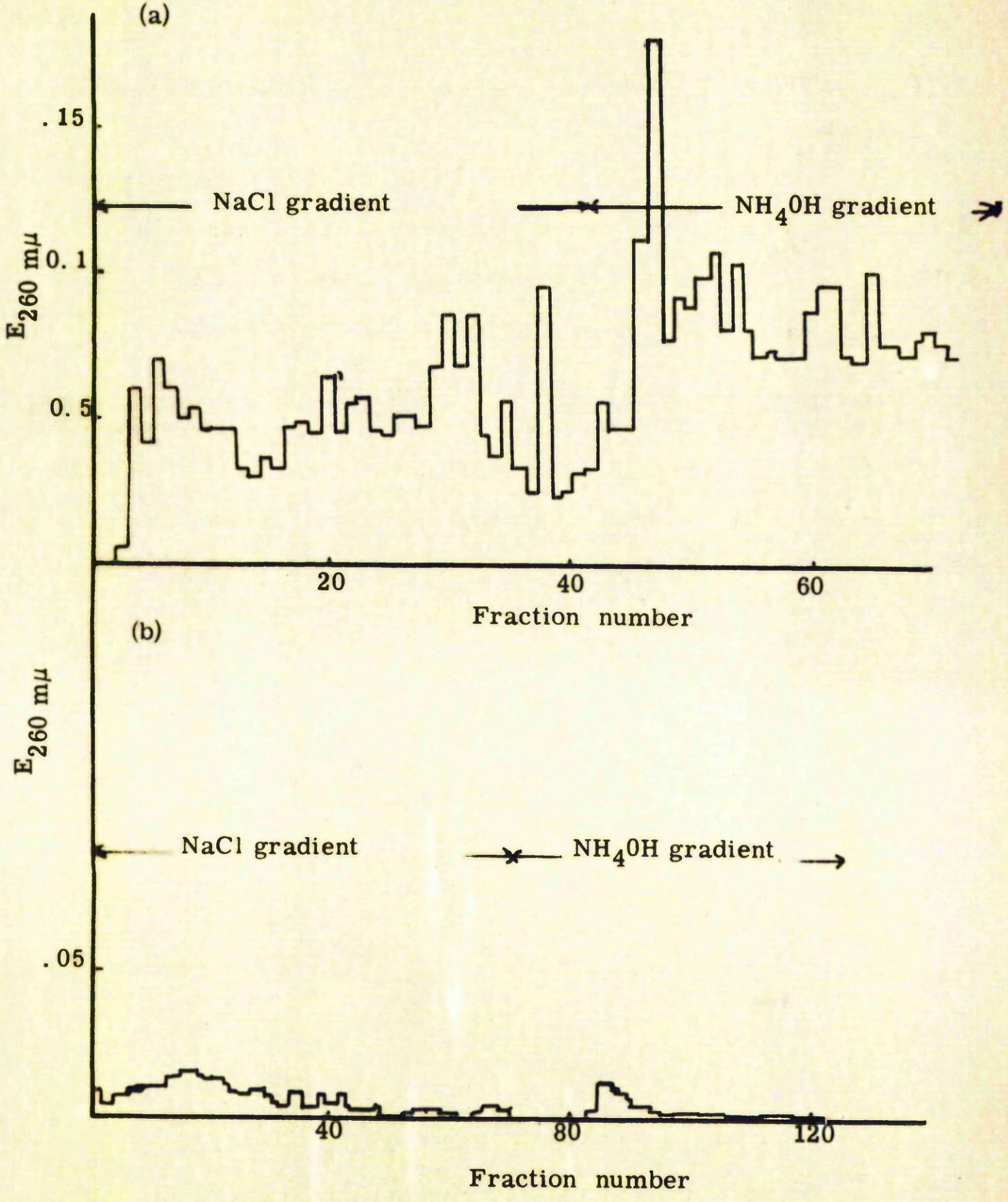
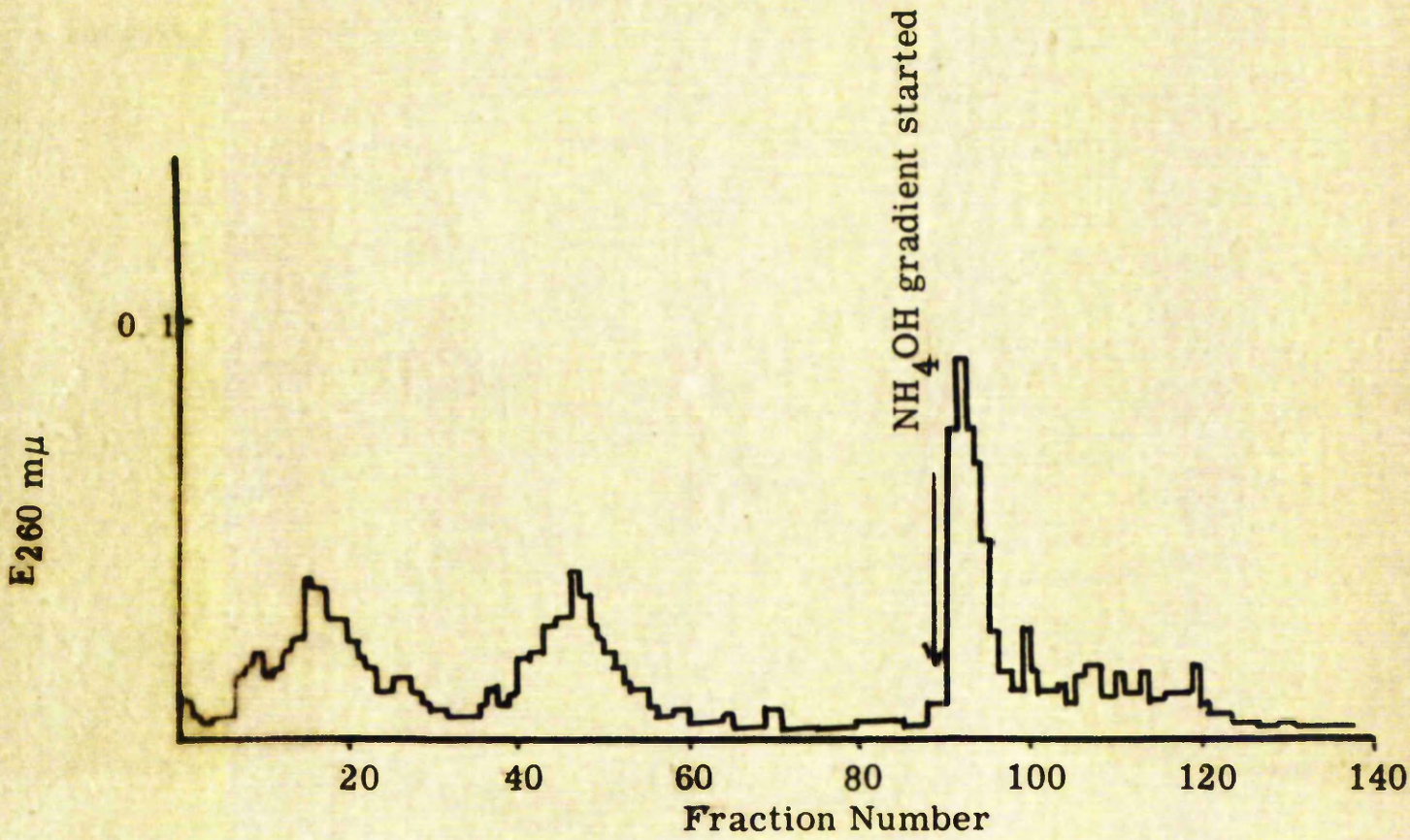

(a)
.15
0.1
0.5
E_{260} mμ
NaCl gradient
NH_4OH gradient
20
40
60
Fraction number
(b)
E_{260} mμ
NaCl gradient
NH_4OH gradient
.05
40
80
120
Fraction number

Figure 8.

Fractionation of RNA by chromatography on DEAE-Sephadex.

RNA was extracted by phenol from whole adrenal glands and applied to a column of DEAE-Sephadex. Linear concentration gradients of NaCl (0 to > 1.0M) and NH_4OH (0 to approx. 0.2M) were employed to elute the RNA. 5ml. fractions were collected.

Figure 8


NH4OH gradient started
0.1
E260 mμ
20
40
60
80
100
120
140
Fraction Number

fractionated on a column of DEAE-Sephadex, the DEAE-Sephadex, after NH_4OH had been passed through the column, was hydrolysed in 12N-HCl and the hydrolysate counted. It was found that less than 2% of the amount of radioactivity originally applied to column was present in this hydrolysate. It was thus concluded that the conditions of elution used were sufficient to elute all the RNA from DEAE-Sephadex, and this means that DEAE-Sephadex is superior to Ecteola-cellulose in this respect as appreciable amounts of RNA can be eluted from Ecteola-cellulose only by N-NaOH.

3. Chromatographic Pattern of the subcellular RNA's.

Typical separations of the RNA of each subcellular fraction are given in Figure 9. These separations were achieved during one of the control experiments. Certain significant differences exist in the chromatographic profiles of the RNA's of these subcellular fractions. In each case the RNA's of each subcellular component are separated into three main peaks which were classified as RNA I, RNA II, and RNA III respectively. RNA I and RNA II are eluted by increasing concentration of NaCl passing through the column, whereas RNA III is not eluted by NaCl but is eluted by NH_4OH. Occasionally a small peak was eluted as the sodium chloride concentration was raised to 1.6M NaCl but the amount

Figure 9.

Fractionation of RNA isolated from the adrenal sub-cellular components on DEAE-Sephadex.

The preparation of the DEAE-Sephadex and the isolation of the RNA are described in the text (pp 56,65). The RNA was eluted from the DEAE-Sephadex as in Figure 8. The $E_{260m\mu}$ of the fractions is represented by the continuous line.

3ml. from selected fractions were heated at 100° for 30min. with 3ml. of orcinol reagent (0.6% (w/v) orcinol in 0.02% (w/v) $FeCl_3.6H_2O$ in conc. HCl). The $E_{665m\mu}$ of these solutions (corr. for blank) is represented by the broken lines.

Under these conditions, E_{665} = 1.000 is given by an RNA solution of a concentration approx. = 55µg./ml.

Figure 9

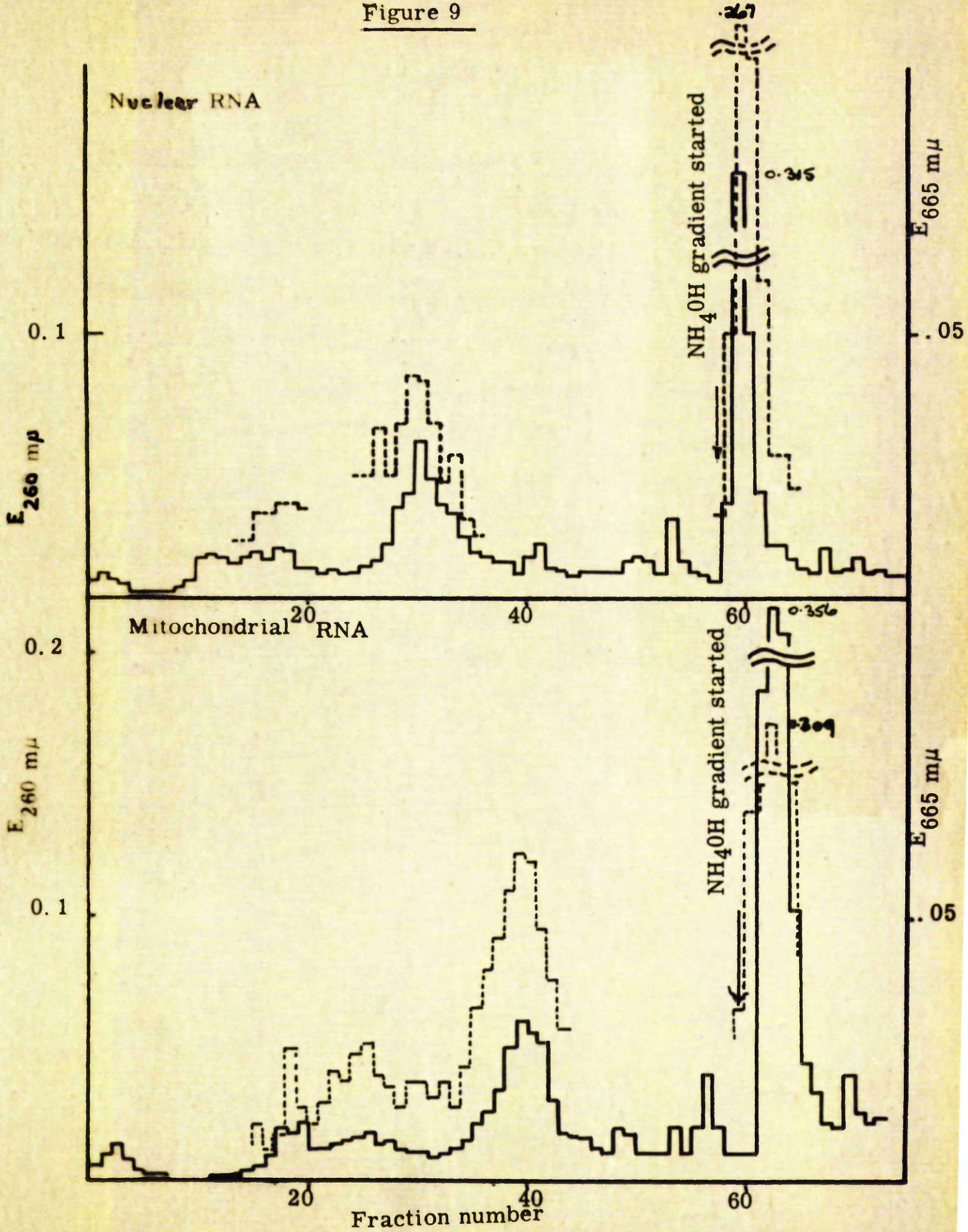

Nuclear RNA
E_{260} mμ
0.1
NH_4OH gradient started
E_{665} mμ
.05
0.345
0.267
20
40
60
Mitochondrial RNA
0.2
0.1
E_{260} mμ
NH_4OH gradient started
E_{665} mμ
.05
0.356
0.209
20
40
60
Fraction number

Figure 9

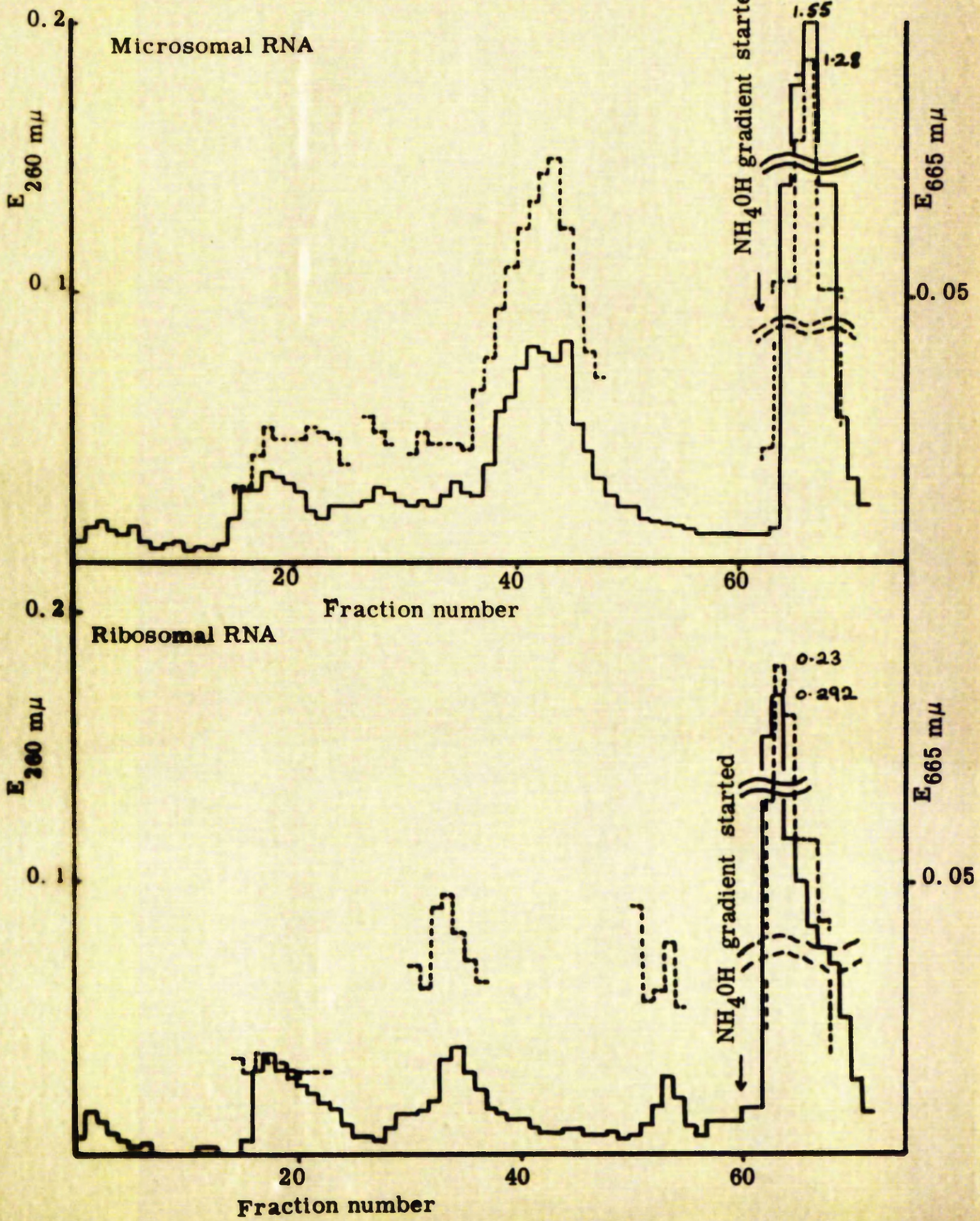

Microsomal RNA
0.2
0.1
E_{260} mμ
E_{665} mμ
0.05
1.55
1.28
NH_4OH gradient started
20
40
60
Fraction number
Ribosomal RNA
0.2
0.1
E_{260} mμ
E_{665} mμ
0.05
0.23
0.292
NH_4OH gradient started
20
40
60
Fraction number

Figure 9

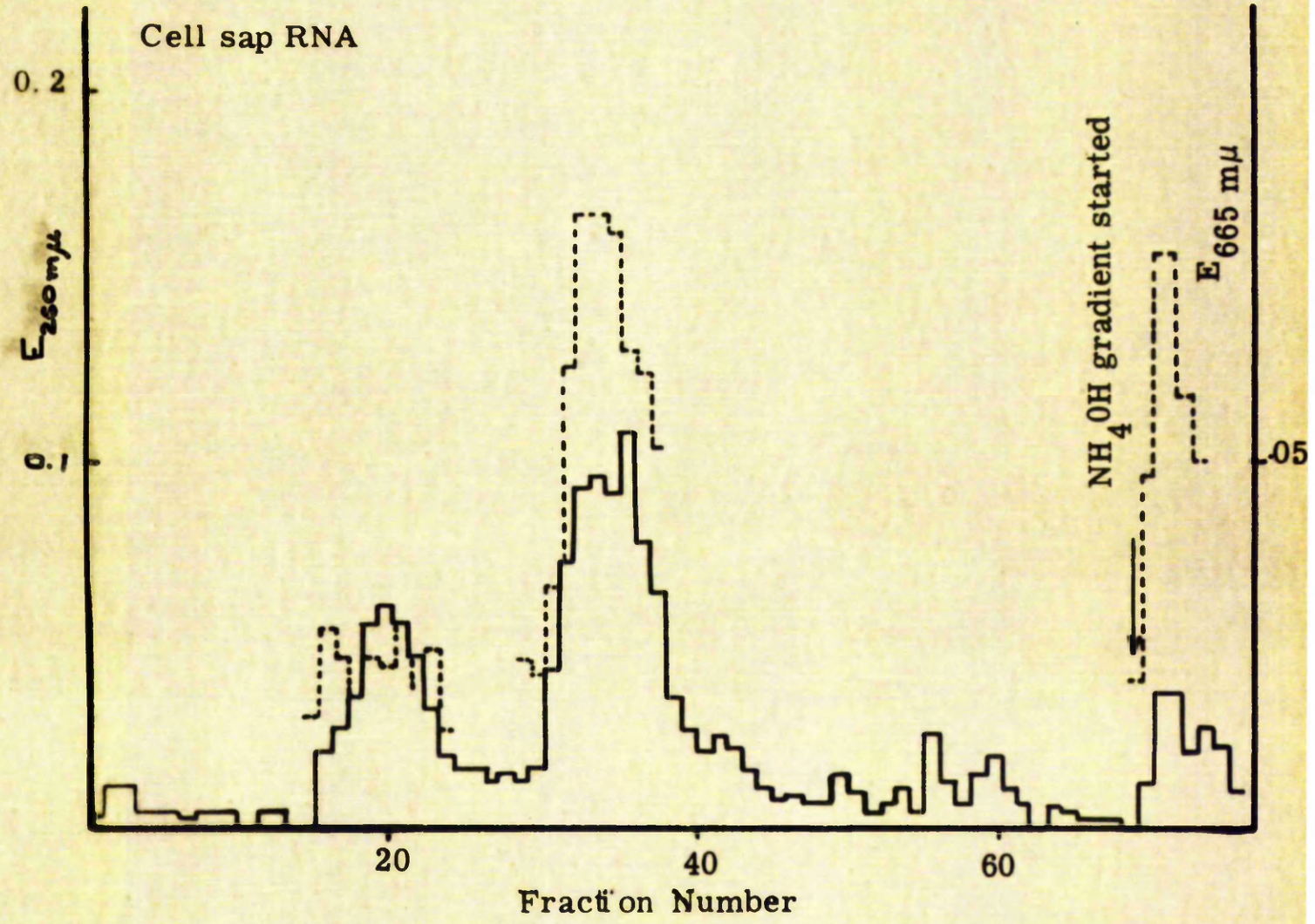

Cell sap RNA
0.2
0.1
$E_{260 m\mu}$
NH_4OH gradient started
$E_{665 m\mu}$
.05
20
40
60
Fraction Number

Table 5.

Relative amounts of RNA in each fraction after separation of individual subcellular RNAs on DEAE-Sephadex.

RNA was isolated and fractionated as described in the text. Ratio I = $\frac{\text{amount of RNA I + amount of RNA II}}{\text{amount of RNA III}}$ and

Ratio II = $\frac{\text{amount of RNA II}}{\text{amount of RNA III}}$

Subcellular Component	Duration of ACTH Treatment (hr.)	Percentage of total RNA recovered in each fraction				
		RNAI	RNAII	RNAIII	RatioI	RatioII
Nuclear	0	56	15	29	2.4	.52
	0	15	30	55	.82	.55
	24	5	16	79	.26	.20
	36	16	27	55	.80	.40
	72	9	26	65	.54	.40
Mitochondria	0	24	33	42	1.86	0.80
	0	40	25	35	1.86	0.71
	0	11	26	63	0.60	0.41
	36	<5	15	80	0.25	0.19
	36	7	29	64	0.44	0.44
	72	5	27	68	0.47	0.40
	72	7	26	67	0.51	0.40

Table 3. (Continued).

Subcellular Component	Duration of ACTH Treatment (hr.)	Percentage of total RNA recovered in each fraction				
		RNAI	RNAII	RNAIII	RatioI	RatioII
Microsomes	0	7	20	73	0.37	0.27
	0	6	8	86	0.16	0.11
	24		11	89		0.12
	36	8	10	83	0.15	0.12
	36	2.5	6.5	91	0.10	0.07
	72	8	6	91	0.10	0.07
	72		10	88	0.14	0.11
Ribosomes	0	14	10	76	0.31	0.13
	0	18	14	68	0.47	0.21
	0	14	17	69	0.45	0.24
	24	4	8	88	0.13	0.08
	36		4	95	0.05	0.04
	36	6	4	90	0.11	0.05
	72	5	14	81	0.23	0.17
	72	4	15	81	0.23	0.18
Cell Sap	0	30	56	14	5.75	3.75
	0	27	50	23	3.5	2.14
	24	31	59	10	9.1	6.0
	36	27	53	20	4.0	2.7
	72	17	40	35	1.85	1.4

was generally small and the specific activity was low. It can be seen that except in the case of cell sap RNA, most of the RNA of the adrenal subcellular components is found in the RNA III fraction.

The amount of orcinol-reacting material in selected fractions was also determined (Figure 9). It can be seen that there is good agreement between the amount of orcinol-reacting material and the $E_{260m\mu}$ of the various fractions, except in the ribosomal RNA I fraction and cell sap RNA III fraction.

The areas under the curves of the elution profiles were calculated for each peak and this was taken to give a semi-quantitative indication of the relative amount of RNA present in each fraction (Table 3). Generally there are greater differences between the elution patterns of the RNA's of the various subcellular components than there are induced by treatment with ACTH. In the nuclei, the amount of RNA II relative to that of RNA III ($\frac{\text{RNA II}}{\text{RNA III}}$ ratio) is 0.55 in control experiments; ACTH administration caused this ratio to fall at all time intervals studied.

The relative content of mitochondrial RNA I is lowered by treatment with ACTH. This effect is apparent at all time intervals studied. Treatment with ACTH causes the relative amount of RNA III to

increase and this is reflected in the $\frac{\text{RNA II}}{\text{RNA III}}$ ratio which falls after ACTH treatment. The fall in $\frac{\text{RNA I + RNA II}}{\text{RNA III}}$ ratio is quite marked on ACTH administration and the greatest decrease is observed after 36hr. of ACTH treatment; it thus seems that ACTH induces an increase in the relative amounts of RNA III or a decrease in the amount of RNA I.

The most striking feature of the microsomal RNA is that there is a very high amount of RNA III relative to the amounts of RNA I and RNA II. This fraction constitutes a high proportion of the total cell RNA. ACTH appears to induce a small increase in the relative amount of this fraction in some of the experiments. The amount of RNA I seems to be lowered and it is difficult to see what changes there are in RNA II, due to the variability in the results.

Administration of ACTH appears to decrease the relative amount of ribosomal RNA I and RNA II, the effect being more marked after the 24 and 36hr. time intervals, this is reflected in the $\frac{\text{RNA I + RNA II}}{\text{RNA III}}$ ratio which is lowered by ACTH treatment. Increases in the relative amounts of RNA III are seen after 4-6hr. ACTH treatment; it is highest after 36hr. and then falls reaching a value after 72hr. which is lower than at 24hr.

The cell sap RNA consists predominantly of the RNA I and RNA II types. This is presumably related to the fact that cell sap RNA is of low molecular weight. The results are so variable that no definite conclusions concerning the effect of ACTH on the distribution of cell sap RNA among these fractions can be drawn.

4. Uptake of ^{32}P by the adrenal RNA fractions.

The effect of administering ACTH for varying lengths of time on the metabolism of RNA fractions obtained by chromatography of RNA of the subcellular components on DEAE-Sephadex is given in Table 4. In some cases the amount of RNA obtained was so small that a reliable determination of the amount could not be made. In such cases, however, an estimate of the maximum possible amount of RNA could be made, knowing the sensitivity limits of ultraviolet determinations, and hence a minimum estimate of the specific activity could be obtained.

The absence of results for the relative specific activity of the nuclear RNA after treatment with ACTH is unfortunate and it is difficult to see what influence ACTH has on the uptake of ^{32}P by these fractions. It does seem, however, that there is a decrease in the specific activity after 72hr. of ACTH administration

Table 4.

Effect of ACTH administration on the uptake of ^{32}P into the RNA of the rat adrenal gland sub-cellular components.

Rats were treated with ACTH for varying periods of time. ^{32}P was administered 2hr. before killing and RNA, isolated from each of the sub-cellular components, was fractionated on columns of DEAE-Sephadex.

Values given represent specific activities of the RNA fractions expressed relative to the blood inorganic phosphate, i.e.

$$\text{Specific activity of RNA} = \frac{\text{cts./min./20 absorbance units at 260m}\mu \times 10^3}{\text{cts./min./100}\mu\text{g. blood inorganic phosphate}}$$

( ) indicates that the value is a minimal one.

* indicates there was no detectable ^{32}P in this fraction.

Table 4.

Origin of RNA	Peak No.	Duration of ACTH treatment (hr.).							
		0	0	0	24	36	36	72	72
Nuclei	I	2.0	1.8	4.8	0.8	-	-	14.3	-
	II	116	32	46	38	48	-	5.7	-
	III	77	22	55	36	36	-	6.7	-
Mitochondria	I	120	25	24	(370)	(100)	(54)	-	-
	II	17.2	10.8	11.6	8.7	7.1	10.8	5.1	5.4
	III	14.8	9.4	11.2	7.1	6.5	6.4	2.1	1.8
Microsomes	I	(46)	-	(97)	(120)	(40)	(36)	(28)	-
	II	23	8.7	9.4	18.5	76	10	5.2	1.8
	III	15.7	10.9	9.9	16.8	5.2	4.9	2.6	3.1
Ribosomes	I	11	(10)	16	(15)	+	6.6	+	+
	II	84	-	25	11.1	11.2	14.4	-	4.2
	III	19.6	10.7	9.7	8.2	7.4	5.8	2.1	3.1
Cell Sap	Ia	(57)	(34)	(27)	(370)	(100)	(200)	(490)	-
	Ib	-	-	-	(12)	(60)	(20)	-	-
	II	54	19.4	27	17.4	13.6	18	24	5.1
	III	47	-	18.1	11.5	-	11	7.8	3.9

in the RNA II and RNA III fractions associated with an increase in the RNA I fraction specific activity.

The microsomal RNA III constitutes the largest proportion of the total cellular RNA, and comparing the specific activity of this fraction with those of the nuclear RNA it can be seen that the nuclear RNA II and RNA III fractions are metabolically more active.

The RNA I of the mitochondria has a high metabolic activity and since the specific activities are minimum estimates no definite conclusion can be drawn with regard to the effect of ACTH treatment. Continued administration of ACTH causes a progressive fall in the specific activities of RNA II and RNA III and after 72hr. the specific activities of RNA II and RNA III are approximately 40% and 20% respectively of their control values. In all experiments, the specific activity of RNA II is greater than that of RNA III and ACTH appears to increase this difference.

The effect of ACTH administration on the metabolism of adrenal microsomal RNA is very similar to that produced on the mitochondrial RNA metabolism and indeed many of the specific activities of the microsomal RNA fractions are of the same order as the corresponding fractions of the mitochondria. This suggests that they are metabolically related. ACTH also causes the specific

activities of the microsomal RNA fraction to fall, this effect being more marked with prolonged administration.

The RNA I of the ribosomes is much less metabolically active than the corresponding fraction of the mitochondria and microsomes. The specific activities of RNA II and RNA III of the ribosomes are slightly higher than those of the corresponding fractions in mitochondria and microsomes in most cases examined. Again ACTH treatment causes a decrease in specific activity, the decrease being quantitatively larger with increased duration of ACTH administration.

The RNA I fraction of the cell sap is heterogeneous and seems to consist of a few metabolically active fractions which only constitute a small proportion of the cell sap RNA. It does seem that ACTH induces an increase in the activity of these fractions; certainly the amount of ^{32}P incorporated into these fractions increases relative to that incorporated into RNA II.

Generally, ACTH administration induces a fall in the specific activity of most of the cell RNA fractions. It may increase the specific activity of the RNA I fraction in the mitochondria, microsomes and cell sap but this cannot be said with certainty.

5. Studies on the RNA in the interphase.

In this experiment, the results of which are given in subsections 3 and 4 above, it was realised that a

significant amount of the total RNA remains in the interphase when a tissue fraction is extracted with phenol. Since reports have been made showing that this "non-extractable" RNA in other tissues has a high metabolic activity (Sibatani, Yamana, Kimura and Okagaki, 1959; Georgiev and Mantleva, 1960; Ramenskaya, Georgiev, Milman, Mantleva and Zbarskii, 1960), it was thought desirable to investigate the nature of the RNA fraction which is found associated with the interphase after phenol extraction of adrenal subcellular components.

The method employed in the early experiments was as follows: after removal of the aqueous phase 3-5 vol. of ethanol:ether (3:1) was added to the phenol phase and interphase (PPI) and allowed to stand overnight at -10°. The precipitate was collected by centrifugation and washed at 0° successively with ethanol:ether (3:1, v/v), 10%(v/v) H_3PO_4, 10%(w/v) trichloroacetic acid (TCA) and the following organic solvents - ethanol:chloroform (3:1, v/v), ethanol:ether (3:1, v/v) and ether. The final dried precipitate was extracted with 10% NaCl at 90° for 30min., and the supernatant, after dialysis, hydrolysed in 0.3N-KOH and separated into the A_2S fraction and the Schmidt-Thannhauser residue by the procedure given in the methods section. The A_2S fraction was found to contain, besides RNA, a high

Table 5.

Relative amounts of nucleic acids in the A_2S fractions and Schmidt-Thannhauser residues of the 10% NaCl extract (RX fraction).

Rat adrenal nuclei were extracted with phenol, the aqueous phase removed and the material in the interphase and phenol phase precipitated with ethanol:ether (3:1, v/v) then washed with 10% H_3PO_4, 10% TCA, followed by a series of lipid solvents. The final residue was extracted with 10% NaCl, dialysed against 1mM-$MgCl_2$, hydrolysed in 0.3N-KOH at 37° for 1hr. and the RNA and DNA separated by acidification and centrifugation. The RNA in the supernatant (the A_2S fraction) was estimated by the orcinol reaction and the DNA in the Schmidt-Thannhauser residue by the method of Ceriotti. The contribution of DNA to the orcinol colour was corrected for.

The figures given in the table below are expressed as percentages of the total RNA in the RX fraction.

Experiment No.	A_2S fraction		Schmidt-Thannhauser residue	
	RNA	DNA	RNA	DNA
1	72	87	28	13
2	91	50	9	50
3	87	7	13	93

content of the total DNA, and the Schmidt-Thannhauser residue contained appreciable amounts of RNA (Table 5). It was realised that the extraction of the interphase derived material with 10% NaCl at 90° may have been carried out at an acid pH, due to traces of acid which had not been extracted by the organic solvents. Since DNA is hydrolysed by acid to acid-soluble products (Schneider, 1946) the possibility of this happening was investigated. 15 pairs of rat adrenals were divided into three groups, homogenised in water and extracted with phenol. The aqueous phase was discarded and the Rx fraction obtained as by method 2.4.5. After dialysis, this fraction was made 0.3N with respect to KOH, incubated for 1hr. at 37°. The alkaline digest was acidified by addition of PCA to give a final concentration of 0.2N. The A_2S fraction was analysed for RNA by measurement of ultraviolet absorption at 260mμ and by the orcinol method. It was found that these estimations yielded values which agreed within 5% of each other. No DNA was detected in the A_2S fraction and it was thus concluded that the acid wash, which was originally carried out as a means of extracting non-RNA phosphorus compounds and especially inorganic phosphorus which would be of high specific activity, was responsible for the breakdown of DNA such that it

was no longer acid-precipitable after 10%-NaCl extraction. The 10% NaCl was also buffered to pH 7 with 0.02M phosphate buffer as a further precaution against DNA being degraded to acid-soluble products.

The A_2S fractions of the RX fraction were assayed for radioactivity and these results are given in Table 6. The A_2S fraction from the RX fraction derived from the nuclei in each experiment is highly radioactive compared to the corresponding A_2S fractions from the other cell constituents. This is demonstrated more clearly when the results in Table 6 are expressed relative to a value of 100 for the radioactivity in the nuclear fraction (Table 7).

Although the nuclear RX, A_2S fractions contain varying amounts of DNA, it is possible to calculate the actual amount of RNA present if the following assumptions are made - firstly that RNA nucleotides do not interfere with the Ceriotti estimation, and secondly that 100µg. DNA reacts with the orcinol reagent to produce a colour equivalent to that produced by 12.5µg. RNA. Thus knowing the amount of RNA and neglecting the radioactivity due to DNA, which is very small, it is possible to calculate the specific activity (Table 8).

When the specific activities of the RNA in the A_2S fraction from the nuclear RX fractionare compared

Table 6.

Radioactivity of A_2S fraction from RX fraction.

Rats were injected with ^{32}P 2hr. before killing and the RX fractions were prepared as in Table 5. The A_2S fractions were assayed for radioactivity. The figures given are expressed as cts./min./total A_2S fraction.

Origin of RX fraction	Experiment No.					
	1	2	3	4	5	6
Nuclei	7,400	4,000	19,000	26,300	22,000	2,400
Mitochondria	63	1,100	2,000	2,000	250	54
Microsomes	304	1,530	2,400	480	270	0
Ribosomes	200	600	260	580	460	0
Cell Sap	140	1,700	1,640	1,270	550	0

Table 7.

Radioactivity of A_2S fraction from RX fraction.

The data given are those of Table 6 expressed relative to the radioactivity of the nuclear A_2S fraction.

Origin of fraction	Experiment No.					
	1	2	3	4	5	6
Nuclei	100	100	100	100	100	100
Mitochondria	0.8	38	11	7.5	1.5	1.4
Microsomes	4.1	37	12.5	1.8	1.2	0
Robosomes	2.6	15	4.6	2.2	2.1	0
Cell Sap	1.9	42.5	8.6	4.8	1.5	0

Table 8.

Specific Activity of RNA in RX fraction from nuclei.

Experimental details are given in the text.

Figures given are expressed as $\frac{\text{cts./min./100}\mu\text{g. RNAP} \times 10^3}{\text{cts./min./100}\mu\text{g. blood inorganic P}}$ so giving the specific activity of the RNA.

Duration of ACTH Treatment (hr.)	Specific Activity
0	350
0	420
36	124
72	105
72	157

with the specific activities of the RNA fractions extracted into the aqueous phase by phenol (Table 4) it can be seen that the nuclear RNA which is not extracted by phenol is very much more active.

6.3. Observations on the effects of ACTH on the metabolism of adrenal RNA fractions isolated by a modified phenol extraction method.

1. General.

The results of the previous experiments which are given in the previous subsection, suggested that the most metabolically active RNA of the adrenal cell was that fraction which becomes associated with interphase material when nuclei are extracted with phenol. A method for the extraction of this RNA suitable for subsequent examination, was developed during the course of the experiments already described.

The nuclear fraction examined in these previous experiments was the fraction centrifuged down at 800g for 10min. from an adrenal homogenate in 0.25M-sucrose containing 3μM-$MgCl_2$/ml. It was thought necessary to purify this nuclear fraction further if the results were to demonstrate conclusively the presence of a nuclear metabolically-active RNA fraction. The reasons for the choice of method used for the purification of nuclei will be given later.

In this series of experiments, rats were treated with ACTH for varying lengths of time and killed 2hr. after administration of ^{32}P. A control group of rats was also killed at the same time as each test group. The number of rats in each group varied between 15 and 18. The weight of 15 pairs of rat adrenals is about 650mg. The adrenals for the control and test groups of rats were separately pooled, fractionated into the subcellular components and the RNA of these components separated into the AqL, AqD and RX fractions as already described.

2. $\frac{RNA}{DNA}$ ratios of nuclei.

The method used for the purification of adrenal nuclei was modified from that of Dingman and Sporn (1962). $\frac{RNA}{DNA}$ ratios of nuclei purified by this modified method are given in Table 9. Due to the fact that different workers have used various methods for the analysis of RNA and DNA, it is difficult to correlate these $\frac{RNA}{DNA}$ ratios (Table 9) with the published values. For this reason, the data of Waddington[s] (personal communication) are included, and it can be seen that the method used yields preparations of liver and adrenal nuclei with $\frac{RNA}{DNA}$ ratios lower than the ratios obtained from nuclei prepared by only differential centrifugation of a 0.25M-sucrose homogenate. This lower ratio can be taken as

Table 2.

RNA
DNA ratios of nuclei of rat liver and adrenal.

Analysis of RNA and DNA were performed using the method in methods section.

'Crude' nuclei refers to the preparation obtained by centrifuging, at 800g for 10min., a tissue homogenate in 0.25M-sucrose containing 1μmole $MgCl_2$/ml.

'Purified' nuclei refers to nuclei obtained by centrifuging the 'crude' nuclei in 2.2M-sucrose (containing 1μmole $MgCl_2$/ml.) at 50,000g for 2hr.

Figures are averages of a number of determinations which are given in brackets.

Preparation	RNA / DNA ratio	Remarks
Whole liver	4.5(4)	
'Crude' liver nuclei	0.40(2)	Own data
'Purified' liver nuclei	0.38(6)	
'Crude' liver nuclei	0.8-1.3	Data of Waddington (Private communication)
'Purified' liver nuclei	0.33-0.39(22)	
Whole adrenal	1.4(8)	
'Crude' adrenal nuclei	0.4-0.6	Own data
'Purified' adrenal nuclei	0.38(6)	

indicative of purification. Adrenal nuclei and liver nuclei have approximately equivalent $\frac{RNA}{DNA}$ ratios. Since the $\frac{RNA}{DNA}$ ratios for whole liver and whole adrenal are 4.5 and 1.4 respectively, this means that whilst approximately 6-7% of liver RNA is found in the nuclei about 20% of the adrenal RNA is found in the nuclei.

5. Absorption Spectra of the various fractions.

(a) Absorption Spectra of the AqL fractions.

The ultraviolet absorption of the RNA of the AqL fractions after dialysis against 1mM-$MgCl_2$ were recorded using a Beckman DB spectrophotometer coupled to a potentiometric recorder. Spectral characteristics of these fractions are given in Table 10. All RNA fractions have a $\lambda_{max.}$ at 258 ± 1mμ and $\lambda_{min.}$ at 231 ± 1mμ. The $\frac{E_{max}}{E_{min}}$ ratios of the RNA in the AqL fractions derived from control adrenal lies between 1.58 and 1.92 and the RNA from the ACTH stimulated adrenals have $\frac{E_{max}}{E_{min}}$ ratios varying between 1.1 and 2.06, although most of the RNA fractions have this ratio greater than 1.6. The $\frac{E_{max}}{E_{280}}$ ratio is higher than the $\frac{E_{max}}{E_{min}}$ ratio in all fractions examined. These spectral chardeteristics confirmed that the AqL fractions contained RNA. In general no marked differences are noted between the RNA of the AqL fractions from control adrenals and test adrenals.

TABLE 10

Characteristics of ultraviolet absorption spectra of AcL fractions of rat adrenals after ACTH treatment.

Experimental details are given in the text. Control values are the average of 4 experiments. Figures for the test experiments were each obtained from a single experiment.

Origin of fraction.	Duration of ACTH Treatment (hr.)															
	None				18				24				36			
	max λ	min λ	Emax/Emin	Emax/E280	max λ	min λ	Emax/Emin	Emax/E280	max λ	min λ	Emax/Emin	Emax/E280	max λ	min λ	Emax/Emin	Emax/E280
Nuclear	258	230	1.53	1.68	255	232	1.1	1.7	258	231	1.2	1.70	258	233	1.60	1.78
Mitochondria	258	230	1.86	1.75	256	230	1.2	1.7	258	230	1.94	2.06	258	232	2.02	2.00
Microsomes	258	231	1.92	2.03	258	230	1.8	1.8	258	231	2.06	2.17	257	231	2.06	2.02
Ribosomes	257	231	1.88	1.91	258	230	1.6	1.7	256	230	1.78	2.15	258	230	2.06	1.94
Cell Sap	259	230	1.70	1.94	256	230	1.4	1.7	258	230	1.85	2.07	259	230	1.98	1.98

TABLE 11.

Characteristics of ultraviolet absorption spectra of AcD fractions of rat adrenals after ACTH treatment.

Experimental details are given in the text. Control value is average of 4 determinations. Figures for the test experiments were each obtained from a single experiment.

Origin of fraction.	Duration of ACTH Treatment (hr.)															
	None				16				24				36			
	λ max	λ min	Emax/Emin	Emax/E280	λ max	λ min	Emax/Emin	Emax/E280	λ max	λ min	Emax/Emin	Emax/E280	λ max	λ min	Emax/Emin	Emax/E280
Nuclear	254	234	1.26	1.38	250	233	1.10	1.80	260	237	1.48	1.72	257	235	1.20	1.45
Mitochondria	253	233	1.42	1.80	254	232	1.13	1.62	258	234	1.68	1.95	257	234	1.32	1.59
Microsomes	257	231	1.59	1.80	254	230	1.27	1.69	258	234	1.64	2.14	258	232	1.70	1.81
Ribosomes	257	234	1.28	1.47	245	229	1.08	1.77	258	236	1.43	2.12	258	234	1.63	1.75
Cell Sap	254	232	1.54	1.99	245	230	1.02	1.80	260	238	1.35	1.69	-	-	-	-

(b) Absorption Spectra of the RNA in the AqD fractions.

The absorption spectra were determined as above and the spectral characteristics are given in Table 11. It can be seen that the RNA in the AqD fraction has a different spectrum from that of the RNA in the AqL fraction. The most obvious difference is that the λ_{min} of the AqD RNA is higher than that of the AqL RNA and this is observed in most cases. The λ_{max} of some of the RNA AqD fractions is 254mμ and is 258mμ-260mμ for others. $\frac{E_{max}}{E_{min}}$ and $\frac{E_{max}}{E_{280}}$ ratios of the RNA in the AqD fractions are lower than the corresponding ratios of the RNA in the AqL fractions. All $\frac{E_{max}}{E_{min}}$ ratios are less than the $\frac{E_{max}}{E_{280}}$ ratios.

(c) Absorption Spectra of the RNA of the RX fractions.

Typical ultraviolet absorption spectra of the RX fractions are given in Figure 10. It can be seen that these are slightly atypical of RNA since the $\frac{E_{260}}{E_{280}}$ ratios are low. This suggests that the RX fractions contain RNA and protein.

Therefore, the RX fractions were shaken up with an equal vol. of 90% phenol, the aqueous phase removed and extracted free of residual phenol with ether. Figure 10 shows the spectra of the resulting aqueous phase, which were determined after dialysis. These spectra demonstrate that an RNA can be released into the aqueous phase on further extraction, with phenol, of a dialysed

Figure 30.

Typical absorption spectra of the RI fractions.

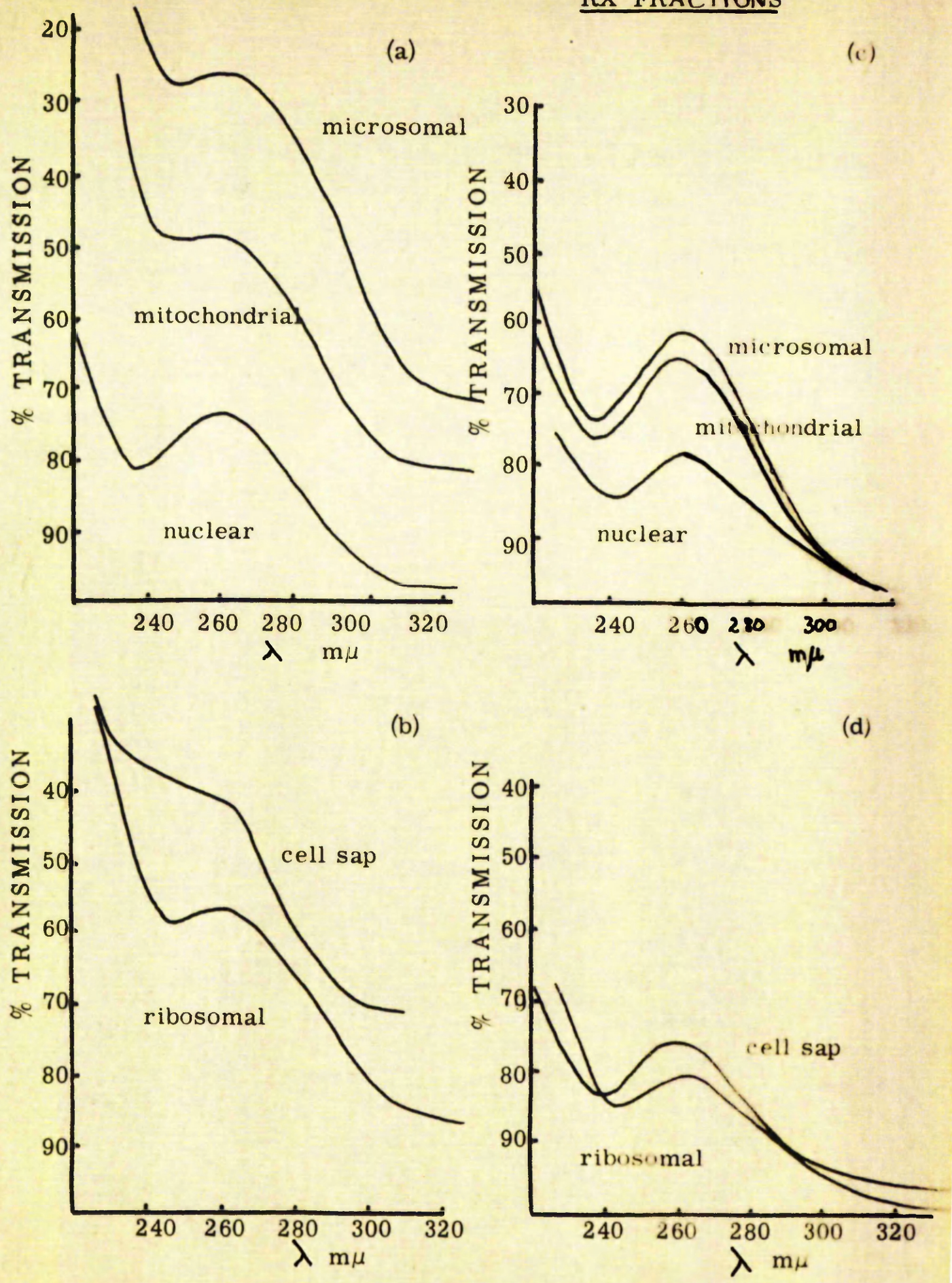
(a)and(b) Ultraviolet absorption spectra of the RI fractions from the adrenal sub-cellular components.

(c)and(d) Ultraviolet absorption spectra of the dialysed aqueous layers from phenol-extracted RI fractions (p 98).

The spectra are reproduced from those recorded by means of millivolt chart-recorder coupled to a Beckman DB spectrophotometer.

Figure 10

SPECTRA OF RX FRACTIONS AND OF PHENOL-EXTRACTED RX FRACTIONS



10% NaCl extract of the interphase material. Presumably, 10% NaCl disrupts an insoluble complex of RNA with protein or DNA. The 10% NaCl may also release small peptides from the protein of the interphase material. This would explain why the spectra of the RX fraction suggest presence of protein and why further extraction of the RX fractions with phenol appears to remove this proteinaceous impurity.

4. Amounts of RNA in the various fractions.

Homogenisation of tissue in 0.25M-sucrose solution with a Potter type of homogeniser is not usually considered capable of disrupting the cells to give a quantitative yield of the subcellular components. Inevitably, the homogenate will contain, besides the "intact" subcellular components, disrupted nuclei and mitochondria, whole cells and cytoplasmic debris. Nevertheless, the bulk of these contaminants will be centrifuged from the homogenate along with the nuclear fraction. The supernatant, after this initial centrifugation, can thus be separated, by differential centrifugation, into subcellular components such that analysis of these subcellular components will allow comparisons to be made of the relative amount of a substance in each components.

Table 12 shows the amount of RNA found in the subcellular components, calculated relative to the amount

TABLE 12.

Distribution of RNA in the subcellular components of the adrenal.

The procedures for isolating the fractions are as given in the text. The figures given below represent the amount of RNA, based on ultraviolet measurements, in the isolated fractions relative to a value of 100 for the RNA in the AqL of the microsomal fraction. The figures given at 0 hr. of ACTH treatment are the averages of 3 values and those for other durations of treatment are the values obtained from a single experiment.

Duration of ACTH Treatment (hr.)	Subcellular component														
	Nuclei			Mitochondria			Microsomes			Ribosomes			Cell Sap		
	AqL	AqD	RX	AqL	AqD	RX	AqL	AqD	RX	AqL	AqD	RX	AqL	AqD	RX
0	10.8	8.5	2.3	31.6	7.0	1.5	100	5.5	4.4	30	6.1	2.3	28	7	1.6
18	9.4	8.7	-	-	13.7	-	100	13.0	-	40	7.8	-	42.7	17.8	-
24	2.2	2.6	0.9	32.4	11.0	0.85	100	5.3	0.7	73	2.6	0.59	36	4.9	-
36	3.1	1.7	2.2	50	4.0	2.0	100	5.8	3.4	70	4.1	2.7	37	1.1	2.6

in the microsomal AqL fraction = 100. The microsomal AqL fraction contains the highest proportion of the RNA of the adrenal cell.

(a) Amount of RNA in AqL fraction.

This fraction contains most of the total cellular RNA of the adrenal and, in the previous experiments, it was this fraction which was chromatographed on DEAE-Sephadex. It thus is known to be heterogeneous with respect to metabolic activity and physico-chemical properties.

There is approximately three times as much RNA in the microsomes of the rat adrenal as there is in the mitochondria. This proportion is not altered by treating rats with ACTH.

The amount of ribosomal RNA relative to that of the microsomal RNA increases from 30% to 70% on prolonged (36hr.) ACTH administration. Similarly the amount of cell sap RNA increases but the magnitude of the increase is not so great. The largest increase is noted after 18hr. administration of ACTH.

(b) AqD fraction.

For all the cell components except the nuclei, the amount of RNA in the AqD fraction is relatively much less than that found in the AqL fraction. After ACTH treatment for 18hr. the amount of RNA in the AqD fraction of the mitochondria, microsomes, ribosomes and

cell sap relative to that in microsomal AqL fraction appears to increase. However, after 24hr. and 36hr. the relative amounts of RNA in the AqD fractions are not changed in the microsomal fraction and are lowered in the ribosomal and cell sap fractions. The relative amount of AqD mitochondrial RNA is increased after 24hr. and decreased after 36hr.

The nuclear AqD fraction contains a large proportion of the total RNA of the nucleus.

(c) RX fraction.

It can be seen from Table 12 that the RX fraction contains only a small proportion of the total cellular RNA.

5. Uptake of ^{32}P into adrenal RNA fractions.

(a) Uptake in RNA of AqL fraction.

The specific activities of the RNA of the AqL fractions are given in Table 13. In the control animals the RNA of the highest specific activity was that of the cell sap. This is presumably due to end-terminal addition of AMP and CMP residues to the sRNA molecules. The specific activity of the other AqL RNA fractions are very similar. ACTH administration results in a fall in all the RNA fractions at all the time intervals studied, the only exception being the nuclear RNA fraction after 36hr. of ACTH treatment.

Effect of ACTH on the uptake of ^{32}P into the RNA of the AqL fractions.

The isolation procedures of the AqL fractions are given in the text. The radioactivity was assayed on the AqL fraction after passing it through a G25 Sephadex column. Values given are relative to the specific activity of blood inorganic phosphate, i.e.,

$$\text{Specific activity} = \frac{\text{cts./min./2ml}_{260\text{m}\mu}^{1\text{cm.}} \text{ units} \times 10^3}{\text{cts./min./100ug. plasma inorganic phosphate.}}$$

Duration of ACTH treatment (hr.)		Sub-cellular components from which AqL fraction was isolated.				
		Nuclei	Mitochondria	Microsomes	Ribosomes	Cell Sap
0		11.2	15.6	11.4	15.6	36.3
12		8.9	8.9	6.7	6.8	24.5
	% change	-27	-43	-41	-40	-33
0		-	15.8	-	14.1	27.6
24		-	12.0	-	8.0	14.0
	% change	-	-24	-	-43	-48
0		-	-	12.7	19.8	26.5
24		-	-	10.6	11.0	8.6
	% change	-	-	-16	-44	-68
0		18	10.7	6.7	9.5	66
36		20.6	5.0	3.2	3.1	16
	% change	+13	-52	-52	-67	-76

which shows a small, and probably insignificant, increase.

The values obtained are in broad agreement with those of the RNA fractions separated by column chromatography on DEAE-Sephadex (Table 4).

(b) Uptake of ^{32}P into the RNA of AqD fractions.

The AqD fractions, after dialysis, were hydrolysed in 0.5N-KOH for 1hr. at 37°, acidified to 0.2N with respect to PCA and any residue formed on acidification removed by centrifugation. The supernatant was diluted one in one, the E_{260} read and assayed for radioactivity. The results are given in Table 14. Administration of ACTH lowers the incorporation of ^{32}P into the RNA in the AqD fraction of all cell fractions at the time intervals examined, except for that of the cell sap 36hr. after treatment with ACTH. The decreases observed range from 4% to 65%.

Certain differences appear where the specific activities of the RNA of the AqD fraction are compared to the specific activities of the RNA of the AqL fraction. The mitochondrial AqD RNA has a higher specific activity than the AqL RNA and this is also observed after ACTH treatment. Conversely the cell sap RNA in the AqD fraction has a lower specific activity than that of cell sap AqL RNA. In the other cell fraction the differences are less apparent. Such

TABLE 14.

Effect of ACTH on the uptake of ^{32}P into the RNA of the AqD fractions.

The isolation procedures of the AqD fractions are given in the text. Radioactivity assays were carried out on the A_2S fractions of the dialysates. The figures given are specific activities relative to blood inorganic phosphate, i.e.,

$$\text{Specific activity of AqD RNA} = \frac{\text{cts./min.} \times 4.75 \times E^{1\text{cm.}}_{260\text{m}\mu} \text{ units of } A_2S \text{ fraction} \times 10^3}{\text{cts./min./100ug. blood inorganic phosphate.}}$$

Duration of ACTH treatment (hr.)		Sub-cellular component from which AqD fraction was isolated.				
		Nuclei	Mitochondria	Microsomes	Ribosomes	Cell Sap
0		22.8	20.6	10.9	6.6	11.9
18		11.7	19.9	8.9	5.5.	11.4
	% change	-49	-4	-18	-15	-4
0		18.0	32.6	10.1	10.3	26.3
24		9.0	18.6	4.5	9.8	17.5
	% change	-50	-43	-55	-5	-32
0		7.7	19.7	6.9	6.5	6.3
36		2.7	8.7	2.9	2.9	6.4
	% change	-65	-56	-58	-55	+2

(c) Uptake into the RX fraction.

The RX fractions were purified by phenol extraction, as already described. The purified RX fraction was hydrolysed in 0.5N-KOH for 1hr. at 57° and the digest separated into its A_2S fraction and Schmidt-Thann-hauser residue. The absorbance of the A_2S fraction was read at 260mμ and assayed for radioactivity. (Table 15). It can be seen that the nuclear RX fraction contains one of the most metabolically active species of RNA in the cell and these values confirm those already found in the previous experiment (Table 8). It must be remembered, however, that the figures in Table 15 were obtained using pure nuclei.

The specific activity of the ribosomal RNA in the RX fraction is also higher than that of the AqL and AqD fractions. There is little difference between the specific activities of the mitochondrial and microsomal RX RNA and the specific activities of the mitochondrial and microsomal AqD RNA. Again it is found that ACTH, in general, causes a decrease in the uptake of ^{32}P into the RNA fractions isolated.

6. Relationship between specific activity of inorganic phosphate of the adrenal gland and the specific activity of the blood inorganic phosphate.

All specific activities given have been calculated

TABLE 15.

Effect of ACTH on the uptake of ^{32}P into the RNA of the RX fractions.

The method of isolation of the RX fractions is given in the text. The RX fractions were extracted with phenol, the aqueous phase freed of phenol, dialysed, subjected to a Schmidt-Thannhauser type of separation and the radioactivity of the A_2S fraction assayed. The values were calculated as for Table 14.

Duration of ACTH treatment (hr.)		Sub-cellular component from which RX fraction was isolated.				
		Nuclei	Mitochondria	Microsomes	Ribosomes	Cell Sap
0		-	34	26.7	24.6	-
18		-	25	17.1	17.9	-
	% change	-	-26	-36	-27	-
0		165	-	-	38	-
24		160	-	-	25	-
	% change	-3	-	-	-34	-
0		254	33	36	33	-
24		110	16	13	10	-
	% change	-57	-52	-64	-70	-
0		154	15.0	6.7	7.4	8.7
36		127	6.3	4.4	4.1	7.8
	% change	-18	-58	-34	-45	-10

relative to the specific activity of the blood inorganic phosphate. It could, therefore, be argued that the changes in specific activity, after ACTH administration, may have been due to changes in rate of transfer of inorganic phosphate from blood into the cell. To test this possibility, the specific activity of the inorganic phosphate of the cell sap was determined (Table 16).

The specific activities of the inorganic phosphate of the blood and of the adrenal cell sap are lower in the ACTH treated animals. However, the ratio of these two specific activities remains constant. Thus, calculating the RNA specific activities relative to the specific activity of the adrenal cell sap inorganic phosphate would have given values quantitatively similar to those obtained by expressing them relative to the blood inorganic phosphate specific activity.

3.4. Studies on the Breakdown of RNA in the Rat Adrenal.

1. General.

The results of the previous experiments suggested that ACTH treatment caused a decrease in the breakdown of adrenal RNA. If this is so, it should be possible to detect changes in the mechanisms involved in the breakdown of RNA.

Table 16.

The specific activities of blood inorganic phosphate and adrenal cell sap inorganic phosphate.

The specific activities are given in cts./min./100µg. inorganic phosphate x 10^{-3}.

	Specific Activities		Ratio of the specific activities
	Plasma	Adrenal Cell Sap	
Control	3,800	3,210	1.22
ACTH 36hr.	3,069	2,535	1.20
Control	211	174	1.12
ACTH 18hr.	186	164	1.13

2. Choice of RNase assay.

There is a wide diversity of assay methods available for RNase (see Anfinsen and White, 1963). The method used by Shortman (1961) offers an assay for RNase which is very sensitive and can be performed, simultaneously, on a large number of tissue homogenate samples. Roth and Milstein (1952) used ^{32}P labelled RNA as a substrate. They measured the amount of radioactivity in the filtrate after acid precipitation of the reaction mixture and this amount was taken as being proportional to activity. This method possesses the advantage that it is twenty five times as sensitive as the method of Shortman (1961) but, on the other hand, is more time consuming and requires the preparation of labelled RNA.

In this series of experiments, the method of Shortman (1961), modified in two aspects, was employed. Tris was used as the buffer for the reaction mixture and the precipitating agent used at the end of the reaction was a mixture of 1vol. 0.75% uranyl acetate in 2.5N-PCA to 10vol. of 76%(v/v) ethanol in N-HCl. Shortman (1961) used veronal buffer but since this buffer has an appreciable absorption at 260mμ, tris was thought to be a better choice.

Roth and Milstein (1952) and Shortman (1961)

used, as a precipitating agent, 76% ethanol in N-HCl but it was found that when this reagent was used as a precipitating agent the $E_{260m\mu}$ of the tissue blanks was high. 0.75% uranyl acetate in 25% PCA was used by Anfinsen, Redfield, Choate, Page and Carroll (1954) but this agent unfortunately absorbs strongly at 260mμ ($E \simeq 6.85$).

To test the suitability of precipitating agents 76% ethanol in N-HCl was mixed in varying proportions with 0.75% uranyl acetate in 2.5N-PCA. 1ml. of 1% yeast RNA was added to 10ml. of the precipitating agent and the $E_{260m\mu}$ determined of a one in five dilution of the supernatant obtained by centrifugation.

The precipitating agent containing 0.075% uranyl acetate appears to precipitate the highest percentage of the yeast RNA (Table 17). Consequently, a mixture of 76% ethanol in N-HCl and 0.75% uranyl acetate in 2.5N-PCA in the ratio 10:1 was used for assay. In practice this proved to give tissue blanks with $E_{260m\mu}$ approximately 0.040-0.060, whereas tissue blanks with $E_{260m\mu}$ approximately 0.080-0.100 were obtained when 76% ethanol in N-HCl was used as the precipitating agent. It was also found that better agreement between replicates was obtained using the composite precipitating agent.

Table 17.

Absorbance of supernatant after addition of precipitating agent to a yeast RNA solution.

0.75% uranyl acetate in 2.5N-PCA and 76% ethanol in N-HCl were mixed together in various proportions and 1ml. of 1% yeast RNA solution added to 10ml. of each mixture. The $E_{260m\mu}$ of the supernatant, diluted 1 in 5 was determined.

Percentage concentration of uranyl acetate added to yeast RNA	$E_{260m\mu}$ of supernatant
0	0.450
0.0075	0.350
0.0375	0.220
0.075	0.130
0.375	0.325
0.75	1.1

3. The levels of Alkaline RNase in adrenal.

(a) "Free" RNase.

The levels of "free" alkaline RNase in rat adrenal was assayed and the results expressed in units of activity as described in the methods section. Amounts of "free" RNase were found to be very low (Table 18) and the levels could just be detected. The "free" RNase activity in a pair of adrenals was thus equivalent to less than 0.0025μg. of bovine pancreatic RNase.

(b) "Latent" RNase.

The RNase, whose activity became apparent on the addition of pCMB to a tissue homogenate, is referred to as "latent" RNase.

Rats were treated with ACTH for times varying between 6hr. and 24hr. The adrenals were removed and the activity of the "latent" RNase assayed. The activities of "latent" RNase was also determined in the adrenals of a control group of rats for each time interval of ACTH examined.

It can be seen that ACTH produces no statistically significant difference in the total amount of this enzyme although it appears that there is a small increase initially, at 6hr. and 12hr. after ACTH administration, followed by a decrease after 18hr. and 24hr. of ACTH treatment (Table 19).

Table 18.

The effect of ACTH treatment on 'free' RNase in the adrenal. Assay mixtures contained 12μmoles tris buffer, pH 7.8, 90μmoles NaCl, 2mg. purified yeast RNA and about 6mg. adrenal tissue in a total volume of 0-6 ml. Incubations were carried out at 37° for 20min. The reactions were stopped, precipitating reagent added and the supernatant diluted 1 in 10 and the $E_{260m\mu}$ determined. The definition of a unit of free RNase is given in the text. The numbers in brackets indicate the number of observations.

Duration of ACTH treatment (hr.)	Units RNase/100mg. tissue
0	no RNase detected (3)
0	0.67 (2)
24	0.75 (2)
48	no RNase detected (2)
72	no RNase detected (2)

Table 10.

The effect of ACTH administration on the level of latent RNase in the rat adrenal.

Rats were treated with ACTH and the levels of latent RNase in the adrenal assayed.

Assay mixtures contained 12/moles tris buffer, pH 7.8, 20/moles NaCl, 1.0 μmoles EDTA and about 5.5-4.5 mg. adrenal tissue and 2mg. purified yeast RNA in a total volume of 0.6ml. For comparison purposes the activity of bovine pancreatic RNase was assayed simultaneously, and the assay mixtures contained 12/moles tris buffer, pH 7.3, 20/moles NaCl, 0.002 μg. bovine pancreatic RNase and 2mg. purified yeast RNA. Incubation was carried out at 37° for 20min. The definition of a unit of latent RNase is given in the text. Values are given as the mean ± standard error of the mean (S.E.M.). The numbers of determinations are given in brackets.

Table 19

The effect of ACTH administration on the levels of "latent" RNase in the rat adrenal

* indicates $P<0.05$

Experiment No.	Duration of ACTH treatment in test animal (hr.)	Units of "latent" RNase in adrenal /100g. body wt.			/100mg. adrenal		
		Control	Test	Difference	Control	Test	Difference
1	6	6.8±1.8(4)	7.7±1.3(4)	+0.9	20.4±4.3	24.0±4.0	+3.6
2	12	4.8±1.2(4)	5.7±1.6(4)	+0.9	16.9±4.1	16.9±6.6	0
3	18	5.5±2.9(4)	2.9±0.5(4)	-2.6	18.1±3.5	7.1±1.3	+11.0*
4	24	8.3±2.2(4)	6.8±0.7(4)	-1.5	23.2±6.4	15.7±1.8	+7.5

Table 20.

The figures below are taken from Table 19 and expressed relative to a control value of 100.

Experiment No.	Duration of ACTH Treatment (hrs.)	Latent RNase	
		Units/100g. body wt.	Units/100mg. adrenal
1	6	113	118
2	12	113	100
3	18	52	59
4	24	61	67

The concentration of "latent" RNase in the adrenal is significantly decreased after 10hr. of ACTH administration ($P < 0.01$), but the differences at other time intervals studied are not statistically significant.

Expressing the results relative to control values (=100) shows the percentage changes in adrenal content and concentration of "latent" RNase as induced by ACTH treatment (Table 20).

4. RNase inhibitor.

(a) Effect of ACTH administration.

The amounts of RNase inhibitor in rat adrenals after treatment with ACTH for varying time intervals have also been determined.

The assay procedure was similar to that of Shortman (1961) and the calculation of the results was carried out using the relationship which he found between inhibitor concentration and activity. This is valid only if the relationship between adrenal RNase inhibitor concentration and activity is similar. To examine if this were the case, four pairs of adrenals (180mg.) were homogenised in 0.25 M-sucrose and centrifuged at 18,000g for 2hr. The supernatant was diluted with 5mM-EDTA, pH 7.0 to give dilutions varying between 1:10 and 1:50. The activity of bovine pancreatic RNase was assayed in presence of these

diluted samples and the percentage of RNase activity relative to the control values determined. From Figure 11, it can be seen that the relationship between amounts of the adrenal RNase inhibitor and percentage inhibition is similar, at the concentrations examined to the corresponding relationship found with rat liver RNase inhibitor. It was concluded that it was justifiable to use Shortman's curve in the calculation of results.

The content of RNase inhibitor is significantly increased in the adrenal 12hr. ($P < 0.05$), 18hr. ($P < 0.01$) and 24hr. ($P < 0.05$) after ACTH treatment (Table 21). Table 22 shows that ACTH treatment increases the content of RNase inhibitor by 86% after 12hr., about 100% after 18hr. and 66% after 24hr. The increase of 83% after 6hr. is not statistically significant but it does seem that even 6hr. after ACTH treatment there may be an increase in the content of RNase inhibitor.

There is a large increase in the concentration of RNase inhibitor in the adrenal 18hr. after injecting rats with ACTH. This increase is significant at the 1% level in one experiment, at the 5% level in another and just fails to reach significance, at the 5% level in experiment No. 4. Although increases were observed

Figure 11.

The activity of bovine pancreatic RNase in presence of various amounts of either rat-liver or rat-adrenal supernatant.

A 0.25M-sucrose homogenate of rat adrenal tissue was centrifuged at 18,000g for 2hr. and the supernatant diluted with 5.0mM-EDTA, pH 7.8

The activity of bovine pancreatic RNase in presence of various amounts of the diluted supernatant was determined.

Assay mixtures contained 12μmoles tris buffer, pH 7.8, 90μmoles NaCl, between 0.45 and 0.5μmoles EDTA, 0.005μg. bovine pancreatic RNase, 2mg. of purified yeast RNA and a portion of the adrenal supernatant in a vol. of 0.6ml.

The inhibitor activity of rat-liver supernatant is from Shortman (1961).

Figure 11

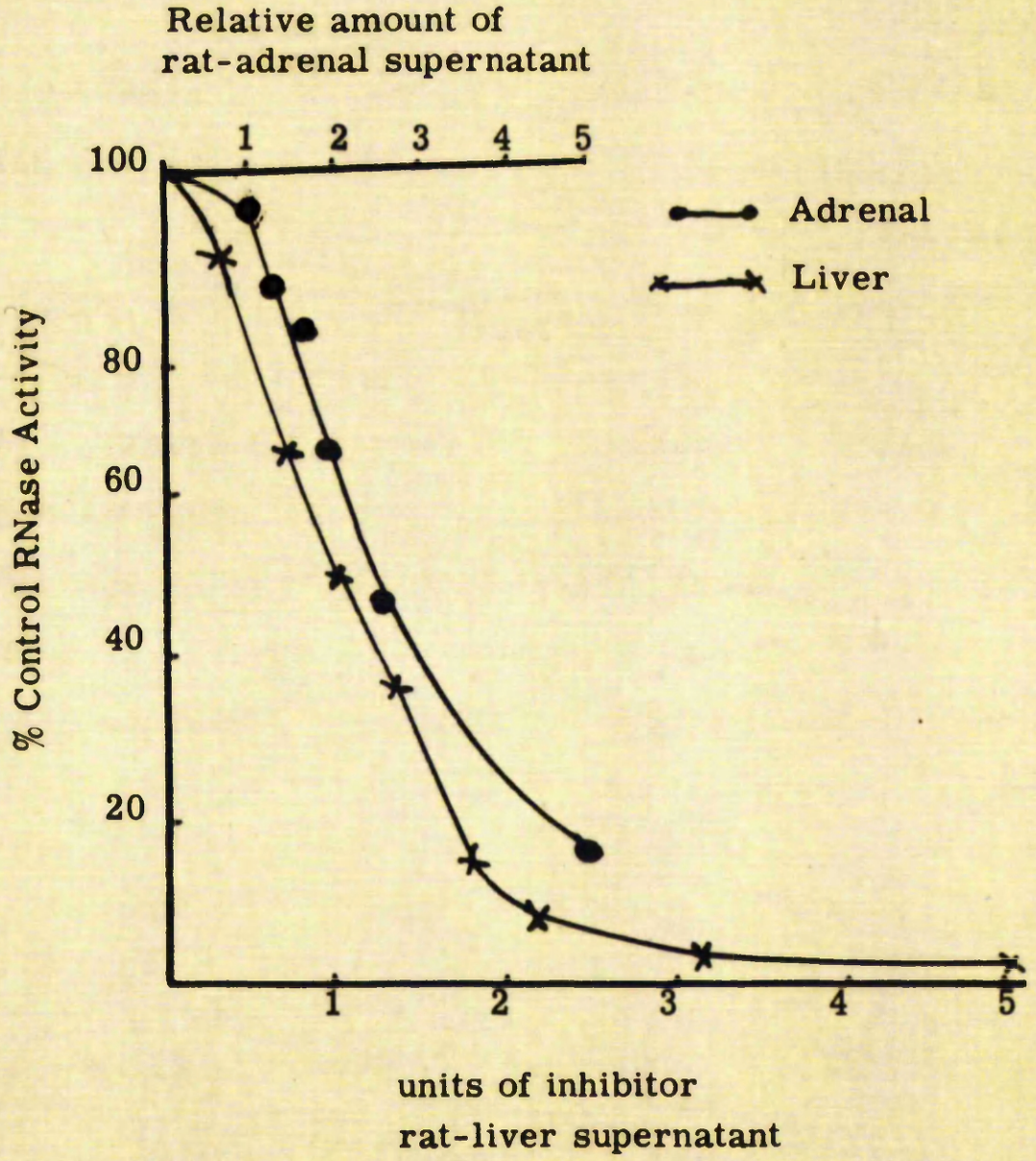

Relative amount of
rat-adrenal supernatant
1
2
3
4
5
100
80
60
40
20
% Control RNase Activity
Adrenal
Liver
1
2
3
4
5
units of inhibitor
rat-liver supernatant

Table 21.

The Effect of ACTH treatment on adrenal RNase inhibitor.

Rats were treated with ACTH for varying durations of time and the activity of RNase inhibitor in the adrenals determined.

Assay mixtures contained 12μmoles tris buffer, pH 7.6, 90μmoles NaCl, 0.45μmoles EDTA, between 0.2 and 0.4mg. of adrenal tissue, 0.005μg. bovine pancreatic RNase and 2mg. purified yeast RNA, in a total volume of 0.6ml. The percentage inhibition of the activity of the pancreatic RNase was related to the number of units of inhibitor using Figure 6.

The values given are the means of 4 determinations ± standard error of the mean (S.E.M.). Student's t-test was applied to the individual figures to determine whether the means are significantly different.

* indicates $P < 0.05$

** indicates $P < 0.01$

TABLE 21.

The effect of ACTH treatment on adrenal RNase Inhibitor.

Experiment No.	Duration of ACTH treatment in test animal (hr.)	Units of RNase Inhibitor. /100g. body wt.			/100mg. adrenal		
		Control	Test	Difference	Control	Test	Difference
1	6	79.3±18.3	93.8±13.7	14.5	236±42.0	296±48.0	60.0
2	12	109.3±4.3	149.3±17.6	39.5*	396±16.4	428±44.8	32.0
3	18	70.0±5.0	166.8±5.2	96.8**	229±53.5	445±26.4	216.0*
4	18	99.0±10.0	181.0±27.0	82.0**	373±45.6	583±58.0	210.0
5	18	51.0±2.4	110.0±2.4	59.0**	172±9.1	332±6.5	160.0**
6	24	66.0±6.4	110.0±20.4	44.0*	179±34.4	257±48.4	78.0

Table 22.

The Effect of ACTH treatment on adrenal RNase inhibitor.

The results below are those of Table 21 expressed relative to values of 100 for the control animals.

Experiment No.	Duration of ACTH treatment (hr.)	Units of RNase Inhibitor	
		/100g. body wt.	/100mg. adrenal
1	6	183	125
2	12	156	108
3	18	288	194
4	18	185	156
5	18	216	198
6	24	166	144

at the other time intervals, these were not significant.

(b) Properties of RNase Inhibitor.

The location of the inhibitor in the adrenal cell was determined. Eight rats were divided into two groups of four and the rats of one group were each given 5i.u. ACTH. The rats were killed 18hr. after injection. The adrenals from each group were pooled, and homogenised in 0.44M-sucrose. After centrifuging the homogenate at 31,000g for 1hr., the supernatants and pellets were assayed for RNase inhibitor (Table 23). It was found that the inhibitor is mainly in the supernatant and the total amount is increased by ACTH. There is only a small proportion of the total content of RNase inhibitor in the cell material centrifuged down at 31,000g for 1hr., but the amount increases after ACTH.

The effect of temperature on the activity of the inhibitor was examined by the following experiment. An adrenal cell sap preparation (supernatant of an homogenate in 0.44M-sucrose spun at 31,000g for 1hr.) was diluted with 5mM-EDTA, pH 7.8 and 2ml. aliquots were either held at 0^{o}, or heated, for 5min., to one of the following temperatures, 45^{o}, 50^{o}, 55^{o}, 60^{o} or 70^{o}. Immediately after heating, the content of RNase inhibitor was determined. The results are given in Table 24.

Table 23.

The location of RNase inhibitor in the adrenal cell both before and after ACTH treatment.

Assay mixtures contained 12μmoles tris buffer, pH 7.8, 90μmoles NaCl, 0.45μmoles EDTA, a suitable amount of the tissue fraction, 0.005μg. bovine pancreatic RNase and 2mg. purified yeast RNA, in a total volume of 0.6ml. The percentage inhibition of the activity of the added bovine pancreatic RNase was related to units of inhibitor using Figure 6.

Duration of ACTH treatment (hr.)	RNase inhibitor			
	/100g. body wt.		/100mg. tissue	
	'cell sap'	'pellet'	'cell sap'	'pellet'
0	46	9.4	104	21.6
18	127	5.2	244	10.0

Table 24.

The Effect of temperature on the activity of RNase inhibitor

Experimental details are given in text. Each assay mixture contained 12μmoles tris buffer, pH 7.6, 90μmoles NaCl, 0.45μmoles EDTA, the cell sap from about 140μg. adrenal tissue, 0.006μg. bovine pancreatic RNase and 2mg. purified yeast RNA, in a total volume of 0.6ml. The percentage inhibition of the activity of the bovine pancreatic RNase was related to the number of units of inhibitor using Figure 6.

Treatment prior to assay	Units of inhibitor	% Decrease
0°	1.5	
45°	1.32	11
50°	0.75	50
55°	0.60	60
60°	0.40	75
70°	0	100

It would seem that the inhibitor is heat labile, but it may be that the decrease in activity on heating is due to the action of degradative enzymes.

3.5. Sedimentation Analysis of Adrenal RNA.

1. Introduction.

Sedimentation analysis of cellular RNA by centrifugation on sucrose density gradients has proved to be a powerful means of separating RNA molecules according to their size. Application of this method has demonstrated the presence in cells of a rapidly-labelled RNA with a high turnover rate. It is believed that this RNA fraction is synthesised in the nucleus on the genes and provides the code for protein synthesis in the cytoplasm (Jacob and Monod, 1961). The possibility of a rapidly-labelled RNA fraction in the adrenal was investigated with the aim of investigating the effect of ACTH on its metabolism.

Adrenal quarters were incubated *in vitro* either alone or in the presence of ACTH and either ^{32}P, as orthophosphate, of [^{3}H]-uridine was added for varying lengths of time at the end of the incubation period. The RNA was isolated and analysed on a sucrose density gradient. The results are given in Figure 12.

2. Output of corticosterone by the adrenal quarters.

In this series of experiments, in order to ascertain

Table 25.

The influence of ACTH on the corticosterone output of adrenals incubated in vitro.

Adrenal quarters were incubated in vitro and aliquots of medium were assayed for corticosterone content.

Experiment No.	ACTH added m. i. u. /ml. of medium	Corticosterone produced µg. /100mg. tissue/hr.
1.	0	5.03
	60	6.5
2	0	2.8
	66	5.1
3	0	3.0
	66	10.2
4	0	3.94
	100	4.72
	332	7.20

that the adrenals incubated were secreting steroids and that they were responding to ACTH, corticosterone estimations were occasionally carried out on the incubating medium. This also showed if the *in vitro* conditions employed were satisfactory. Representative results of these investigations are given in Table 25. These values show that under the conditions used, the adrenal quarters responded, in terms of increased corticosteroid output, to the addition of ACTH to the medium, and thus the technique was judged to be satisfactory.

5. Sedimentation coefficients of the adrenal RNA.

Sedimentation analysis of adrenal RNA by centrifugation on sucrose density gradients showed that the adrenal RNA sedimented into three main peaks (Figure 13). The sedimentation rates of the two principal RNA peaks were examined in the Spinco model E analytical ultracentrifuge. The s value of the fastest sedimenting peak was calculated to be 34.1 and the next fastest peak on s value of 19.4. The slowest sedimenting peak was assumed to have an s value of 4. In the subsequent paragraphs the three principal RNA peaks are referred to as 34s, 19s and 4s RNA respectively. These s values of adrenal RNA are similar to those of liver RNA as found by Spohn and Dingman (1965) but

higher than the values quoted for liver microsomal RNA by Hall and Doty (1959).

4. Sedimentation patterns of adrenal RNA.

Sedimentation analysis of RNA isolated from adrenals incubated *in vitro* are given in Figure 12. It must be emphasised that due to slight differences in the centrifugal forces and times employed in the various experiments, the patterns of RNA distribution in the sucrose density gradient cannot be directly compared unless, of course, the preparations of RNA were centrifuged on sucrose density gradients simultaneously, that is, by using the same ultracentrifuge at the same time. For this reason, only the patterns of each control and the respective test can be compared with respect to sedimentation rates. Thus if a RNA fraction peaks at, say, tube No.14 for both test and control RNAs then it may be assumed that these fractions have equivalent s values.

The RNA from both ACTH-treated and non-treated adrenal quarters is fractionated into three main u.v. peaks, although in some cases it seems as if ACTH induces formation of a peak sedimenting faster than 4s and appearing as a shoulder of the 4s peak (Figure 12a,d), but in one case the converse applied (Figure 12g).

The relative amounts of 54s RNA and 19s RNA agrees fairly well with that found in other mammalian tissues (Hiatt, 1962; Scherrer and Darnell, 1962; Spohn and Bingman, 1965), but it appears that the amount of 4s RNA is higher than that found by the above investigators.

Originally it was believed that this large amount of 4s RNA was due to enzymatic and chemical breakdown of the RNA during the extraction procedure. To test this possibility RNA was isolated from Krebs II ascites tumour cells by the same method used to extract the adrenal RNA. Sedimentation analysis of the Krebs II ascites tumour cellular RNA (Figure 13 e) showed that the amount of 4s RNA was low and the pattern was similar to that obtained by Mason, Cline, and Smellie (1963). It was thus concluded that breakdown did not occur during the isolation procedure, but the possibility remains that there is an adrenal RNase which is not inactivated by bentonite.

However, when it is considered that the RNA analysed was isolated from adrenals incubated <u>in vitro</u>, it becomes apparent that the large 4s RNA peak may be due to degradation products of RNA from cells dying during incubation. This hypothesis is substantiated by the observation that the 4s RNA peak is smaller in the RNA of adrenals incubated for a shorter time

interval, 5.25hr., (Figure 12 d) than in the RNA of adrenals incubated for a much longer time interval, 6hr. to 6.5hr., (Figure 12a,b,c,f,g).

5. Distribution of Radioactivity.

(a) Using ^{32}P as RNA precursor.

The labelling patterns for RNA of adrenals exposed to ^{32}P for 60 and 100min. are also given in Figure 12. Several features are evident under the conditions used,

(i) adrenal RNA is heterogeneous with respect to uptake of ^{32}P

(ii) a high proportion of the radioactivity is associated with the 4s RNA

(iii) in some experiments the 4s RNA from ACTH stimulated adrenals is less actively labelled than the RNA from the corresponding controls.

(iv) ACTH appears to induce an increase in the proportion of radioactivity associated with a very fast sedimenting fraction. It can be seen that the radioactivity of this fraction is spread over at least four fractions; this rules out the possibility of this fraction being merely precipitated material.

(v) in the 100min. ^{32}P labelling experiments the radioactive peaks and the ultraviolet peaks of the control adrenal RNA coincide, but a

radioactive peak precedes the ultraviolet peak of the 34s RNA fraction from the ACTH stimulated adrenals. This would suggest that ACTH is stimulating the production of a small RNA fraction of high specific activity, with an s value slightly greater than 34.

(b) Using [^{3}H]-uridine as RNA precursor.

Figure 12,f,g shows that a markedly heterogeneous labelled RNA was extracted from adrenal RNA incubated in presence of [^{3}H]-uridine for both 10 and 20min. It is clear, however, that after 10min. very little isotope is incorporated into the adrenal RNA. A different pattern emerges after a 20min. period of incorporation. It is evident that most of the radioactivity is contained in fraction s sedimenting faster than the 34s RNA. The pattern is not dissimilar to that of liver nuclear RNA of rats killed 10min. after injection of [^{14}C]-orotic acid (Hiatt, 1962).

The rapidly sedimenting, rapidly labelled RNA is polydisperse in both ACTH-stimulated and non-stimulated adrenal quarters.

Figure 12.

Sedimentation analysis of RNA from adrenals incubated *in vitro*.

Quartered rat adrenals were incubated *in vitro* (p 52) for varying lengths of time before addition of either ^{32}P or $[^{3}H]$ uridine to the incubation medium. The RNA was isolated from the adrenal quarters (p 60) and, layered on a 5% to 20% sucrose density gradient, and centrifuged (p 65).

——— represents the $E_{260m\mu}$ of the collected fractions (diluted approx. 1 in 8).

..... represents cts./min./ml. of the diluted fractions.

(a) control: quarters incubated for total of 6hr. Period of ^{32}P pulse, 60min. gradient centrifuged at 42,000g for 11.8hr.

test: as control except that quarters were incubated in the presence of ACTH (500 m.i.u. per 8 quarters).

(b) control: quarters incubated for total of 6.5hr. Period of ^{32}P pulse, 100min. gradient centrifuged at 42,000g for 11.5hr.

test: as control except that quarters were incubated in the presence of ACTH (500 m.i.u. per 8 quarters).

(c) control: quarters incubated for total of 6.0hr. Period of ^{32}P pulse, 100min. gradient centrifuged at 42,000g for 12.0hr.

test: as control except that quarters were incubated in presence of ACTH (500 m.i.u. per 8 quarters).

Figure 12

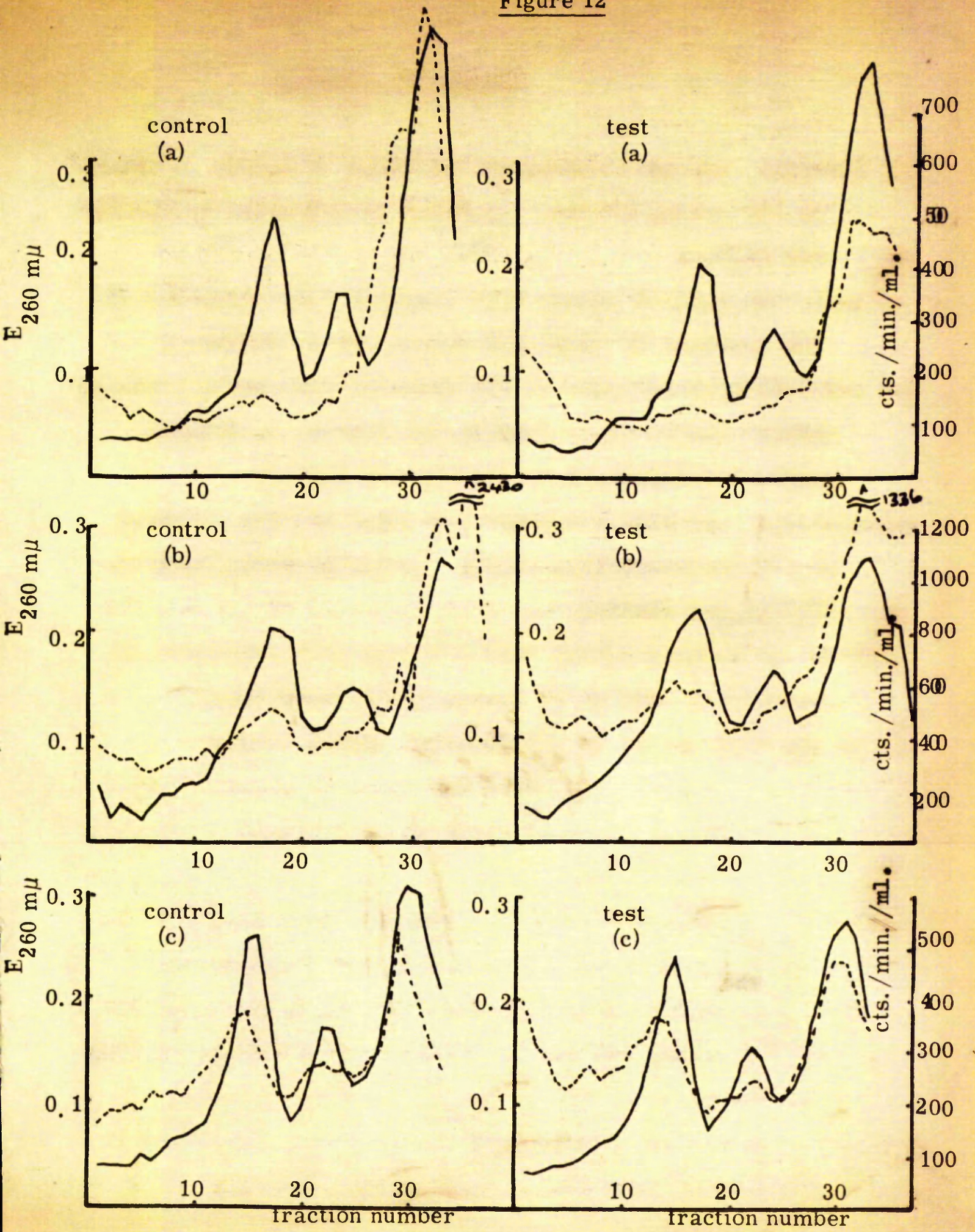

control
(a)
test
(a)
control
(b)
test
(b)
control
(c)
test
(c)
$E_{260\ m\mu}$
cts./min./ml.
fraction number
10
20
30
0.1
0.2
0.3
100
200
300
400
500
600
700
800
1000
1200
~2420
~336

Figure 12. (Cont.).

(d) control: quarters incubated for total of 4.5hr. Period of ^{32}P pulse, 100min. gradient centrifuged at 42,000g for 11.0hr.

test: as control except that quarters were incubated in the presence of ACTH (200 m.i.u. per 8 quarters).

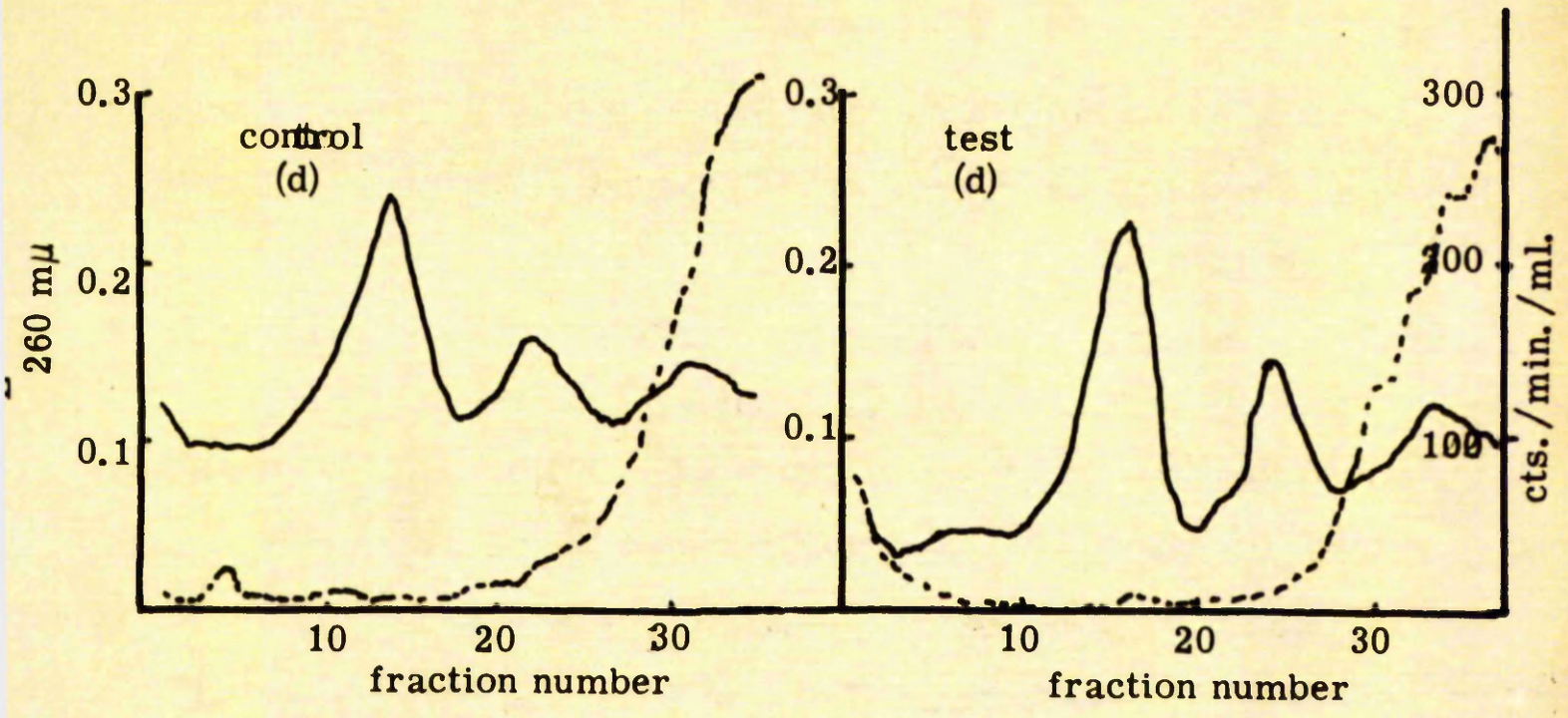
(e) sedimentation analysis of RNA isolated from mouse Krebs II ascites tumour cells (kindly supplied by R. Eason). gradient centrifuged at 42,000g for 11.0hr.

(f) control: quarters incubated for total of 6hr. Period of $[^{3}H]$-uridine pulse, 10min. gradient centrifuged at 37,000g for 11.0hr.

test: as control except that adrenals were incubated in presence of ACTH (5000 m i.u. per 8 quarters).

(g) as (f) with period of $[^{3}H]$-uridine pulse, 20min.

Figure 12


0.3
0.2
0.1
260 mμ
control
(d)
10
20
30
fraction number
0.3
0.2
0.1
test
(d)
300
200
100
cts./min./ml.
10
20
30
fraction number

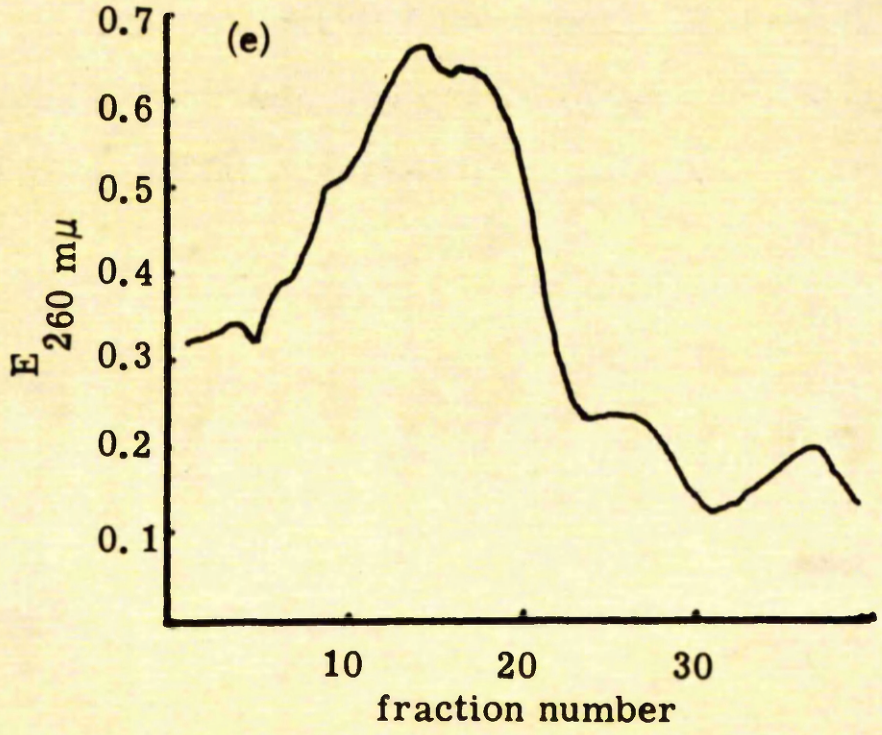
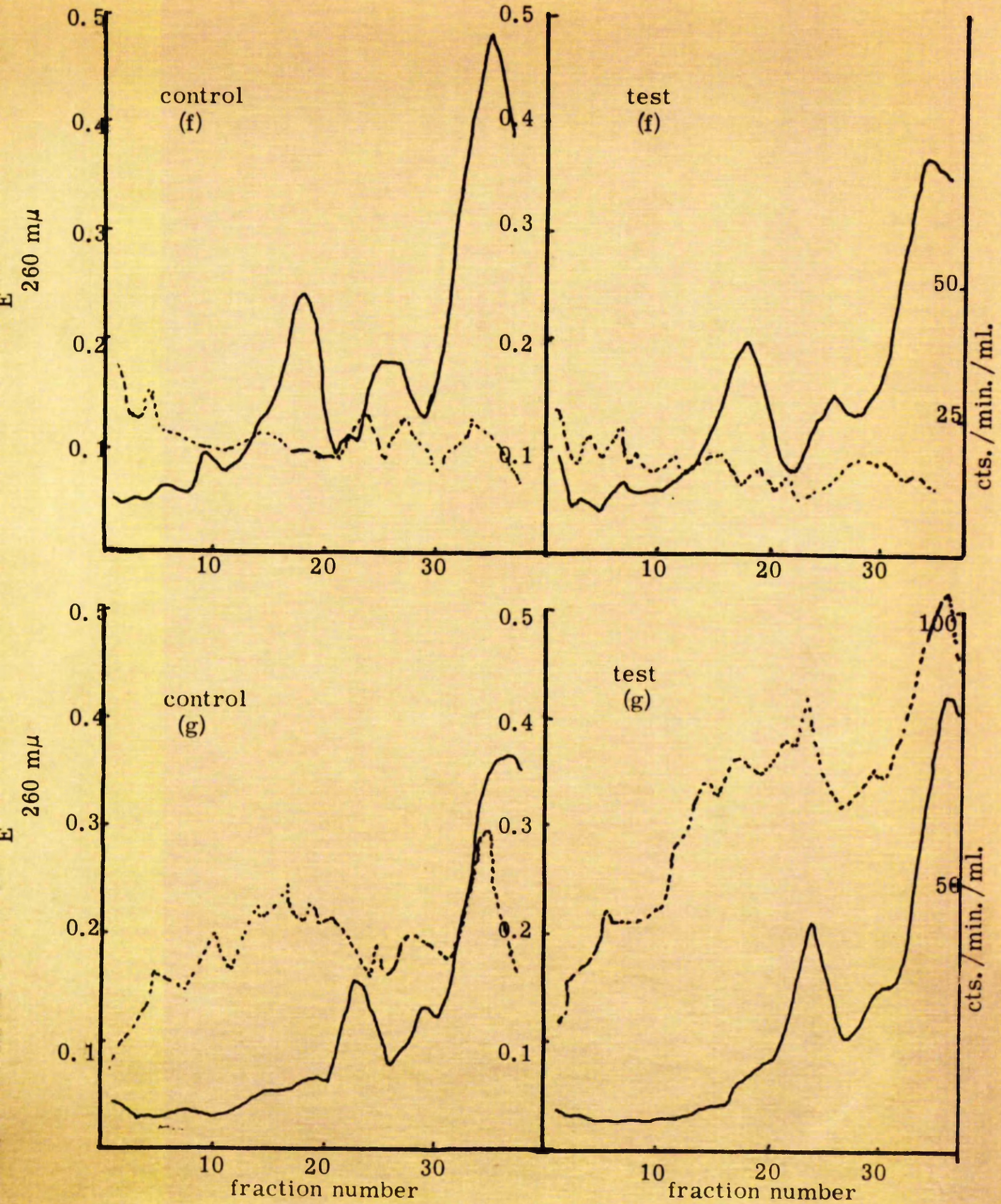

0.7
0.6
0.5
0.4
0.3
0.2
0.1
E 260 mμ
(e)
10
20
30
fraction number

Figure 12


control
(f)
test
(f)
control
(g)
test
(g)
E 260 mμ
cts./min./ml.
0.5
0.4
0.3
0.2
0.1
50
25
100
50
10
20
30
fraction number

SECTION 4

D I S C U S S I O N

4.1. The nature of the sub-cellular components examined.

In the experiments reported in this thesis, the rat adrenals were fractionated into their sub-cellular components essentially by the methods of Schneider (1948). Objections to the use of cell fractionation and the limitations of this technique have been reviewed by Hogeboom and Schneider (1955). There is a further difficulty encountered in the use of cell fractionation of the adrenal, as the adrenal gland is known to consist of at least four different cell types. Thus any particular sub-cellular fraction obtained will consist of a mixture of these fractions from each cell type. Thus, if analysis of a particular sub-cellular fraction reveals a difference induced by ACTH, it will not be possible to state which zone of cells has been affected. It should be noted that mammalian liver also consists of a number of cell types (Wilson, Stowell, Yokoyama and Tsuboi, 1953) and thus the problem of correct interpretation of results also exists when this organ is being studied.

Ideally, it would be more satisfactory if it were possible to separate the adrenal cells according to their morphological appearance prior to sub-cellular fractionation. However, it is not possible, by present day techniques, to obtain sufficient material

for the type of experiments described here without using prohibitive numbers of rats, and these available methods are also very time-consuming.

The isolation of pure nuclei suitable for biochemical studies has attracted the attention of many investigators and much effort has been applied in attempts to obtain nuclei free from cytoplasmic impurities and containing all the constituents as present *in vivo*. Since there does not seem to be a method capable of yielding such nuclei, the choice of method is thus dependent upon the nature of the investigation. During one of the experiments in this series, a procedure of isolating nuclei free from cytoplasmic impurities was required. A survey of the literature showed that there were three main types of methods for the isolation of nuclei from mammalian cells. The first of these is based on the use of non-aqueous solvents to separate the nuclei from the other cytoplasmic material of lyophilised tissue. This procedure was originated by Behrens (1932) and has been modified by others (Bounce, Fishkoff, Barnett and Frear, 1950; Allfrey, Stern, Mirsky and Saetren, 1953; Bounce, 1955). This method yields nuclei of doubtful purity (see Bounce 1955; Roodyn, 1963), and is laborious to perform. Also, it

is impossible to sub-fractionate the cytoplasmic material after removal of the nuclei. It was clear that this type of method was unsuitable for the isolation of adrenal nuclei of the required purity. Nuclei have been isolated from solutions of an acid pH. (Dounce, 1943; 1955). However, objections similar to those of the Behrens procedure arise. There is also a suspicion that RNA may be leached out of nuclei prepared in acid solution (Dounce et al., 1950; Allfrey et al., 1952; Kay, Smellie, Humphrey and Davidson, 1956).

Methods for the isolation of nuclei from homogenates of tissue in aqueous sucrose solution have also been used by many workers, but, as pointed out by Chaveau, Moulé and Rouiller (1956) it is impossible to obtain nuclei free from cytoplasmic material using only differential centrifugation of the tissue homogenate in 0.25M-sucrose. These workers described a method which yielded pure rat liver nuclei. This method was based on the fact that liver nuclei have a higher density than that of the cytoplasmic components, of whole cells, and of red blood cells (Chaveau, 1952). Thus, centrifugation of a tissue homogenate in a sucrose solution of an intermediate density will separate the nuclei from the other cytoplasmic components.

Chaveau et al. (1956) homogenised the liver in 2.2M-sucrose but Dingman and Sporn (1962) found that nuclei obtained by differential centrifugation of a tissue homogenate in 0.32M-sucrose could be purified by centrifuging these nuclei in 2.2M-sucrose. This modification allows the other cell fractions to be isolated. The nuclei obtained by these methods from tissues, such as rat liver, rat brain and guinea pig liver have been shown to be free of cytoplasmic impurities and to be morphologically well preserved (Chaveau et al., 1956; Sporn, Wanko and Dingman, 1962; Maggio, Siekevitz and Palade, 1963).

It therefore appeared that this type of method was the most suitable for isolating the adrenal nuclei for the present investigation.

The $\frac{RNA}{DNA}$ ratios obtained for the adrenal nuclei cannot be readily compared with other published data as slightly different methods were used for the estimation of the content of RNA and DNA. It is known (Tables 1,2) that variations in the conditions of the Schmidt-Thannhauser assay procedure can lead to false values of RNA and DNA content being obtained (see also Hutchison and Munro, 1961).

The mitochondria from a wide variety of cells have been found to possess similar structures as

observed by means of the electron microscope (Palade, 1952; 1955; Sjöstrand and Rhodin, 1953), but the mitochondria of the rat adrenal cells have been found to have a different structure from those of other tissues (Lever, 1955; 1956; Ashworth, Race, and Mollenhauer, 1959; Sabatini and De Robertis, 1961). Moreover, the structure of the mitochondria differ according to the cell type to which they belong (Sabatini and De Robertis, 1961). These findings emphasise the heterogeneity of the mitochondrial fraction examined in the present work.

Microsomes do not exist as such in the cell and are really artefacts of the isolation procedure (Palade and Siekevitz 1956a,b; Siekevitz, 1963). A system of tubules and vesicles pervades the cytoplasm of the mammalian cell and the name endoplasmic reticulum has been given to this structure (Porter and Kallman, 1952; Porter, 1953).

The ribosomal fractions in this investigation were assumed to be "free" ribosomes, i.e. ribosomes, which prior to homogenisation were not attached to the endoplasmic reticulum.

4.2. Distribution of RNA in the adrenal sub-cellular components.

There is much less RNA found in the adrenal cell than is found in the liver cell. In fact, the RNA

content of the "average" liver cell is approximately three times that of the "average" adrenal cell (Table 9). This is almost certainly related to the fact that the liver is predominately a protein-synthesising tissue and the adrenal is predominately a steroid-synthesising tissue.

Examination of the distribution of RNA in the sub-cellular fractions reveals that, in normal and in ACTH-stimulated adrenal glands, the microsomes contain about three times as much RNA as do the mitochondria (Table 12). Estimations of the content of RNA in the subcellular fractions of the adrenals of dogs (Brannemo and Reddy, 1961), of young rats (Fiala et al., 1956) and of humans (Laird and Barton, 1956) have also shown the amount of RNA in the adrenal mitochondria to be about a third of that in the microsomes.

It is known that administration of ACTH causes the amount of RNA in the rat adrenal to increase (Fiala et al., 1956; Ramaiah, 1959; Munro, Hutchison, Ramaiah and Neilson, 1959) and from the present investigations it is apparent that amounts of RNA in the microsomes and mitochondria increase in parallel (Table 12). This is compatible with the increase in the number of mitochondria and perhaps with the increase in the amount of endoplasmic reticulum noted

in the rat adrenal cells after administration of ACTH (Ashworth *et al.*, 1959).

It appears that the RNA content of the ribosomal fraction is sensitive to ACTH. This preferential increase in the ribosomal RNA is not easy to explain since very little is known about the control of the number of ribosomes or their synthesis or degradation. Whether or not the increase in the number of ribosomes, as reflected by the increases in RNA is a function of active protein synthesis remains to be seen. However, ACTH certainly stimulates adrenal protein synthesis *in vivo* and *in vitro* (Bransome and Reddy, 1965) and increases the content of protein in the adrenal (Fiala *et al.*, 1956; Munro *et al.*, 1962). Relevant to these findings are the results of Ferguson (1963) who found that puromycin, which inhibits protein synthesis, will block the increase in steroid production (induced by ACTH) in rat adrenal quarters.

The content of the RNA in the cell sap also increases at a greater rate than does the microsomal RNA although the increase is not as great as observed in ribosomal RNA.

In conclusion, it may be said that ACTH administration increases the amount of adrenal RNA and that it induces changes in the distribution of RNA in the cytoplasmic fractions.

4.3. Heterogenity of Adrenal RNA.

(a) as shown by column chromatography.

Preparations of RNA were first chromatographed on Ecteola-cellulose by Bradley andRich (1956). This adsorbant has been subsequently used many times as a means of fractionating RNA (Goldthwait, 1959; Bloemendal, Bosch and Sluyser, 1960; Bosch, Bloemendal and Sluyser, 1960; Goldthwait and Starr, 1960; Rafelson, 1960; Bosch, Van der Wende and Blooemendal, 1961a; Bosch, Van der Wende, Sluyser and Bloemendal, 1961b; Goldthwait and Kerr, 1962; Köteles, Antoni and Hidvegi, 1962).

Some of the limitations of the use of Ecteola-cellulose as a means of separating RNA have already been given in the results section (5.2) and others have been discussed by Bradley and Rich (1956) and Goldthwait (1959). More recently, two serious disadvantages have been reported by Goldthwait and Kerr (1962). These authors found that a variable percentage of high molecular weight ribosomal RNA was not absorbed by Ecteola-cellulose and furthermore that the RNA which was adsorbed and subsequently eluted by 2M-NaCl at pH 9.7 was degraded due to interaction with Ecteola-cellulose. These observations together with those of Bosch et al. (1960; 1961a,b) and of

Bloemendal et al. (1960) strongly suggest that the use of Ecteola-cellulose is limited for the fractionation of nucleic acids.

DEAE-cellulose and DEAE-Sephadex have been less extensively used as anion-exchangers for chromatography of RNA (Osawa, 1960; Nishiyama, Okamoto, Watanbe and Takanami, 1961; Kawade, Okamoto and Yamamoto, 1963) but their use appears to have been confined to fractionation of sRNA.

An exchanger of similar composition to DEAE-cellulose or DEAE-Sephadex is "Cato-2", described as "an ungelatinized tertiary amino alkyl ether of starch", (Smith, Rebun and Kaplan, 1960). The behaviour of ribonucleic acids isolated from isologous newborn, adult and neoplastic mouse thymus tissues have each been found to be significantly different (Smith and Kaplan, 1961). However, the chromatographic patterns of these ribonucleic acids on "Cato-2" are dissimilar to those of adrenal ribonucleic acids on DEAE-Sephadex. These dissimilarities are probably due to differences in the RNA of the two tissues and to the slight differences in the composition and hence properties of the two exchangers.

Rat liver sRNA, but not high molecular weight RNA is eluted by M-LiCl from DEAE-cellulose (Nishiyama et al.,

1961) and Kamado et al., (1965) eluted over 92% of sRNA from DEAE-Sephadex by M-NaCl. In the present studies most of the adrenal cell sap RNA is eluted by M-NaCl from DEAE-Sephadex (Figure 9 and Table 5). From these three facts, it may be concluded that the RNA I and RNA II fractions isolated on DEAE-Sephadex in the present investigations are of low molecular weight and that the RNA III fraction which is eluted by NH_4OH from DEAE-Sephadex, is of high molecular weight. The question thus arises - what is the origin of these low molecular weight RNA fractions found in the mitochondria, microsomes and ribosomes? There are three possibilities,

(i) that these sub-cellular fractions do actually contain small amounts of low molecular weight RNA,

(ii) that they have arisen by breakdown of RNA during the extraction procedure,

(iii) that the RNA I and RNA II fractions are found exclusively in the cell sap and their presence in the cytoplasmic components is due to contamination.

The first possibility is borne out by recent work, as low molecular weight RNA has been found associated with E. coli ribosomes (Elson, 1962; 1964; Tal and

Elson, 1961; Rosset and Monier, 1963). The function of this low molecular weight RNA is still in doubt. Rosset and Monier (1963) have demonstrated that the RNA fraction which they isolated possessed properties incompatible with those of transfer RNA but Elson (1964) has provided strong evidence that this RNA fraction is transfer RNA (sRNA). An RNA fraction which is similar in some respects to sRNA but which does not possess any amino-acid acceptor ability has also been isolated from rat liver microsomes (Bosch et al., 1961) Nevertheless, all these authors are agreed that this low molecular weight RNA may be metabolically very important.

The other possibilities, namely that the RNA I and RNA II fractions may have arisen from partial degradation or may be due to contamination cannot be ruled out.

Bearing these considerations in mind, it can be stated that the chromatographic pattern of each adrenal sub-cellular RNA is different and also that the effect of ACTH appears to be one of increasing the relative amount of high molecular weight RNA, RNA III. (Table 3).

(b) as shown by phenol extraction.

Modifications of the Kirby phenol extraction

method for RNA have provided a valuable means for fractionating cellular RNA. The most active workers in this field are Georgiev and his colleagues and Sibatani and his collaborators. It was first realised by these investigators that, on shaking tissue with phenol, the RNA extracted into the aqueous layer is metabolically different from that which becomes associated with the interphase material (Sibatani, Yamana, Kimura and Okagaki, 1959; Georgiev and Mantéva, 1960; Ramenskaya, Georgiev, Milman, Mantéva and Zbarskii, 1960).

The "interphase RNA" fraction from rabbit lymphatic cells and from rat liver were shown to incorporate ^{32}P at a much faster rate than does the "aqueous RNA" (Sibatani et al., 1959; Yamana and Sibatani, 1960). Georgiev and Mantéva (1960) and Ramenskaya et al., (1960) carried out a more thorough investigation. They found that the "interphase RNA" from nuclei was much more rapidly labelled than the "aqueous RNA". On the other hand, the "interphase RNA" of the cytoplasmic components were found to be only slightly more metabolically active than the corresponding "aqueous RNA" fractions. Similar results were obtained in the present investigation on the adrenal (Tables 13, 14 and 15).

The methods used for isolating RNA from the interphase after phenol extraction include extraction of the interphase with 10% NaCl at 100° (Georgiev and Manteva, 1960) or the use of a modified Schmidt-Thannhauser separation (Sibatani et al., 1959; Sibatani and Kimura, 1960) or the use of elevated temperatures during phenol extraction (Georgiev and Manteva, 1962). During the course of this investigation, the early attempts in obtaining RNA from the interphase-phenol phase, essentially by the method of Georgiev and Manteva (1960), were unsuccessful. Part of the difficulty encountered in repeating the methods of Georgiev and Manteva (1960) lay in the lack of experimental detail given by these authors.

Presumably, the fractionation of RNA by the methods used in the current study depends upon the nature of the bonds between the ribonucleic acids and the protein in the cell. It may thus be assumed that the RNA in the interphase (RX, RNA) after phenol-SDS treatment of tissue is tightly bound to protein or to deoxyribonucleoprotein. It is significant in this context that the RNA released by 10% NaCl from the interphase material is extracted into the aqueous phase when re-extracted with phenol. This

shows that the fact that this RNA was originally not extracted by phenol was not because of some unusual property of this RNA but was because of the nature of the bond between this RNA and protein or deoxyribonucleoprotein.

The nature of the RNA extracted by phenol-SDS, i.e. the RNA of the AqD fraction, is not easy to visualise. The data suggest that it is dissimilar to the AqL RNA with respect to the uptake of ^{32}P (Tables 13 and 14) and to its composition (Tables 10 and 11), and therefore it may be concluded that the AqD RNA has a different function to that of AqL RNA. RNA, in small amounts, has been detected in the microsomal membrane (= endoplasmic reticulum) (Shigeura and Chargaff, 1958; Moulé, Rouiller and Chauveau, 1950; Chauveau, Moulé, Rouiller and Schneebeli, 1962) and the AqD RNA or indeed the RL-RNA from the cytoplasmic components may be analagous to this RNA fraction.

4.4. Metabolism of Adrenal RNA.

The uptake of ^{32}P into the adrenal RNA fractions can be considered in two ways.

(i) how the pattern of labelling in the RNA of the adrenal compares with that found in other tissues.

(ii) The effect of ACTH on the incorporation of ^{32}P into adrenal RNA.

The RNA of each sub-cellular fraction is metabolically heterogeneous and thus it is difficult to compare the labelling of the adrenal sub-cellular fractions with much of the published data on the uptake of ^{32}P into the total RNA of the sub-cellular fractions from other tissues. Nevertheless, the specific activities of each of the AqL RNA fractions, of the mitochondria, microsomes and ribosomes are all of the same order and similar results were obtained from studies of mouse liver and mouse mammary carcinoma (Barnum and Huseby, 1950; Barnum, Huseby and Vermund, 1955). On the other hand small differences have been noted in the uptake of ^{32}P into the RNA of the sub-cellular components of rabbit liver (Marshak and Calvet, 1949) and of rat liver (Jeener, 1949; Jeener and Szafarz, 1950; Davidson, McIndoe and Smellie, 1951; Smellie, McIndoe, Logan and Davidson and Dawson, 1953). However, all these authors find a greater incorporation of ^{32}P into the RNA from the cell sap than into the RNA found in the mitochondria, microsomes and ribosomes. It can be seen from Table 13 that, in the adrenal, specific activity of the cell sap AqL RNA fraction is greater

than the specific activity of the AqL RNA of the other cytoplasmic components. The pattern of labelling in the adrenal cytoplasmic components is thus similar to that found in other tissues.

It has been known for a long time that the RNA of the nucleus becomes more actively labelled than the RNA of the cytoplasm (Bergstrand et al., 1948; Marshak and Calvet, 1949). Many other investigators have confirmed these findings in a multitude of tissues (see Smellie, 1955). Later, it was found that the nuclei isolated from a wide variety of cells, contained several types of RNA which differed in metabolic activity (Allfrey, Mirsky and Osawa, 1955; Brown, Davies, Colter, Logan and Kritchevsky, 1957; Logan and Davidson, 1957; Osawa et al., 1957; 1958; Georgiev and Mantseva, 1960; Sibatani et al., 1962; Hiatt, 1962; Harris, Fisher, Rodgers, Spencer and Watts, 1965). It can be seen that the RNA of the adrenal nucleus is likewise metabolically heterogeneous (Tables 4, 8, 13, 14 and 15).

The metabolically active RNA fraction found in the adrenal nucleus (Tables 8 and 15) is probably comparable to the rapidly labelled RNA fraction found in the nuclei of Krebs II ascites tumour cells (Georgiev and Mantseva, 1960) of liver cells (Hiatt, 1962)

and of Hela cells (Harris, et al., 1963). The question arises as to the function of this rapidly labelled RNA fraction. It is believed by many investigators (Hiatt, 1962; Georgiev and Mantova, 1962; Sibatani et al., 1962; Scherrer and Darnell, 1962) that the rapidly labelled RNA is composed of two fractions. One is thought to be analogous to messenger RNA as found in bacteria (Jacob and Monod, 1961; Brenner et al., 1961; Hayashi and Spiegelman 1961) and the other is thought to be a precursor of ribosomal RNA. These concepts have been repeatedly questioned by Harris (see Harris 1965a). He believes that the rapidly labelled RNA of the mammalian cell nucleus is broken down in the nucleus and does, therefore, not migrate to the cytoplasm (Harris et al., 1963; Harris, 1965b). Since his evidence is partially based upon the fact that a rapidly labelled RNA is not detected in the cytoplasm (see also Hiatt, 1962), the demonstration of such a fraction in the cytoplasm of mouse liver (Royer, McCarthy and Bolton, 1963) and of rat liver (Munro and Korner, 1964) throws doubt upon his interpretations. Thus it may be assumed that the RX fraction from the adrenal nuclei containsmessenger RNA and ribosomal RNA precursor. It can be seen that ACTH causes the

specific activity of these very important fractions to be lowered, and it may be that ACTH "protects" specific messenger RNA from breakdown. Thus, an increase in the number of molecules of messenger RNA migrating to the cytoplasm would possibly result and this in turn would be expected to cause an increase in the synthesis of proteins. This hypothesis is consistent with the discovery that puromycin inhibits the ACTH-induced increase in steroid output of the adrenal *in vitro* (Ferguson, 1963). However, before this adrenal RNA fraction (RX fraction) can be said to contain messenger RNA, it will be necessary to demonstrate that it is capable of stimulating the synthesis of specific adrenal proteins. Before this can be done, a gentler means of isolating adrenal messenger RNA will have to be evolved, since the RNA of the RX fraction is almost certainly partially degraded.

Other rapidly labelled RNA fractions are found in the adrenal gland. These are the RNA I fractions of the mitochondria and microsomes (Table 4). For reasons already given, it is believed that the RNA I and RNA II fractions may be transfer RNA (sRNA). It has been shown that, before an sRNA molecule can accept its amino acid adenylate (Hecht *et al.*, 1958a),

it first accepts AMP and CMP from the corresponding triphosphates so that the end terminal sequence is pCpCpA (Hecht et al., 1958b; 1959). Hence it may be expected that the sRNA molecules will be highly labelled. Current hypotheses of protein synthesis demand that a small amount of sRNA will be attached to the ribosomes (of the microsomes) at any given instant (Gilbert, 1963b; Staehelin, Wettstein, Oura and Noll, 1964). Thus a small amount of highly active RNA will be expected to be found associated with the microsomes. It is significant in this respect that the RNA I fraction of the "free" ribosomes has a much lower specific activity than the RNA I fraction of the microsomes (Table 4). This is compatible with the idea that in mammalian systems ribosomes themselves cannot synthesise protein but must be attached to the endoplasmic reticulum and to messenger RNA (Henshaw, Bojowski and Hiatt, 1965; Korner and Munro, 1965). It is noteworthy that this interpretation of the data infers that the adrenal mitochondria are engaged in protein synthesis.

Perhaps the most significant feature of the present studies on the uptake of ^{32}P into the rat adrenal RNA is that administration of ACTH causes a fall in the specific activity of the RNA. The

decrease appears to be directly proportional to the duration of ACTH treatment (Tables 13,14 and 15) and occurs in all the RNA fractions isolated and examined. These findings are similar to those of Ramaiah (1959) but are at variance with other findings. Bransome and Reddy (1965b) studied the uptake of [^{14}C] labelled amino acids into rat adrenal nucleic acids. They found that the incorporation of [^{14}C]-U-glycine into the nucleic acids was increased both *in vivo* and *in vitro* by ACTH. They, however, isolated the nucleic acid fraction for radioactive assay with hot trichloroacetic acid, the quantitativeness of which procedure is not beyond suspicion (Hutchison and Munro 1961; Hutchison *et al.*, 1962). By this method, the fraction obtained will thus consist of RNA, DNA and perhaps protein which will be of high specific activity. Thus, a true estimate of the effect of ACTH on the incorporation into adrenal RNA cannot be obtained.

Also these authors were examining the adrenals from larger rats, i.e. of 200-250g. body weight, and the ACTH was given for a shorter time interval, namely 6hr., than was used in the present investigations. Their results may therefore not be directly comparable to ours as the two investigations differ with respect

to the sex and age of the rats, to the duration of ACTH treatment and to the actual RNA precursor used.

The decrease in specific activity of adrenal RNA observed as a result of ACTH administration is also in contrast to the results of Nicholls et al., (1956a) who, it will be recalled, showed that exposure of male rats to a cold stress for 16hr. raised the incorporation of ^{32}P into the adrenal ribonucleotides. However in a later experiment the effect of a longer duration of cold stress (8 days) was found to cause a decrease in the uptake of ^{32}P into the adrenal ribonucleotides (Nicholls et al., 1956b).

This decreased uptake of ^{32}P into adrenal RNA fractions as noted in the present studies, resulting from administration of ACTH can be explained in two ways. The first is that the pool of adrenal inorganic phosphate is perhaps enlarged and secondly that the breakdown mechanisms of RNA may be under the influence of ACTH. The results presented in Table 16 rule out the first possibility as an increased pool size of inorganic phosphate would be reflected in a decrease in the specific activity. After ACTH treatment there is actually a fall in the specific activity of the adrenal inorganic phosphate but this decrease is directly comparable with the fall in the specific

activity of the blood inorganic phosphate (caused by the increase in levels of blood corticosterone) as the ratio of these two activities remains constant. If the pool size of the adrenal inorganic phosphate was altered under the influence of ACTH this ratio would be expected to alter. Similar observations have been made by Nicholls et al., (1956b). If ACTH does control the breakdown of RNA it may do so by either or both of the following two ways.

(i) the rate of breakdown of RNA may be slowed down, thus, provided the rate of synthesis remains constant, the amount of RNA will increase and the specific activity of the RNA will be lowered,

(ii) the breakdown products of RNA, as 5' mononucleotides, may be more efficiently reincorporated into newly synthesised RNA; in this scheme the rate of synthesis of RNA will be increased by ACTH but the rate of breakdown will remain constant.

It will be seen that although these mechanisms are quite different, it would be difficult to differentiate between them by only kinetic data on the uptake of RNA precursors. As will be discussed in the following subsection, the former mechanism is

thought to be the operable one. However, further studies of, say, the influence of ACTH on adrenal RNA polymerase activity and on the enzymes hydrolysing the 5' mononucleotides could provide evidence which may allow the selection of the correct mechanism.

4.5. Breakdown of Adrenal RNA.

In this section of the present studies, the effect of ACTH on both the RNase content of the adrenal and the activity of the RNase inhibitor have been investigated. After ACTH treatment, changes were observed in both of these but the effect on the inhibitor was more marked than on the RNase activity (Tables 19 and 21).

The observed significant decrease in adrenal RNase after 18hr. of ACTH treatment indicates that a possible mechanism whereby ACTH causes the amount of RNA to increase is by lowering the amount of RNase in the cell.

A decrease in the level of alkaline RNase as the RNA content increases has also been observed in the Walker 256 tumour (Allison, Wannemacher, Parmer and Gomez, 1961). More recently it has been shown that administration of N,N' diethylene-N"-phenethylphosphoramide to rats bearing this tumour resulted in a reduced rate of growth of the tumour concomitant with

reduced RNA concentration and an increase in the concentration of alkaline RNase (Wannemacher, Allison, Chu and Crossley, 1962). Whether these relationships are more than casual ones remains to be established. Moreover these findings appear to be the only known examples where the RNA level changes inversely to RNase activity, as other work suggests that there is a positive correlation between the RNA content and RNase activity of tissue.

Brody (1957) found that during the period of logarithmic growth in the developing human placenta, the $\frac{RNA}{DNA}$ ratio and the activity of the alkaline RNase are high and as the organ develops both the $\frac{RNA}{DNA}$ ratio and the activity of RNase falls.

A similar correlation has also been observed between the RNA content and the alkaline RNase activity of the rat mammary gland at various stages of the lactation cycle (Slater, 1961).

A direct relationship also exists between the RNA content and the acid RNase level in many normal aminal tissues (Ledoux, Pileri, Vanderhaege and Brändli, 1957).

The relationship between the amounts of RNA and RNase activity has been particularily well studied in plant tissues. In general, it is found that increases in the level of RNA are accompanied by

elevated levels of RNase (acid or alkaline) (Ledoux, Galand and Huart, 1962a,b; Kessler and Engelberg, 1963).

It thus appears that the picture with respect to RNA and RNase in the adrenal is different to that observed in other tissues. The reason for this is not clear, but neither is the reason why there should be a positive correlation between RNase and RNA levels. The answer to this apparent paradox will probably lie in the solution of the biological role of the intra-cellular nucleases.

Adrenal alkaline RNase is largely if not all "latent" (Tables 18 and 19). This is almost certainly due to the presence of an intra-cellular RNase inhibitor. It must be emphasised, however, that the results reported in this series of experiments were obtained from observations of the RNase activity in whole adrenal homogenate. Consequently, it is possible that, whilst the RNase inhibitor and the alkaline RNase may be in separate cell compartments in the intact cell, they are allowed to combine during homogenisation, thus leading to low values of "free" RNase being estimated (Table 18). If this were to prove to be the case *in vivo*, it would indicate that a possible function of the RNase inhibitor is to inactivate

the RNase released into the cytoplasm from disrupted lysosomes (Roth, 1960a).

Perhaps the adrenal latent RNase is similar to the "latent" RNases demonstrated in E.Coli ribosomes (Elson, 1958, 1959; Elson and Tal, 1959; Spahr and Hollingworth, 1961) and in rat liver microsomes (Tashiro, 1958; Roth, 1960a,b). The possibility of these enzymes being absorbed to the ribosomes during extraction procedures was ruled out by these authors. It also seems that these enzymes are involved in protein synthesis (Roth, 1960b; Tal and Elson, 1963; Spahr and Hollingworth, 1961), and it has been suggested that they take part in the breakdown of messenger RNA.

ACTH administration dramatically increases the activity of RNase inhibitor in the rat adrenal gland (Table 21). The presence of an RNase inhibitor in mammalian cells has been known since the work of Pirotte and Desreux in 1952 and its presence has been demonstrated in a wide variety of other cells (see 1.2.4e). Nevertheless, the relationship between the RNase inhibitor and the RNA metabolism has attracted little attention.

The only studies known to the author, demonstrating a relationship between changes in RNase inhibitor

activity and changes in RNA content are those of Shortman (1962b) and of Roth, Hilton and Morris, (1962). Shortman (1962b) discovered that the activity of rat liver RNase inhibitor increased after partial hepatectomy and was about 140% above the control level 48hr. after the operation. It was noted that the increase in the level of the inhibitor precedes the increase in RNA after partial hepatectomy.

An analogous situation exists in the rat adrenal. It has been shown that dose levels of ACTH similar to those used in the investigation of the RNase inhibitor also increase the RNA content of the female rat adrenal gland but that no measurable increase occurs until the adrenals have been stimulated for at least 24hr. (Figure 1). It is thus significant that the increase in RNase inhibitor activity occurs prior to the increase in adrenal RNA. Thus, the RNase inhibitor by stopping the breakdown of RNA may cause the amount of RNA in the adrenal to increase provided the rate of synthesis remains constant. This hypothesis is strongly supported by the metabolic data discussed in the preceding sub-section.

4.6. Effect of ACTH, *in vitro*, on adrenal RNA.

The *in vitro* experiments carried out in this series of investigations were intended to be complementary to

the *in vivo* metabolic studies. It was hoped that sedimentation analysis of RNA isolated from adrenal glands incorporating an RNA precursor *in vitro* would provide an alternative means of studying the rapidly labelled RNA as found in other mammalian cells and in the adrenal (Tables 8 and 15). Using these techniques the differences observed in RNA metabolism as a result of ACTH stimulation are small but they may be significant. When ^{32}P was used as the RNA precursor ACTH was found to increase the proportion of radioactivity sedimenting near the bottom of the tube (Figures 12a-d). If this RNA fraction is messenger RNA, this would infer that ACTH is either increasing the rate of production of messenger RNA or is protecting, in some way, the messenger RNA from degradation. This fraction may actually consist of ribosomal RNA precursor and messenger RNA, as recent work has shown that the presence of 1mM-Mg^{++}, which was used in the present study, causes partial aggregation of the rapidly labelled RNA found in rat liver cytoplasm (Monnor and Kurnor, 1964). Thus further investigation as to whether the adrenal rapidly labelled RNA also aggregates at a concentration of 1mM-Mg^{++} is required.

An observation which may prove to be significant is that ACTH causes the formation of a small RNA peak sedimenting slightly faster than the main 54s RNA peak (Figures 12b,c). Certainly it at least demonstrates that the 54s RNA may be composed of RNA types of differing metabolic activity.

The labelling patterns of the adrenal RNA were markedly different when [^{3}H]-uridine was used as a precursor compared to those obtained with ^{32}P as a precursor. This is perhaps a function of the duration of incorporation. However, about 40-50% of the total radioactivity incorporated into the RNA (from [^{3}H]-uridine) is found in the RNA fraction sedimenting faster than the main ribosomal RNA peak (Figures 12f,g). Similar patterns of labelling of RNA have been observed in Hela cells (Scherrer and Darnell, 1962; [illegible] 1963; Girard, Penman and Darnell, 1964) and it has been suggested that this rapidly labelled RNA is of nuclear origin and consists of the precursor of structural RNA and of messenger RNA. Thus it seems that analogous RNA fractions are present in the rat adrenal although the present evidence is not sufficient to conclude if ACTH exerts any effect on the metabolism of these ribonucleic acids.

Perhaps the failure to find any marked effects of ACTH on the labelling patterns of adrenal RNA can be accounted for by one of the following facts.

(i) that there are no marked changes and the changes observed are a reflection of the true picture.

(ii) recently Bransome and Reddy (1963c) have found that glucose inhibits the increase in *in vitro* incorporation of [^{14}C]U-glycine into adrenal protein caused by the ACTH. It should be noted that glucose was added to the incubation medium used in the present studies. However, the composition of the incubation medium for optimal incorporation of suitable radioactive precursors into adrenal RNA is different to that suitable for incorporation into adrenal protein (Bransome and Reddy, 1963b,c)

(iii) that part of the rapidly labelled adrenal RNA is not being extracted by the phenol method. This possibility is consistent with results of Table 15.

4.7. *General conclusions, and a proposed mechanism of action of ACTH on the RNA metabolism of the adrenal.*

In conclusion, it can be said that the metabolism of rat adrenal RNA with respect to the uptake of ^{32}P into various RNA fractions is not dissimilar to that of other mammalian tissues. On the other hand,

the means by which ACTH appears to regulate the amount of RNA in the rat adrenal gland is by a mechanism hitherto little suspected as a metabolic control mechanism. The question as to why ACTH should affect RNA metabolism at all still remains unanswered. It is easy to understand why other hormones such as oestrogens, growth hormone and TSH should affect the RNA metabolism of their target organs since the action of these hormones is one of induction of protein synthesis. On the other hand, the accepted function of ACTH is to increase the rate of secretion of corticoids from the adrenal cortex, a process which on first examination does not necessarily require *de novo* protein synthesis. However recent work suggests that continued protein synthesis is necessary for the stimulatory effect of ACTH on steroid production *in vitro* (Ferguson 1963).

It does seem that it is only on prolonged ACTH stimulation that the amount of adrenal RNA increases. Thus the question arises as to whether the effect of ACTH on RNA metabolism is a direct one.

The sequence of events arising from continuous ACTH stimulation of the adrenal gland could be as follows.

(1) Let A→B→C→D etc. (Figure 15) denote the biosynthetic pathway of corticosterone in the

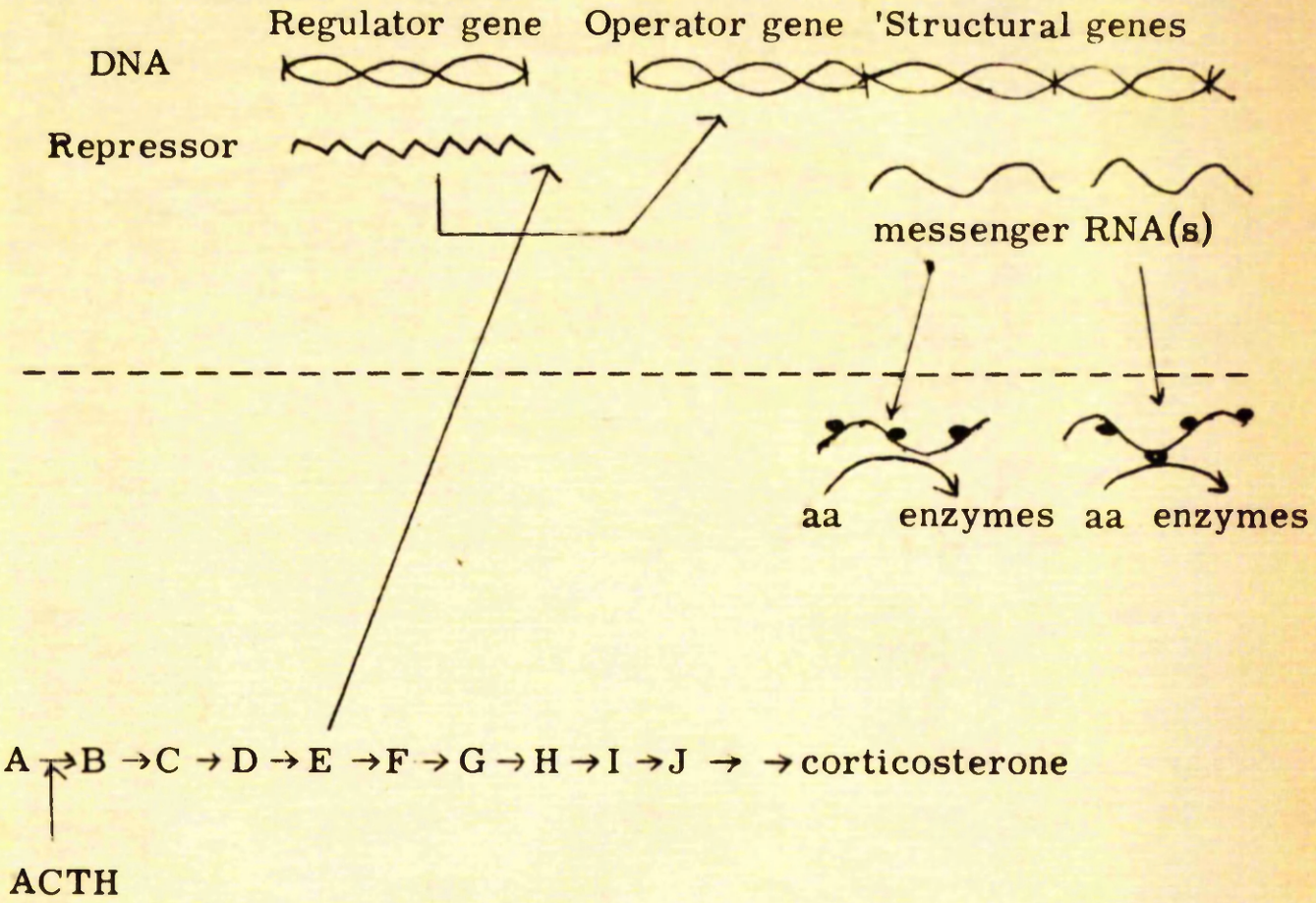
adrenal cortical cells and suppose the enzyme system catalysing the production of B from A is stimulated by ACTH. On ACTH stimulation of the adrenal an increased production of corticosterone will result.

(2) As the rate of secretion increases, as caused by ACTH it is likely that one of the enzyme-catalysed steps becomes rate-limiting and the production of corticosterone becomes maximal. Thus the only way by which ACTH can produce a further increase in the rate of corticosterone synthesis is by increasing the amount of enzymes produced.

(3) Suppose the step E→F (Figure 15) is the rate limiting one. In this situation the level of precursor E will increase (it may not, but for the present discussion we will assume that it is E and not, say C or D which accumulates). If E now acts as an "inducer", it will combine with a specific "repressor" (protein?) which is synthesised at a regulator gene (Jacob and Monod, 1961; Monod, Jacob and Gros, 1962). This repressor, in the absence of E, acts at another gene locus (the "structural" genes) and prevents the formation of, or release of, messenger RNA which is synthesised at this gene. In this situation there is no messenger RNA for the enzyme migrating to the cytoplasm and hence no de novo enzyme synthesis.

Figure 13

OUTLINE OF A POSSIBLE MECHANISM OF ACTION OF ACTH ON ADRENAL RNA METABOLISM



However, when E combines with the repressor, an allosteric transition of the repressor takes place (Monod, Changeux and Jacob, 1963) such that it no longer can inhibit the release of messenger RNA from the gene(s) which it controls. Therefore messenger RNA migrates to the cytoplasm and there it codes for new enzyme production.

4. If the enzymes are contained in say the mitochondria then the increased levels of enzymes will require an increase in the structural proteins and thus the messenger ribonucleic acids for these proteins will also be released into the cytoplasm. It may be that the number of ribosomes in a cell is governed by the amount of messenger RNA present in the cytoplasm and therefore as the amount of messenger RNA i.e. demand for protein, increases so will the number of ribosomes. The data from the present investigation suggest this is brought about by a decreased rate of degradation. The net result is that the enzymes are synthesised and the rate of production of corticosterone increases, provided ACTH is present.

This hypothesis would explain why low levels of ACTH do not increase the RNA levels as it is only when the gland is stimulated above a certain level that RNA metabolism is affected.

This picture is undoubtedly a gross simplification of the operating one but it is one which lends itself to test by the three following experiments.

(i) Injection of E into rats should lead to increased RNA and protein levels and the amount of corticosterone secreted should increase. Thus the E will have an ACTH-like action

(ii) Administration of a compound inhibiting the enzyme catalysing formation of G from F should lead to raised levels of E which will in turn cause an increase in enzyme and RNA content. In this situation the secretion of corticosterone will stop.

(iii) Administration of a chemical inhibiting the formation of D from C would be expected to have no effect on the RNA metabolism of the adrenal.

SUMMARY

Previously it had been shown that the uptake of ^{32}P into rat adrenal RNA was diminished by ACTH treatment, with a simultaneous increase in the actual content of (adrenal) RNA over a period of three days. The present work was carried out in attempts to elucidate the mechanisms behind this apparent paradox.

1. The effects of ACTH treatment for times varying between 15hr. and 72hr. on the RNA metabolism of the female rat adrenal gland have been studied. Rats were killed 2hr. after administration of ^{32}P, the RNA was isolated from the sub-cellular components of the adrenal glands and subsequently fractionated into three main types on columns of DEAE-Sephadex. These RNA types proved to be metabolically heterogeneous. It was found that ACTH markedly lowers the specific activity (relative to blood inorganic phosphate) of these RNA fractions at all durations of ACTH treatment studied.

2. The total RNA of each sub-cellular fraction was fractionated into three classes by a modification of Kirby's phenol extraction procedure. Incorporation studies indicated that these three classes were metabolically different, and again it was found that

the effect of ACTH was to lower the specific activities of these fractions.

8. It was suggested that the metabolic data indicated that either the rate of breakdown of RNA was lowered by ACTH or that, after ACTH treatment, the degradation products of RNA were more efficiently re-incorporated into RNA.

4. Adrenal nuclei were isolated by a method which yields nuclei of a high degree of purity and it was shown the nuclei contained the most metabolically active RNA fraction of the adrenal. This RNA fraction is not extracted by phenol.

5. It appears that the distribution of RNA between the sub-cellular components is altered by ACTH treatment.

6. The concentration of adrenal "latent" alkaline RNase is significantly lowered after 18hr. of ACTH treatment.

7. Evidence is presented indicating that ACTH stimulation increases the activity of an adrenal RNase inhibitor. The increase is especially marked 18hr. after ACTH administration. These results were interpreted as indicating that at least part of the mechanism whereby ACTH increases adrenal RNA is by raising the level of this RNase inhibitor.

8. The adrenal RNase inhibitor is located in the cell sap and is heat-labile.

9. Sedimentation analysis of RNA from adrenals incubated *in vitro* were carried out. Using ^{32}P as an RNA precursor, evidence was obtained suggesting that the pattern of labelling in the various ribonucleic acids was altered by ACTH. When adrenals were incubated in presence of [^{3}H]-uridine for 20min. *in vitro* 40-50% of the radioactivity incorporated into RNA was sedimented in advance of the 5-4s RNA peak.

10. These results are discussed, and a possible mechanism whereby ACTH exerts its action on adrenal RNA metabolism is proposed.

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