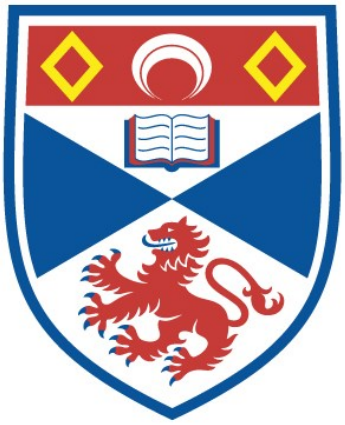
RNA METABOLISM IN THE MUSCLE AND LIVER OF PREDNISOLONE-TREATED RATS

Francis N. Onyezili

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1979

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by

Francis N. Onyezili

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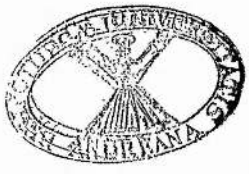

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April, 1979

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the University of St. Andrews, under the direction of Dr. G.A.J. Goodlad.

C E R T I F I C A T E

I hereby certify that Francis N. Onyezili has spent nine terms in research work under my supervision, that he has fulfilled the conditions of Ordinance No. 1 (St. Andrews) and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

A C K N O W L E D G E M E N T S

I thank my supervisor, Dr. G.A.J. Goodlad, for his advice throughout this work.

I am also very grateful to Dr. C.M. Goodlad for her invaluable suggestions on sucrose gradient analyses.

Mr. Ellis Jaffray helped in duplicating the diagrams and Mr. J.H. Oliver cared for the experimental animals.

Finally I owe immense gratitude to Professor A.E. Boyo of the College of Medicine, University of Lagos for his moral support and for his faith in me.

C O N T E N T S

I. INTRODUCTION

Inflammation, as a subject, has dominated the history of medicine as far back as Hippocrates (about 460-377 B.C.) who saw a close connection between it and fever (Menkin, 1961). It was, however, not until the nineteenth century that serious investigation of this phenomenon started with the pioneering endeavours of Cohnheim (1889). Inflammation has been defined by Menkin (1950) as the vascular lymphatic and local tissue reaction elicited in higher animals by the presence of viable or non-viable irritants. It is a protective and quite normal response to a stimulus that threatens the well-being of the host. An inflammatory process is initiated by a disturbance in the local fluid exchange which is manifested by increased capillary permeability (Cohnheim, 1889). With this enhanced passage of plasma protein, fibrinogen accumulates in the area of injury. Thrombi then occlude the draining lymphatics, the site of the inflammation is circumscribed and the phagocytic capacity of leukocytes proceeds to dispose of the irritant. This inflammatory response may be beneficial. Thus, a thermal or chemical burn triggers off the inflammatory and reparative process which proceeds smoothly from injury to healing. However, when tissue injury is caused by a self-replicating parasite (bacterium, virum, or neoplasm) the ensuing inflammatory response becomes more complex as the inflammatory process seeks to isolate the noxius agents from the rest of the organism. This may lead to chronic disabling conditions that affect single or multiple organ systems of the body. Examples of these disease conditions include gout, tetanus, serum sickness and rheumatoid arthritis.

When a patient presents with any of the conditions mentioned

above, clinicians usually direct treatment at two therapeutically independent aspects of his disease firstly to relieve the pain resulting from inflammation and, secondly, to destroy the etiological factor. One of the most effective groups of therapeutic agents against inflammation are the corticosteroids and their synthetic analogues. Corticosteroids are secreted by the adrenal cortex and the essential features of their biosynthesis, summarised in Fig. 1, involve the transformation of acetyl-CoA by several enzyme-catalysed conversions and through several intermediaries to Pregnenolone, the 21-carbon compound from which corticosteroids, as well as androgens and estrogens are derived (Schulster et al, 1976).

Corticosteroids were first isolated from adrenal cortical tissue by Mason et al, (1938) and it soon became obvious that various corticosteroids differed in their biological activity; some, like aldosterone, showed mineralocorticoid effects and influenced sodium retention while others, like cortisol, had minimal impact on salt and water metabolism but protected against stress and showed effects on carbohydrate metabolism (increased glucose production and glycogen deposition). Clinical interest in the isolation and, particularly, synthesis of adrenal steroids arose from the rumours, during World War II, that German airmen were receiving compound E (cortisone) to minimise fatigue and give them extra stamina. It was not long before this compound was synthesised from desoxycholic acid, obtained from ox bile (Sarett, 1946). Soon, additional uses were found for this steroid, its anti-inflammatory powers became recognised and, by 1950, hydrocortisone, the physiologically active

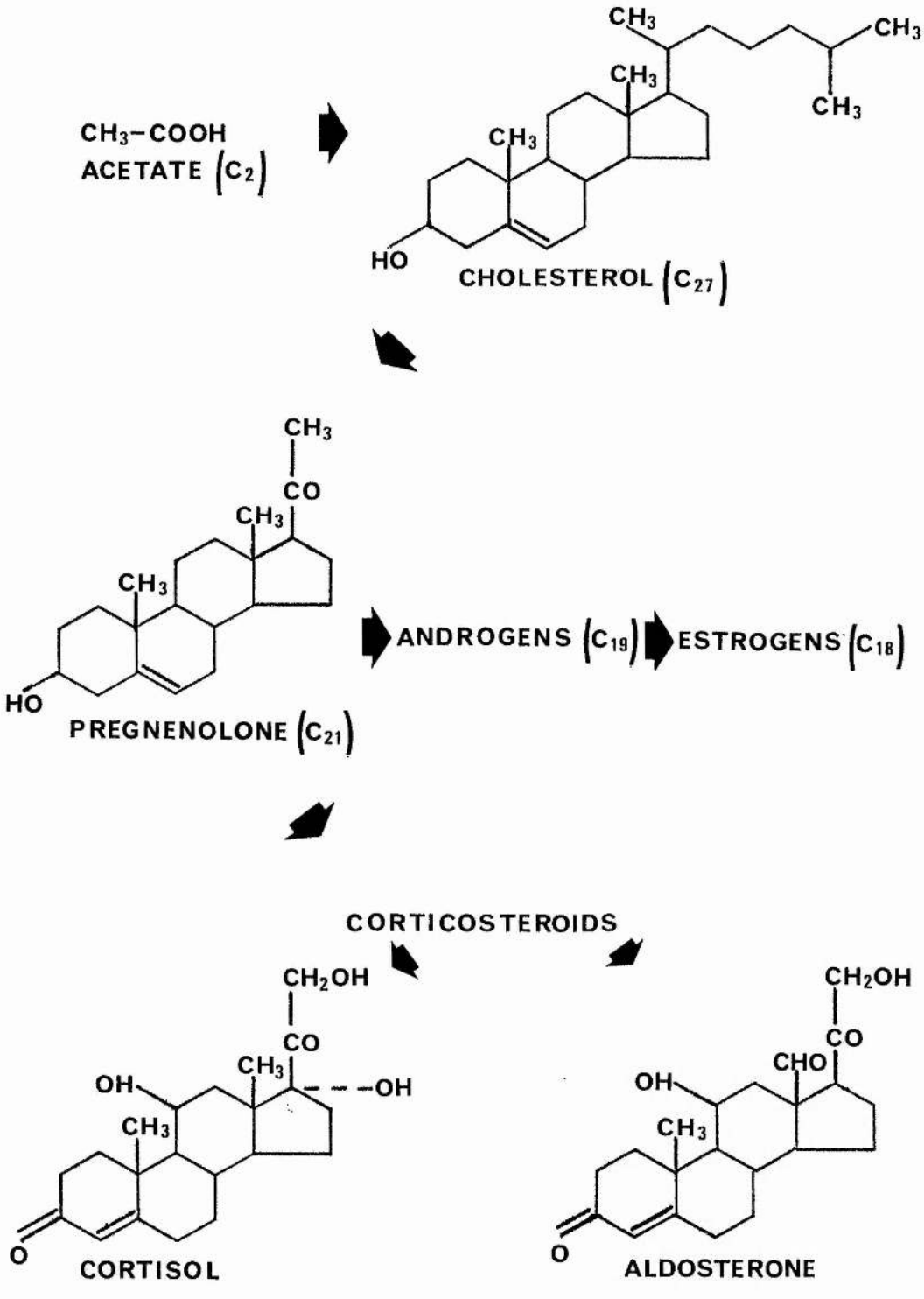
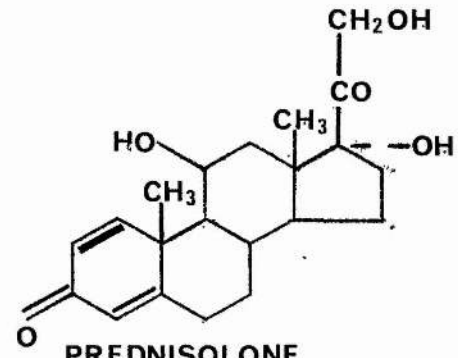
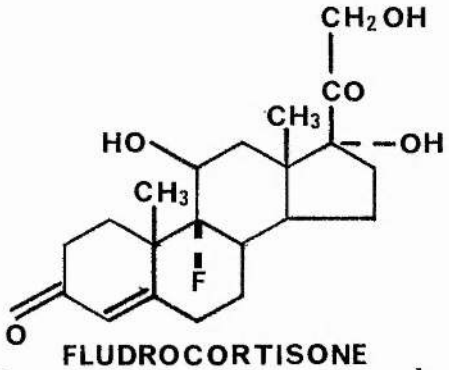


FIG. 1 Outline for the biosynthesis of corticosteroids

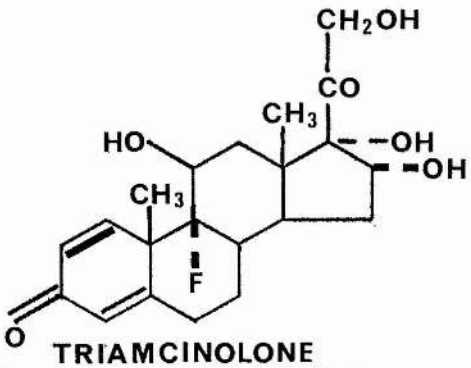
form of cortisone, had been chemically synthesised. However, it was evident that, despite dramatic clinical responses, prolonged administration of cortisone or hydrocortisone exaggerated the physiological effects of the steroids leading to untoward effects, the most frequent and troublesome of which was excessive retention of salt and water. These 'side-effects' limited the usefulness of these steroids and prompted the search for synthetic analogues. Prednisolone (Δ^1-hydrocortisone) was developed and was found to possess four times the anti-inflammatory potency (as measured by its ability to prevent granuloma formation, induced by the presence of cotton wool, in experimental animals) of cortisone or hydrocortisone (Liddle and Fox, 1961). Furthermore, the new steroid produced less retention of sodium and water while still retaining strong glucocorticoid properties (as measured by the ability to deposit glycogen in the liver). Thus, by modifying the steroid nucleus, at least partial dissociation of mineralocorticoid and glucocorticoid activities had been achieved and efforts aimed at eliminating side-effects by further modification of the steroid nucleus (Fig. 2) increased. The introduction of a halogen atom at the C_9 position of hydrocortisone produced 9α -fluorohydrocortisone which possessed a greatly enhanced anti-inflammatory power; the addition of a hydroxyl group at the C_{16} position, to produce triamcinolone, appeared to cancel out the sodium-retaining effects inherent in 9α -fluorohydrocortisone; methylation at the same position produced dexamethasone which, in addition to its powerful anti-inflammatory properties, enhanced sodium excretion rather than its retention.



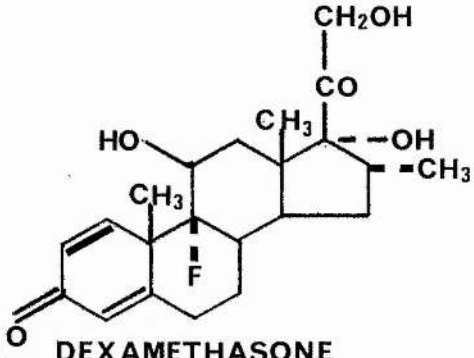
PREDNISOLONE
(Δ¹-hydrocortisone)



FLUDROCORTISONE
(9α - fluorohydrocortisone)



TRIAMCINOLONE
(16α- hydroxy- 9α- fluoro-
Δ¹-hydroxycortisone)



DEXAMETHASONE
(16α-methyl - 9α- fluoro-
Δ¹-hydroxycortisone)

FIG. 2 Various corticosteroid analogues (significant alterations from the hydrocortisone molecule are shown in thicker lines)

The reason for the enhanced biological activity of these hydrocortisone analogues is not clear but it has been suggested (Jailer, 1960) that the substituted compounds are not easily degraded by hepatic enzymes. Consequently, they remain intact, in circulation and, perhaps, in target tissues for longer periods thus exerting a more prolonged effect. Also, there appears to be no general agreement on the anti-inflammatory mechanism of these steroids. Menkin (1950) suggested that corticosteroids repressed protein synthesis in the target cells causing a suppression of cellular activity and, finally, cell lysis; Allison et al (1955) found that these steroids inhibited leukocyte accumulation; Grant et al (1960) concluded that corticosteroids inhibited the adhesion of white blood cells to blood vessel walls; and Mason and Reid (1971) demonstrated that platelet aggregation was inhibited by the steroids. Despite the failure to explain their mode of action in combatting inflammation, corticosteroids remain high on the clinician's prescription list because they promptly suppress many forms of inflammation and are life-saving in conditions in which the inflammatory process per se is life-threatening. There is, however, considerable concern over the medical complications which are known to arise from prolonged corticosteroid therapy. These complications include a decreased ability to resist bacterial or viral infections, appearance of oedema and related changes in electrolyte balance, interference with adrenal-pitiutary relationship, occurrence of peptic ulcers, mental stimulation, diabetes, alterations in glucose metabolism and development of osteoporosis, to mention but a few. Prednisolone, in particular,

causes severe muscle wasting in patients (Faludi et al, 1964). The elimination of these undesirable effects can only be achieved through a better understanding of the biochemical events following the administration of corticosteroids.

Possible Mechanisms of Corticosteroid Action

The general lack of success in eliminating the side-effects resulting from corticosteroid therapy may be reflective of the fact that both their anti-inflammatory action and side-effects could be only different biological expressions of a common mechanism of corticosteroid action. There have been several suggestions that a single activity may account for the entire spectrum of biologic response. Munck (1971) claimed that corticosteroids decrease glucose utilisation by peripherial tissue by blocking its transport into the cell, presumably by preventing phosphorylation and Schayer (1964) had previously suggested that both the physiological and the pharmacological actions of corticosteroids result from the passive attachment to microvascular smooth muscle cells causing interference with the dilation of small blood vessels. This effect on blood flow could presumably decrease the available substrates and lead to the onset of catabolic action. Munck and Wira (1971 and 1972) take the view that the events which precede the phenotypic response of respective target tissues begin with the rapid entry of the corticosteroids into the cell and proceed via an initial binding of the hormone to specific cytoplasmic receptors which constitute a carrier system designed to transport the steroids to their ultimate site of action. This primary interaction serves in some way, possibly through an allosteric mechanism to direct the

hormone-receptor complex to the nucleus at which level the action of the steroid may govern the specific responses observed in the target tissue. Munck's hypothesis is illustrated in Fig. 3 in relation to the atrophying effect of glucocorticoids on rat thymus cells.

Another mechanism through which a hormone can act is a membrane-mediated process involving cyclic AMP. This is the 'secondary messenger' theory proposed by Sutherland (1965) from his studies of the stimulation of glycogenolysis by epinephrine and glucagon. Although this theory is broad enough to embrace the possibility that corticosteroids too may act by altering intracellular cAMP levels, there is no evidence for cAMP involvement in corticosteroid action (Granner et al, 1968; Soifer and Hechter, 1971; Szego 1971; Bakken et al, 1972). However, as a consequence of the observation that there is a dose-related effect of added prostaglandins on cAMP levels in mouse ovary (Kuehl, 1973) it has been suggested (Ramay, 1975) that corticosteroids may act by controlling prostaglandin formation. There is however no experimental support, at the present time, for this hypothesis.

Corticosteroid Actions at the Transcriptional Level

Although they were thought of initially as inhibitors of protein synthesis and stimulators of protein breakdown (Long et al, 1960; Mayer et al, 1976; Millward et al, 1976) corticosteroids have also been shown to cause elevation of liver weight, protein, and RNA content in rats and mice (Amaral and Moriba 1967; Ottolenghi and Barnabei, 1970). Are there then significant differences

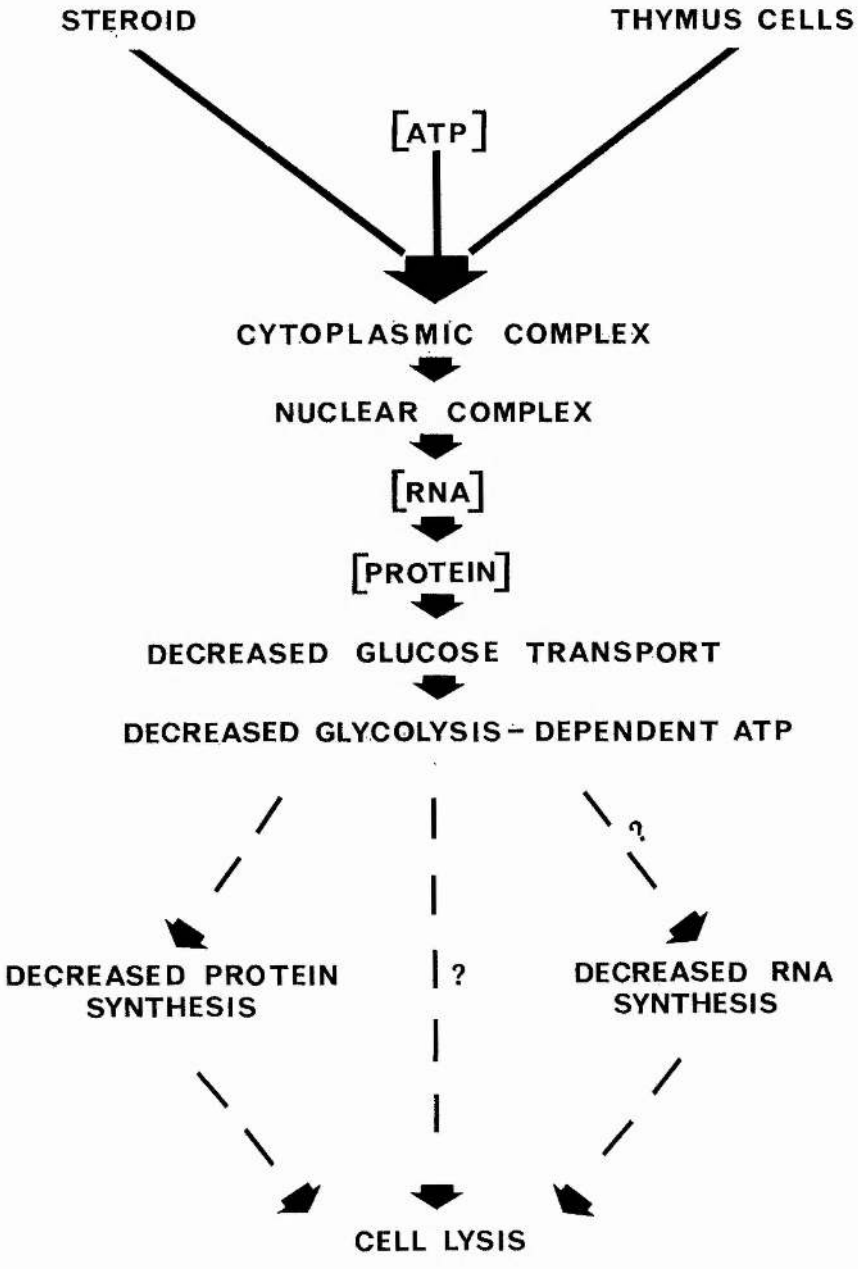


FIG. 3 Interaction of glucocorticoids with rat thymus cells and its relationship with effects on target cells (Munck and Wira, 1971).
(Dashed lines and parentheses indicate relationships for which evidence is still circumstancial).

between catabolic and anabolic sites of corticosteroid action in terms of events which precede the response of respective tissues or are there unifying principles underlying the preliminary steps prior to these responses? The answer to this question is still elusive despite the extensive investigative work in this field. A full biochemical understanding of these responses would require elucidation of each step leading up to the observed response, a task which, at the present time, does not seem to have been accomplished for any corticosteroid.

It has been known for some time (Long et al, 1940) that adreno-cortical hormones stimulate hepatic gluconeogenesis at the expense of protein derived from other tissues, principally skeletal muscle. Since then, Smith and Long (1967) have demonstrated an increased mobilisation of amino-acids from skeletal muscle and a decreased incorporation of these amino-acids into muscle protein following the treatment of rats with cortisol. This decreased incorporation of amino-acids into protein occurs in spite of the enrichment of the intracellular and plasma concentrations of free amino-acids a few hours after corticosteroid treatment (Kaplan and Shimizu, 1963; Betheil et al, 1965) and cannot, therefore, be attributed to some corticosteroid-mediated inhibition of amino-acid transport (Manchester et al, 1959; Kostyo and Redmond, 1966; Makman et al, 1968). If, on the other hand, corticosteroids acted to reduce the rate of muscle protein synthesis, such an action would explain the intracellular accumulation of free amino-acids and their subsequent passage into the plasma could lead to increased levels of free amino-acids. Amino-acid metabolism can be

simplified by writing:

$$\text{Protein} \underset{\longrightarrow}{\overset{(1)}{\longleftarrow}} \text{Amino-acid} \xrightarrow{(2)} \text{Urea} + Co_2 + H_2O$$

where (1) is the anabolic fate and (2) the catabolic fate of the amino-acid. The increased urea formation in the liver following corticosteroid treatment (Smith and Long, 1967) would suggest, therefore, that in the liver, as in the muscle, corticosteroids have a catabolic effect causing some loss of protein. The question then must be whether the increased incorporation (anabolic fate) of amino-acids into liver protein following corticosteroid treatment (Korner, 1960) is due to increased availability of amino-acid from other tissues and whether this anabolic fate is secondary to a general catabolic effect of corticosteroids on all tissues. An alternative view, that the catabolic action of a corticosteroid on muscle or other extra-hepatic tissue was due to a primary stimulation of protein synthesis in the liver and a resultant hepatic demand for muscle-derived metabolites, has been suggested by Dunn et al, (1971). In an investigation of this possibility, Mayer and Rosen (1975) established the presence of corticosteroid receptors in muscle tissue. Other workers had previously demonstrated the presence of such receptors in the liver (Litwack et al, 1973). It would appear, therefore, that the muscle and the liver are independent and direct targets for corticosteroid action and that attention should now be concentrated on finding out how corticosteroids affect protein synthesis and breakdown in each of these tissues independently.

Could a corticosteroid exert selective effects on gene

transcription? In experiments with actinomycin D, Garren et al (1964) showed that the hydrocortisone-induced synthesis of trytophan pyrrolase and tyrosine transaminase in rat liver did not occur when actinomycin D was administered together with the hormone. Actinomycin D inhibits RNA synthesis by binding selectively to DNA (Barnabei et al, 1965) and these findings of Garren and his co-workers are, therefore, consistent with hormonal regulation at a transcriptional level. Given the present understanding of the process of genetic transcription to form messenger RNA and the role of this RNA in polysome formation and coding of amino-acid sequences, hormonal regulation of protein synthesis at this level, by a gene-hormone interaction, is an attractive proposition (Monod et al, 1963; Beato et al, 1973; Rousseau et al, 1975; Simons et al, 1976). It is now accepted that DNA binds steroid hormone-receptor complexes (Rousseau et al, 1975) and that there are different nuclear- and DNA- binding forms of the hormone-receptor complexes (Simons et al, 1977). If corticosteroids do control biological responses at this level, the different responses in the liver and muscle may reflect the ability of the hormone to cause induction and repression at different regions of the same genome. In this regard it is of interest that administration of cortisol was shown to induce formation of new messenger RNA and to stimulate synthesis of primary transcription products of the ribosomal RNA gene, in general, in livers of control rats and rats which had undergone partial hepatectomy (Drews and Brawerman, 1967).

In the lymphoid cells there are indications that

glucocorticoids act initially to initiate nuclear synthesis of a specific protein that causes cell atrophy by inhibiting glucose transport (Munck et al, 1972). A selective inhibition of the synthesis of RNA molecules as well as an inhibition of RNA polymerase (E.C. 2.7.7.6) activity has been demonstrated in rat thymus cells (Abraham and Sekeris, 1971). The view that corticosteroids exert their primary action within the confines of the thymus cell nucleus is also supported by Drews and Wagner (1970) who showed that prednisolone decreased the ability of rat thymic nuclei to synthesise ribosomal RNA.

It has been known for some time that a rise in liver RNA polymerase is an early response to corticosteroid administration (Barnabei and Florini, 1964; Barnabei et al, 1965; Yu and Feigelson, 1971). There is evidence too that RNA polymerase activities in the rat thymus are altered following corticosteroid treatment (Drews and Wagner, 1970; Bell and Borthwick 1976). It would seem a reasonable assumption, therefore, that alterations in this enzyme may be involved in the corticosteroid control of RNA synthesis.

A major advance in the understanding of the mechanism of transcription control in nuclei of eukaryotes has been the discovery of multiple forms of nuclear DNA- dependent RNA polymerase which differ in their structure, location and function (Jacob 1973; Chambon, 1975). Widnell and Tata (1964 and 1966) described two different RNA polymerase reactions in rat liver nuclei, an Mg^{++}- activated enzyme reaction resulting in rRNA- like RNA production and an $Mn^{++}+(NH_4)_2SO_4$- activated enzyme producing messenger-like

RNA. Other workers have identified classes of RNA polymerase using methods based on the order of their elution from DEAE-Sephadex (Roeder and Rutter, 1969) and on their resistance to inhibition by α-amanitin (Kedinger et al, 1970). α-Amanitin is a poison isolated from toadstools and, in vitro, it inhibits the RNA polymerase involved in the synthesis of DNA-like RNA whereas the enzyme transcribing the ribosomal genes is not affected (Kedinger et al, 1970). Eukaryotic RNA polymerases are now usually classified with respect to their response to this substance and studies by high-resolution autoradiographic (Maul and Hamilton, 1967) and cell fractionation (Yu and Feigelson, 1971; Barbiroli et al, 1975) techniques have established that the amanitin-resistant enzyme is preferentially located in the nucleolus while the amanitin-sensitive enzymes are found in the nucleoplasm. This would suggest that the synthesis of ribosomal RNA occurs in the nucleolus while messenger-like RNA is produced in the nucleoplasm. The existence of multiple RNA polymerase forms with distinct subcellular localisation and with specific functions strengthens the hypothesis that these enzymes may regulate gene expression by specific recognition and transcription of different genes or different classes of genes. Several lines of biochemical, autoradiographic and electron microscopic evidence (Jacob et al, 1970; Tata et al, 1972; Hastie and Mahy, 1973) support this view. Although there is, at present, no evidence that eukaryotic genomes contain regulatory sites similar to the prokaryotic promoter and termination regions (Losick, 1972; Chamberlain, 1974), the similarity between the polymeric molecular structure of the

prokaryotic and eukaryotic RNA polymerases (Chambon, 1975) suggest that regulatory mechanisms in prokaryotes might have their counterparts in eukaryotic cells. The different eukaryotic RNA polymerases could, therefore play a positive role in regulating the expression of genetic information. Corticosteroids might control these processes by causing alterations in the number of DNA sites available to the enzyme for transcription (Dahmus and Bonner, 1965), by acting directly on the enzyme and possibly causing allosteric alterations to change the enzymic activity (Sadjel and Jacob, 1971) or by a combination of both of these factors (Yu and Feigelson, 1971).

In an effort to localise the site of cortisol action, in vivo, in rat thymus cells, Nakagawa and White (1970) investigated some properties of 'aggregate' RNA polymerase preparations from cortisol-treated animals. 'Aggregate' RNA polymerase is a polymerase-DNA-protein complex, obtained by lysing the isolated nuclei with hypotonic buffer, selectively precipitating the complex with $MgCl_2$ and, finally, collecting the complex by centrifugation. In preparations from hormone-treated animals there was a general depression of RNA polymerase activities associated with a depression of synthesis of all types of RNA. From investigations of the ability of E. Coli RNA polymerase to utilise the DNA template in the 'aggregate' enzyme preparations for RNA synthesis, Nakagawa and White (1970) concluded that cortisol altered the efficiency of the DNA template through alterations in the nuclear ionic environment. In the soleus muscle of rats in which compensatory hypertrophy had been induced by tenotomy of the

gastrocnemius and plantaris muscles, Sobel and Kaufman (1970) reported a biphasic alteration of RNA metabolism: an early activation of RNA polymerase caused by some yet unidentified but possibly ionic change in the intracellular environment preceded an increased synthesis of the enzyme after 24 h. It would appear therefore that in the muscle, as in the thymus and liver, RNA synthesis may be regulated by varying levels of RNA polymerase activity. Two separate studies of RNA and protein synthesis in dystrophic chick muscle reported apparently conflicting findings (Baieve and Florini, 1970; Battelle and Florini, 1973). This discrepancy has since been attributed to artefactual alterations in Mn^{++}- activated RNA polymerase activity caused by changes in the integrity of the endogeneous DNA template (Neal and Florini, 1975). More rapid degradation of the template was believed to occur in the dystrophic animal and there was, therefore, differential mechanical damage of nuclei during isolation procedures. The results with these isolated nuclei were, therefore, critically dependent on the RNA polymerase assay method and the length of the incubation time in these assays (Neal and Florini, 1975).

Corticosteroid Control of Translational Processes

There are strong indications that, at least in certain specific cases, corticosteroids may also exert control of protein synthesis at the translational level. Bullock et al (1968) showed that the capacity of isolated preparations of muscle ribosomes to synthesize proteins was reduced following the injection of corticosteroids into rats and rabbits. A corticosteroid could influence cellular

protein synthesis by modulating initiation (Young et al, 1968). This view is not, however, supported by the work of Peters et al (1970) who showed no apparent difference in polysomal profiles isolated from normal or triamcinolone-treated rats, although the latter polysomes showed a much decreased capacity for protein synthesis. Furthermore, Clark and Goodlad (1975) have shown, from a study of the translational process in the gastrocnemius muscle of normal and tumour-bearing rats that, where a tissue suffers a preferential depletion of protein, protein synthesis could be affected at some post-initiation stage of translation, possibly translocation. It would seem, therefore, that corticosteroids might control factors other than the free/bound ribosome ratio to elicit the responses they produce in target organs. When actinomycin D was administered to rats 5 - 9 h after hydrocortisone injection, there was an increased synthesis of tyrosine transaminase in the liver. This increase was not observed in the absence of the antibiotic and this observation supported the view that, following steroid treatment, a repressor appears which inhibits further protein synthesis by inhibiting the function of messenger RNA (Garren et al, 1964). Actinomycin D presumably blocked the transcription of the message for this repressor.

These findings are in accordance with the model proposed by Tomkins et al (1969 and 1972). This model acknowledges that the eukaryotic cell does not express its full genetic potential and that different cell types may express different portions of their genome. In all cases DNA remains the primary genetic material and genetic information is expressed by DNA transcription into

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RNA. According to this model, illustrated in Fig. 4, the hormonal regulation of the synthesis of a specific protein entails two genes, a structural gene for the specific protein and a regulatory gene whose protein product (repressor) inhibits translation and promotes the degradation of messenger RNA. For the synthesis of a specific protein, the structural gene is transcribed and its messenger (M) translated to produce the protein. The regulatory gene is similarly transcribed and its messenger translated to produce a labile protein, the repressor, which combines reversibly with M to produce an inactive messenger-repressor complex. A steroid can act as an inducer which, by an unknown mechanism, inactivates the repressor preventing the conversion of the messenger to a repressed form. Thus the steroid inhibits the degradation of the messenger allowing its concentrations to increase and thereby augmenting the rate of synthesis of the specific protein. In principle, the Tomkins model relates the hormonal control of gene expression to a post-transcriptional repressor mechanism.

Corticosteroid Effects on Ribosomal RNA Turnover

Some attention has been focussed on the possibility that corticosteroids may exert their effects by interfering with RNA synthesis (Makman et al, 1966 and 1968; Abraham and Sekeris, 1971; Bell and Borthwick, 1976). In particular, it has been shown that an alteration in ribosomal RNA synthesis is one of the earliest responses to corticosteroids in rat liver (Yu and Feigelson, 1971) and in rat thymus cells (Drews and Wagner, 1970).

In eukaryotic cells, ribosomal RNA, like other cytoplasmic

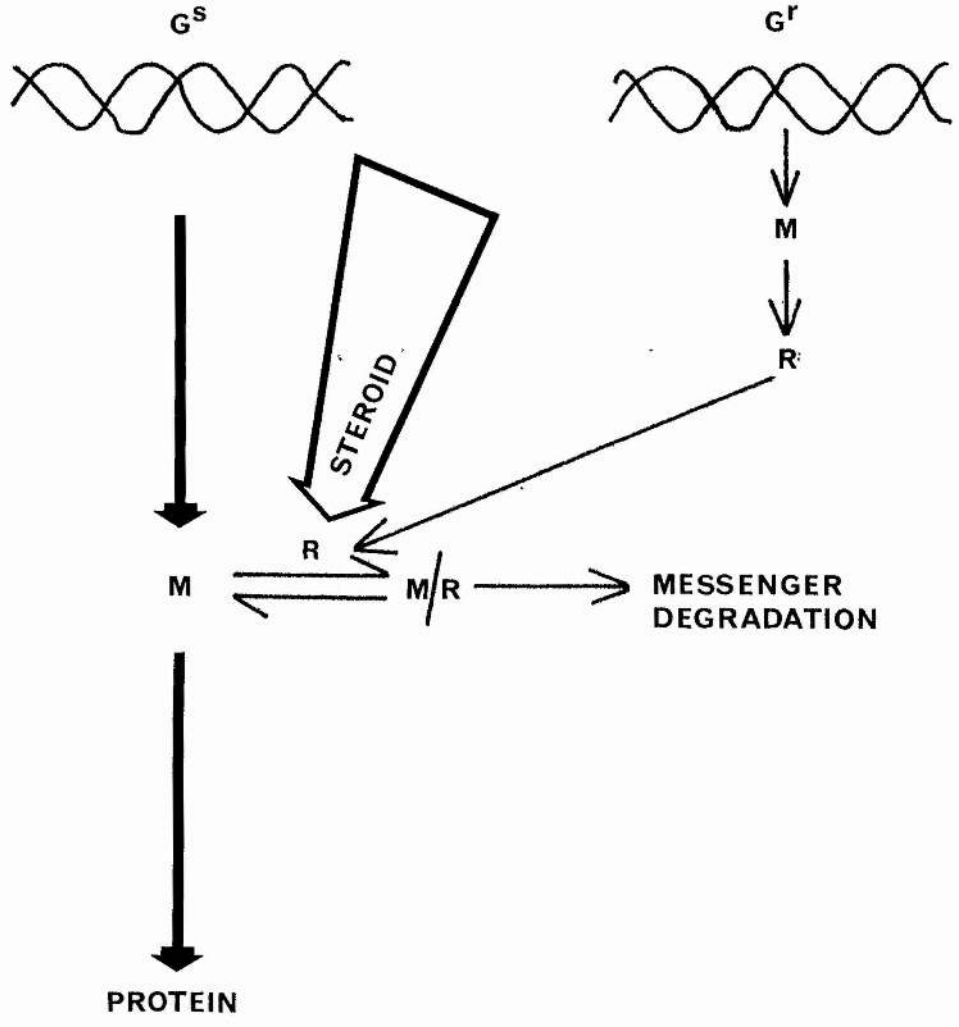


FIG. 4 Hormonal control of messenger RNA function (after Tomkins et al, 1969).

G^s = Structural gene

G^r = Regulatory gene

M = Messenger RNA

R = Repressor protein

M/R = Inactive messenger/repressor protein complex

RNA species originates from the transcription of nuclear DNA (Maden, 1971) and is transcribed from DNA sequences located in the nucleolus (Evans, 1973). The primary transcription product is a 45S-sedimenting species which undergoes a series of 'tailoring' and base modifications to yield the 28S and 18S RNA found in eukaryotic cytoplasmic ribosomes (Adams et al, 1976). It is generally agreed that the bulk of mammalian cellular ribonucleic acid is ribosomal (Hirsch, 1967; Millward et al, 1973) and the high complexity of the architecture of eukaryotic ribosomes supports the idea that expression of genetic information in animal cells can be controlled at the ribosomal level (Bielka, 1978; Rogers, 1978). The activity and turnover of ribosomes and ribosomal RNA are, therefore, important considerations in the study of the expression of genetic information in eukaryotes.

There is increasing evidence that dietary manipulations of mammalian cells play an important role in regulating ribosomal RNA synthesis. Hela cells starved for lysine showed decreased ribosomal RNA synthesis (Maden, 1969) and the lack of amino-acid inhibited the transport, from nucleus to cytoplasm, of ribosomal RNA of Ascites tumour cells (Shields and Korner, 1970). Dietary protein intake (Clark et al, 1957; Shaw and Filios, 1968; Wannemacher et al, 1971) and food deprivation (Enwonwu et al, 1971) have also been shown to affect ribosomal RNA turnover in the rat liver. Such changes in the rat liver may be due to changes in the rate of RNA synthesis (Hirsch and Hiatt, 1966; Kawada et al, 1977; Lewis and Winick, 1978) or to changes in the translation of RNA (Enwonwu and Munro, 1970). Dietary conditions have also been shown

to alter the rate of total RNA synthesis in the rat gastrocnemius muscle (Howarth and Baldwin, 1971). If it is accepted that more than 80% of muscle RNA is ribosomal (Manchester, 1967; Young, 1970), a change in total RNA serves as a good indication of a change in ribosome content. There is also evidence that the activity of muscle polyribosomes are altered by dietary conditions (Young and Alexis, 1968; Van der Deken and Olmstedt, 1970).

Corticosteroids too can alter ribosomal activity and both corticosterone and hydrocortisone have been shown to restore to normal, the decreased turnover rate of ribosomes and polysomes in the liver of adrenalectomised rats (Mishra and Feltham, 1973). These workers concluded that these hormones acted to increase ribosomal RNA synthesis, a view which is supported by the findings of Schmid and Sekeris (1975) who showed that nucleolar RNA synthesis in rat liver was significantly enhanced 4 h after cortisol administration. Izawa and Ichii (1972 and 1973), however, hold the view that the increase half-life of free and membrane-bound ribosomes of rat liver, following cortisol treatment, was due to a decreased degradation of ribosomal RNA. Ottolenghi and Barnabei (1970) had attributed the cortisone-mediated increase in rat liver ribosomal RNA to decreases in both the synthesis and the degradation of this RNA. There are no comparable results of the effects of corticosteroids on muscle tissue but hormonal influence on muscular ribosomal RNA metabolism has been demonstrated by King and King (1978) from studies of thyroid hormone effects in the chick embryo. Previously, Peters et al (1970) had shown that ribosomal activity in rat gastrocnemius muscle was decreased by

treatment with triamcinolone acetonide. These workers attributed this alteration to a decreased RNA synthesis, a finding which agreed with that of Drews (1969) who investigated the effect of the same steroid on rat thymus cells. Ferguson and Wool (1962) had, however, reported no change in RNA synthesis in isolated diaphragm muscle in rats given cortisol for four days.

It would appear, therefore, that glucocorticoids can affect different tissues in different manners (Werthamer et al, 1969, Izawa and Ichii, 1972) and that the effects of the hormones on ribonucleic acid turnover and on the turnover of ribosomal RNA in particular might be important factors in this respect.

Corticosteroid Action on Ribonuclease Activities

Several workers (Barnabei and Ottolenghi, 1968; Kraft and Shortman, 1970; Rosso et al, 1973; Goodlad and Ma, 1974) have demonstrated a relationship between the level of tissue RNA and the level of ribonuclease activity in the tissue. Some (Wiernick and Macleod, 1965; Barnabei and Ottolenghi, 1968; Groves and Sells, 1971) have shown that, where alterations in RNA levels occur in response to steroid treatment, there are related changes in the activity of ribonucleases. A steroid might therefore control RNA metabolism by regulating ribonuclease activity.

Although mammalian ribonucleases have been studied extensively, their physiological roles are still poorly understood. Here the term 'ribonuclease' will be used to describe an enzyme which degrades macromolecular RNA but will not include enzymes which have the general capability to split phosphodiester bonds (phosphodiesterases) or enzymes which hydrolyse both RNA and DNA.

Three main types of ribonucleases have been reported in rat liver subcellular particles and some of their properties have been studied. Acid ribonucleases (E.C. 3.1.4.23) is predominantly located in lysosomes although Roth (1957) found a similar enzymic activity in the rat liver mitochondria. It has a pH optimum at 5.8, is generally believed to have a purely digestive function and is released under conditions of injury or stress or by dead or dying cells to degrade RNA to products which can be re-utilised. Two types of alkaline, ribonucleases (E.C. 3.1.4.-) with pH optimas between 7.0 - 8.0 and 9.0 - 9.5, respectively, have been isolated although controversy persists about their subcellular location. The mitochondria (Roth, 1965; Baudhuin et al, 1975) the post-mitochondrial supernatant (Roth, 1967; Baudhuin et al, 1975) and a ribosomal subunit (Krechetova et al, 1975) have all been cited as locations for alkaline ribonuclease activity. It seems clear, however, that most of the alkaline ribonuclease activity is found predominantly in the post-mitochondrial fraction (Rahman, 1967), that the enzyme has no particular metal requirement and that it can withstand a temperature of 70°C for five minutes. An enzyme which shows optimal activity at pH 9.0 has been shown to occur mostly in the mitochondrial membrane, is strongly inhibited by Na^+ and K^+ and is very heat-labile.

The existence of a ribonuclease inhibitor which depresses the activity of the pH 7.8 enzyme has been reported (Shortman, 1961; Roth, 1962). This inhibitor has been shown to be a labile protein readily inactivated by heat and extremes of pH. It is dependent for its activity on the integrity of one or more -SH groups and

shows optimal inhibition in the pH 8.0 region. It would appear, therefore, that although the pH 7.8 ribonuclease is readily available to digest cellular RNA, an effective and controllable means of regulating its activity is also present in the inhibitor. The practical details of RNAse estimation in the presence of the inhibitor have been described by Shortman (1961). By performing the enzyme assays in the presence or absence of p-chloromercuribenzoate (PCMB), a reagent which inactivates the inhibitor by destroying its sulphydryl groups, he was able to quantitate the free RNAse activity in the cell as well as the 'latent' enzyme activity which was released only when the inhibitor had been inactivated by PCMB. In regenerating rat liver Shortman (1962) noted increasing levels of this inhibitor. High levels of the inhibitor activity were found in a variety of circumstances where cell proliferation or protein synthesis were increased and these levels dropped during ageing (Kraft and Shortman, 1970). Ma (1973) also noted decreased alkaline ribonuclease activity and increased RNAse inhibitor activity in the liver of the pregnant rat. These reports along with those of Brewer et al (1969) suggest that increased catabolic activity in cell could be associated with a decreased inhibitor/RNAse ratio and that a high ratio is observed in increased anabolism.

In summary, it seems that RNAse activity is detectable in most subcellular fractions, that this activity varies with the level of inhibitor activity present, and that the ribonuclease/inhibitor system may be a means of controlling cellular RNA and hence protein, metabolism. Various relationships

between hormone administration and ribonuclease activities have been demonstrated (Terner et al, 1967; Barnabei and Ottolenghi, 1968; Groves and Sells, 1971; Maor et al, 1973) and direct hormone effects on mitochondrial concentrations of RNAses have been suggested (Allard et al, 1956). It is conceivable too that there might be a tissue specificity about the hormonal control of ribonuclease activity. Cortisone has been shown to increase alkaline ribonuclease activity in the thymus, spleen and lymph nodes of mouse but not in the liver (Maor et al, 1973 and 1976) and a wide variety of glucocorticoids, including prednisolone, have been shown to decrease the activities of neutral ribonuclease in mouse liver and skeletal muscle (Kershner and Mayer, 1976). Thus, variation of ribonuclease activity must be considered as a possible factor in the action of corticosteroids on muscle and liver RNA.

Role of Receptors in Corticosteroid Action

If corticosteroids do interact directly with target tissues, they must first be carried to these tissues, a function which for cortisol in human plasma is known to be fulfilled by transcortin, a specific alpha globulin (Sanberg and Slaunwhite, 1963). Werthamer et al (1969 and 1971) who examined the in vitro effects of cortisol and prednisolone on separate populations of lymphocytes and leukocytes have demonstrated the dependence of these hormones on the presence of transcortin. It would seem reasonable, therefore, to suggest a mediatory role for this carrier protein in the action of a corticosteroid. However, dexamethasone, a potent glucocorticoid, does not bind to transcortin (Rousseau et al, 1972) and there are no reports of prednisolone binding to

the protein *in vivo*. Therefore, the evidence that the biologic activity of a corticosteroid is regulated by transcortin is by no means unequivocal.

As a result of the increasing evidence for the existence of specific binding sites for hormones in their target tissues (Hackney *et al*, 1970; Munck *et al*, 1972; Simons, 1977) considerable attention is now being focussed on the interaction of a hormone with receptors located in the target tissue. Classical pharmacology defines a receptor as a transducer which converts input information (i.e. the circulating hormone) into biological response. Some of these receptors have been isolated and their structure shown to be protein. Thus hormone-binding activity was destroyed by extreme pH, by proteolytic enzyme and by -SH group-inactivating reagents (Munck *et al*, 1972; Thompson and Lippman, 1974). Receptors appear to be the determinants of target tissue specificity (Gorski *et al*, 1968) and the hormone-receptor interaction is believed to be the initial event in the sequence of changes leading up to the observed biological response (Lippman *et al*, 1973; Baxter and Forsham, 1974; Kondo *et al*, 1975; Yamamoto and Alberts, 1976; O'Malley and Buller, 1977). Since the receptors are not retained by nuclei in the absence of the hormone (Buller *et al*, 1975) it is probable that the hormone induces some alteration in the conformation of the receptor which facilitates the translocation of the hormone-receptor complex to the nuclear compartment where it is believed to act directly on the genetic apparatus of the cell (Baxter and Forsham, 1974; Yamamoto and Alberts, 1971; O'Malley and Buller, 1977).

Over the past decade, many experimental data relating to these theoretical considerations have accumulated (Munck et al, 1972; Buller et al, 1975; Mayer et al, 1976; O'Malley and Buller, 1977). In particular, the experimental data on estrogen and progesterone activities in the rat uterus and chick oviduct, respectively, (O'Malley and Means, 1974; O'Malley and Buller, 1977) have improved current understanding of the action of steroid hormones and their receptors, in general. It was accepted that (a), the proteins which bind these hormones were limited to their respective target tissues and attracted only the biological active hormone, either naturally occurring or synthetic; (b), the cytoplasmic hormone-receptor complex had a sedimentation coefficient of about 4S but was capable of association with other binding entities, in conditions of low ionic strength, to form an 8S complex; (c), an estrogen-induced conformational change occurred in the uterine cytoplasmic receptor protein and this was followed by the translocation of the hormone-receptor complex to the nucleus were it induced changes in gene expression; (d), the translocation process was temperature dependent (37°C) and required the alteration of the hormone-receptor complex to a 5S-sedimenting form; (e), in the nucleus, the hormone-receptor complex interacted with nuclear chromatin binding firmly but non-covalently in two parts, to the chromatin DNA and to non-histone (acidic) acceptor proteins of the chromatin; (f), the binding to the genome induced alterations in the composition, steric conformation, or both of the chromatin resulting in the changes which constitute the hormone-mediated biologic response.

The conceptual relationship which might exist between the hormone, its receptor and the genetic apparatus is illustrated in Fig. 5. This sequence of events, although largely derived from work with oestrogens, is believed to apply to steroid hormones in general. It has not been demonstrated directly that the binding of the hormone-receptor complex to chromatin resulted in alterations of rates of nuclear transcription but indirect evidence (Hamilton et al, 1968; Luck and Hamilton, 1972; Glasser et al, 1972) support the view that synthesis of RNA plays a major role in the subsequent events which culminate in the biological response.

Scope of the Present Work

The present work constitutes an attempt to explain the diverse effects which corticosteroids elicit in mammalian tissues and, in particular, the action of prednisolone on skeletal muscle RNA. It seems unlikely that a single biochemical alteration in all tissues can conclusively explain all these effects. However, all phenotypic expression require some generation of RNA and protein. Since the events leading up to the formation of RNA and protein is common to all target tissues, this work will concentrate on these transcriptional and translational events. In an attempt to understand how prednisolone elicits its opposite effects on mammalian liver and muscle, the effect of treatment with this steroid or RNA metabolism in these two tissues in the rat, will be examined. The influence of prednisolone on various aspects of RNA synthesis and breakdown in these tissues will be investigated and information will be sought on how prednisolone receptors in the cytosols of these tissues may confer organ specificity to prednisolone-mediated responses.

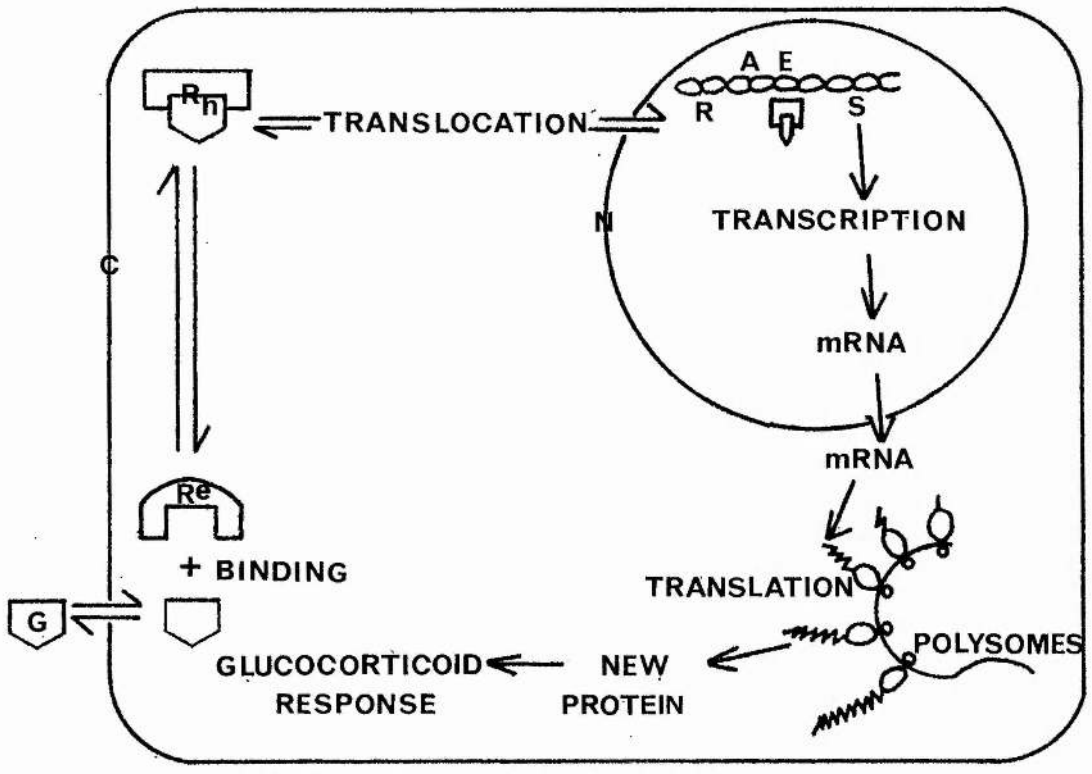


FIG. 5 The Hormone - receptor - chromatin relationship in glucocorticoid action (after Baxter and Forsham, 1974).

G = Glucocorticoid

Re = Receptor

R_h = Receptor/glucocorticoid complex (with altered receptor conformation)

R = Repressed gene
A = Acceptor site
E = Effector site
S = Structural gene
} Chromatin

C = Cytoplasmic membrane of 'target' cell

N = Nuclear membrane of 'target' cell

A B S T R A C T

It has been demonstrated that administration of prednisolone to rats causes loss of RNA and protein in the gastrocnemius muscle and increases in RNA and protein levels in the liver.

These changes have been shown to involve alterations in RNA turnover in the two tissues. In liver, rate of synthesis of ribosomal RNA was shown to be increased and its rate of breakdown decreased following administration of prednisolone. In muscle, the steroid caused net decreases in rates of synthesis and breakdown of ribosomal RNA. These prednisolone-induced alterations in RNA turnover were not the result of changes in the RNA precursor pool in the tissues.

Prednisolone treatment was shown to cause an increase in the activity of the RNA polymerase believed to be responsible for ribosomal RNA synthesis in the liver. This enzyme activity in the muscle was decreased following administration of prednisolone. Ribonuclease activity was decreased in the liver and muscle of prednisolone-treated animals. These alterations of enzymic activities could explain the observed changes in RNA turnover in prednisolone-treated animals.

More prednisolone binding occurred in the liver cytosol than occurred in the muscle cytosol. Prednisolone receptors in the muscle appeared to be of a smaller molecular size than receptors in the liver. The prednisolone-receptor complexes from muscle were less stable in a low ionic environment than complexes from the liver. These quantitative and qualitative differences may have dictated the response of each tissue to prednisolone.

In the course of this work conditions have been established for isolating cytoplasmic RNA from rat gastrocnemius muscle in an essentially undegraded form and in good yield.

2. MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Wherever possible 'Analar' grade chemicals and reagents were used in these studies.

Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate was obtained as a white powder from British Drug Houses (Poole, England). Before use it was purified by extraction in boiling ethanol. 100g of the powder were poured into 3 l of boiling ethanol and boiled for 10 min. with continuous stirring. Thereafter it was filtered, hot, through a ¼-inch thick Hyflo supercel pad supported on a Whatman's No. 1 filter paper on a Buchner funnel. The filtrate was allowed to stand overnight at 4°C to allow crystallisation of SDS. The crystals were collected by filtration and the material dried by washing with diethyl ether, filtering again, and finally dried at 37°C overnight. The yield was 60% of the starting material.

Hyflo supercel

Hyflo supercel was obtained as a white powder from Koch Light Laboratories (Bucks, England). Before use it was washed with 2 vol of 1.0M NaOH and collected on Whatman's No. 1 filter paper by vacuum filtration. Thereafter the material was washed with 3 vol of 1.0M NaOH followed by several washes with distilled water until the filtrate was neutral with respect to pH. The preparation was next washed with 5 vol 1.0M HCl and then with distilled water to a neutral pH. The material was dried at 37°C and stored at 20°C until required.

Bentonite

Technical grade bentonite was obtained as a brown powder from

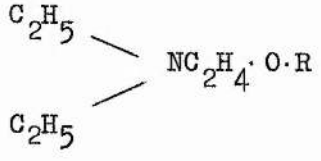
British Drug Houses (Poole, England). Before use it was purified by suspending 5g in 100ml of distilled water. The suspension was stirred vigorously for 20 min. at 20°C and coarse particles were sedimented by centrifugation at 100g on the MSE 6L centrifuge for 10 min. at 10°C and discarded. The fine clay suspension was decanted off and centrifuged at 9000g for 20 min. at 10°C. The supernatant was discarded and the sediment was resuspended in 40ml of 0.01M sodium acetate buffer, pH 6.0. After the suspension was stirred vigorously for 20 min. at 20°C, it was centrifuged at 9000g for 10 min. and the supernatant was discarded. This buffer wash cycle was repeated until the supernatant liquid registered a negligible optical density (< 0.04) at 260nm. Finally the material was dried at 37°C and stored at 20°C.

Activated charcoal

Animal charcoal was obtained as a black powder from British Drug Houses (Poole, England). Before use it was activated by washing 180g successively with 5 l of 0.1M HCl, 2 l 5% (w/v) KCl, 200ml 0.1M $Ba(OH)_2$ and 2 l 5% (w/v) KCl. After each wash the material was sucked dry over a Whatman's No. 42 filter paper in a Buchner funnel. Following the last wash, the material was incubated at 100°C overnight. The activated charcoal was stored in an air-tight jar at 20°C.

DE-52 Cellulose

DE-52 cellulose is the anion exchange resin diethylaminoethyl cellulose whose active group is of the following composition :



where R represents a glucose unit in the cellulose chain.

The resin was obtained from Whatman Biochemicals Ltd. (Maidstone, England) and before use freed of 'fines' by suspending the desired quantity in 10 vol of distilled water and pouring off particles which did not settle under gravity after the suspension had stood for 1 h at 4°C. The resin was then washed with gentle stirring in 10 vol 2.0M NaOH and, thereafter, in 10 vol 4.0M HCl. Each wash was maintained for 30 min. The resin was finally washed repeatedly with 0.001M sodium phosphate buffer, pH 7.4 until the pH of the supernatant liquid rose to 7.4. The resin was equilibrated with the buffer overnight at 4°C before use.

Cirrasol

Cirrasol was a gift from Imperial Chemical Industries Ltd. (Manchester, England). It was stored at 20°C and used without further purification.

Folin and Ciocalteau phenol reagent

Folin and Ciocalteau phenol reagent was obtained from British Drug Houses (Poole, England). It was stored in a dark bottle at 4°C.

Bovine Serum Albumin

Bovine serum albumin (Fraction V powder) was obtained from Sigma Chemical Co. Ltd. (London, England). It was stored at -15°C in an airtight jar containing granules of silica gel and was used without further purification.

Desoxyribonucleic Acid (DNA) standard

DNA from calf thymus was obtained from Sigma Chemical Co. Ltd. (London, England). It was stored at -15°C and was used as an aqueous

solution at a concentration of 40μg per ml.

Ribonucleic Acid (RNA) Standard

RNA isolated from bakers' yeast (Saccharomyces cerevisiae) was used as the standard RNA in this study. The choice of bakers' yeast RNA as a suitable standard was based on its easy extraction in essentially pure form from this readily-available source. Significant differences in purine/pyrimidine ratios have been shown to exist between RNAs from different sources (Adams et al, 1976) and it is acknowledged that in colorimetric determinations of RNA which measure only purine-bound ribose (as in Orcinol reaction) considerable error is introduced if the base ratio of the standard differs markedly from that of the material being assayed. However, in this study, such errors affect RNA levels in 'control' and 'test' animals to the same extent and should not influence any differences detected between these two groups.

The extraction procedure was that described by Crestfield et al, (1955). This method depends on the ability of SDS to dissociate RNA from its accompanying protein without depolymerising the nucleic acid. The detergent-protein complex is salted out and removed by centrifugation. The RNA is then subjected to further purification steps.

300g of bakers' yeast purchased from a local bakery were cut into fine pieces which were then poured into 1.0 l of an aqueous solution, at 100°C, containing 2% (w/v) SDS, 4.5% (v/v) ethanol, 12.5 mM NaH_2PO_4 and 12.5 mM Na_2HPO_4. The mixture was stirred constantly with an overhead motor-driven stirrer for 2 min. during which the temperature was maintained between 85° and 90°C. To

reduce foaming the beaker and its contents were transferred to a boiling water bath and heating and stirring were continued for a further 5 min. This combination of heat and detergent treatment is believed to inactivate completely any ribonucleases which could cause enzymic degradation of the RNA (Crestfield et al, 1955). At the end of the heating period the hot mixture was poured into a 2-l beaker cooled in ice. The suspension was stirred in the cold room until the temperature dropped to 4°C after which the mixture was centrifuged at 600g ave. for 30 min. at 2°C to sediment the debris. Crude RNA was precipitated from the supernatant fractions by pouring the pooled fractions into 2 vol of ice-cold ethanol. The precipitate was collected by centrifugation at 600g ave. for 15 min. at 2°C and washed twice with 300ml portions of ice-cold 67% (v/v) ethanol containing 2ml 2.0M NaCl. Maximal removal of SDS and protein material during this washing was ensured by stirring the precipitate continuously with a glass rod while the wash solution was added slowly. NaCl was necessary to facilitate floculation and recovery of RNA. The crude RNA was suspended in 300ml of 80% (v/v) ethanol and the mixture kept overnight at 4°C.

The crude RNA was then sedimented by centrifugation at 600g ave. for 15 min. at 2°C and dissolved in 300ml of distilled water. The turbid solution was titrated to a neutral pH with 1.0M acetic acid and clarified by centrifugation at 35,000g ave. for 1 h at 2°C on the MSE super 18 centrifuge. Sufficient solid NaCl was added to the pooled supernatant fraction to make the medium 1.0M with respect to the salt in order to dissociate histones from the RNA (Marko and Butler, 1951). The mixture was kept in ice for 1 h during which

time a gel of RNA was formed. The yellow supernatant fluid was separated by centrifugation at 600g ave. for 1 h at 2°C and discarded. The gel was washed 3 times with 200ml portions of ice-cold 67% (v/v) ethanol containing 2ml 2.0M NaCl and dissolved in 200ml of ice-cold distilled water by stirring. This solution was transferred to a dialysis bag and dialysed against 6 changes of distilled water in the cold room at 4°C for 36 h. The turbid solution was filtered by vacuum through a ¼-inch thick pad of Hyflo supercel. This filtration step removed any residual protein leaving a clear colourless filtrate which was lyophilised. 1.9g of RNA was obtained from 300g of the bakers' yeast and the white fluffy material was stored at 0°C in an airtight glass bottle inside a plastic container containing granules of silica gel. The protocol for the isolation of RNA from bakers' yeast is presented in Fig. 6.

Water-saturated phenol reagent

Water-saturated phenol reagent was prepared from phenol crystals obtained from British Drug Houses (Poole, England). 300g of the crystals were dissolved in 600ml of distilled water by stirring the mixture at 20°C for 45 min. The cloudy solution was allowed to stand at 4°C in the dark for 18 h. The lower phenol phase which separated out during this time was made 0.1% (w/v) with respect to 8-hydroxyquinoline (British Drug Houses, Poole, England) to prevent atmospheric oxidation of the phenol (Kirby, 1964). The reagent was stored in a dark bottle at 4°C.

2-methoxyethanol

2-methoxyethanol was obtained from British Drug Houses (Poole, England) as a technical grade solvent and was freed of contaminating

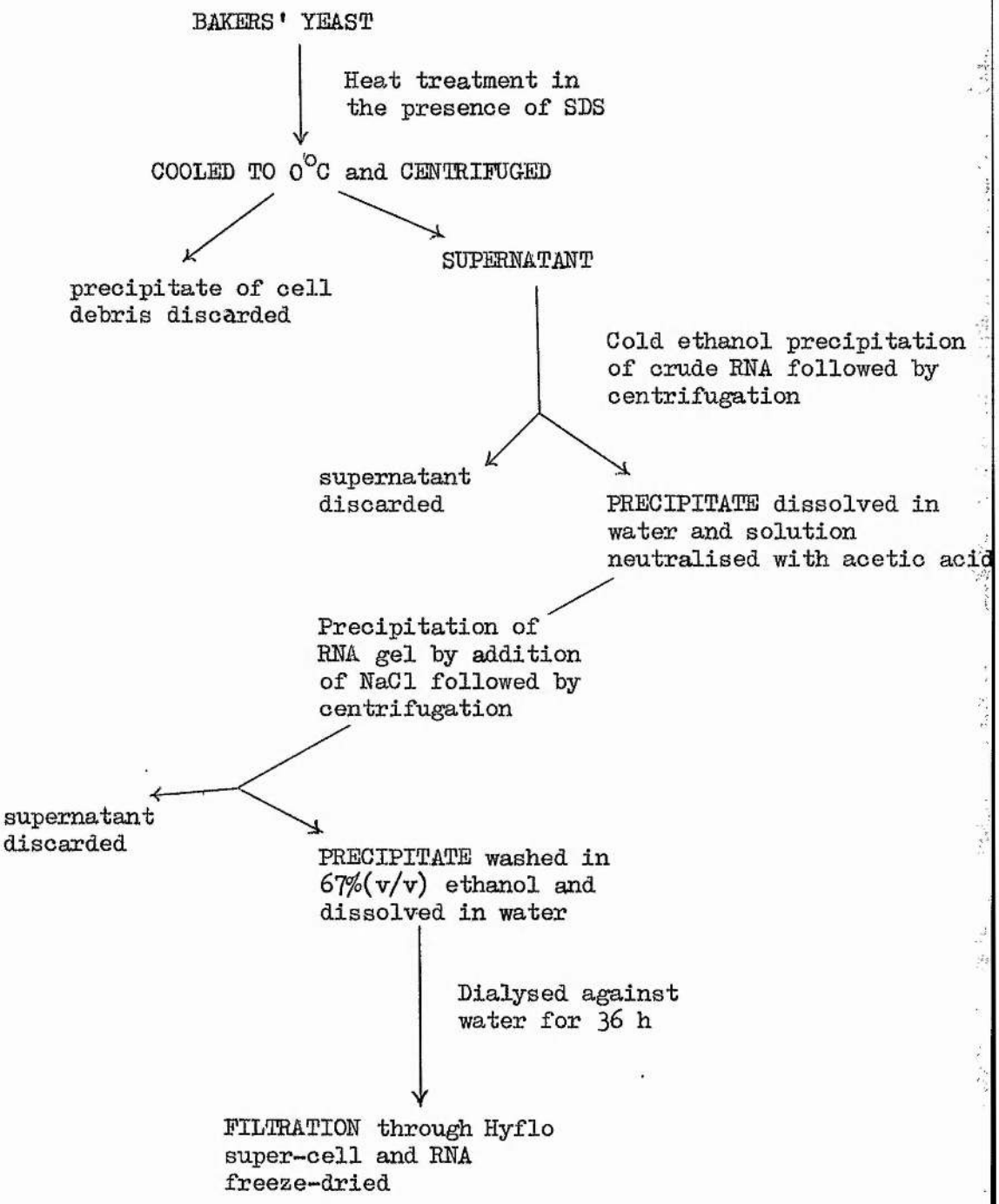


FIG. 6 Protocol followed for the isolation of RNA from bakers' yeast. Experimental details are described on page 34.

peroxides by distillation at 124°C. For this process, an aqueous solution containing 30g hydrated ferrous sulphate and 3ml conc H_2SO_4 was added to 3 l of the solvent. The mixture was distilled and the first 5ml of the distillate and the last 100ml of the solvent were not collected. The material collected showed no absorption at 280nm and had a negligible absorption (< 0.05) at 260nm. It was stored in a dark bottle at 4°C to minimise oxidation.

RADIOISOTOPES

All radioisotopes used in this study were obtained from the Radiochemical Centre (Amersham, England) and they were stored in borosilicate vials shielded with polythene. Radioactivity was counted in glass vials with aluminium-lined cork caps. These vials contained 5ml of the scintillation fluid and gave background counts ranging from 18 to 23cpm in the pre-set ^3H window. The scintillation fluids used were obtained from British Drug Houses (Poole, England) and were toluene- or dioxane-based. 0.6% (w/v) 2, 5-diphenyloxazole (PPO) was the primary scintillator in these fluids and 0.02% (w/v) 1, 4 di-2-(5-phenyloxazolyl benzone) (POPOP) served as the secondary scintillator necessary to shift the wavelength of photon emission from around 370nm to 420nm, where the photomultiplier tubes were more responsive.

[5-^3H] Orotic Acid

[5-^3H] Orotic Acid was obtained as an aqueous solution in borosilicate multidose vials ("Duoseal" vial) and was stored at 0°C. Portions withdrawn with a sterile needle and syringe and diluted as desired just before use. Specific activities of the material showed batchwise variation ranging from 19 to 25 Ci per mmol.

The stability and the radiochemical purity of the material on storage was tested by paper chromatography. 20µl of a sample which had been stored for 9 months were spotted onto the chromatography paper (Whatman No. 33 cut into a square 19 x 19cm) and dried immediately with an air drier. As a marker 20µl of freshly-prepared unlabelled Orotic acid (100mg/ml in distilled water) were spotted on the same horizontal line as the ^{3}H-labelled sample and similarly dried. Ascending chromatography using a mixture of isopropanol/water/HCl (130:37:33) as developing solvent was allowed to proceed for 7 h at 20°C in a sealed chromatography unit. At the end of this period the paper was removed and dried in a hot air chamber. The position of the marker was detected by examination under ultraviolet light and its distance from the origin was measured. For the sample, strips, each 0.5cm wide, were cut sequentially along the direction of development from the origin to the solvent front. The radioactivity on each strip was counted in a toluene-based scintillation fluid (NE 233) using an Intertechnique liquid scintillation spectrometer (Model SL30). The highest radioactivity count was obtained from the strip cut 7.5cm from the origin which coincided with the position of the marker (Fig. 7). It was therefore concluded that almost all the tritium label was present as orotic acid and that the material had not undergone any significant deterioration.

[6, 7(n) - ^{3}H] Prednisolone

[6, 7(n)-^{3}H] Prednisolone was obtained in a toluene/ethanol (9:1) mixture. It was stored at -15°C and, after each use was resealed under nitrogen. The specific activity of the material was 46 Ci per mmol.

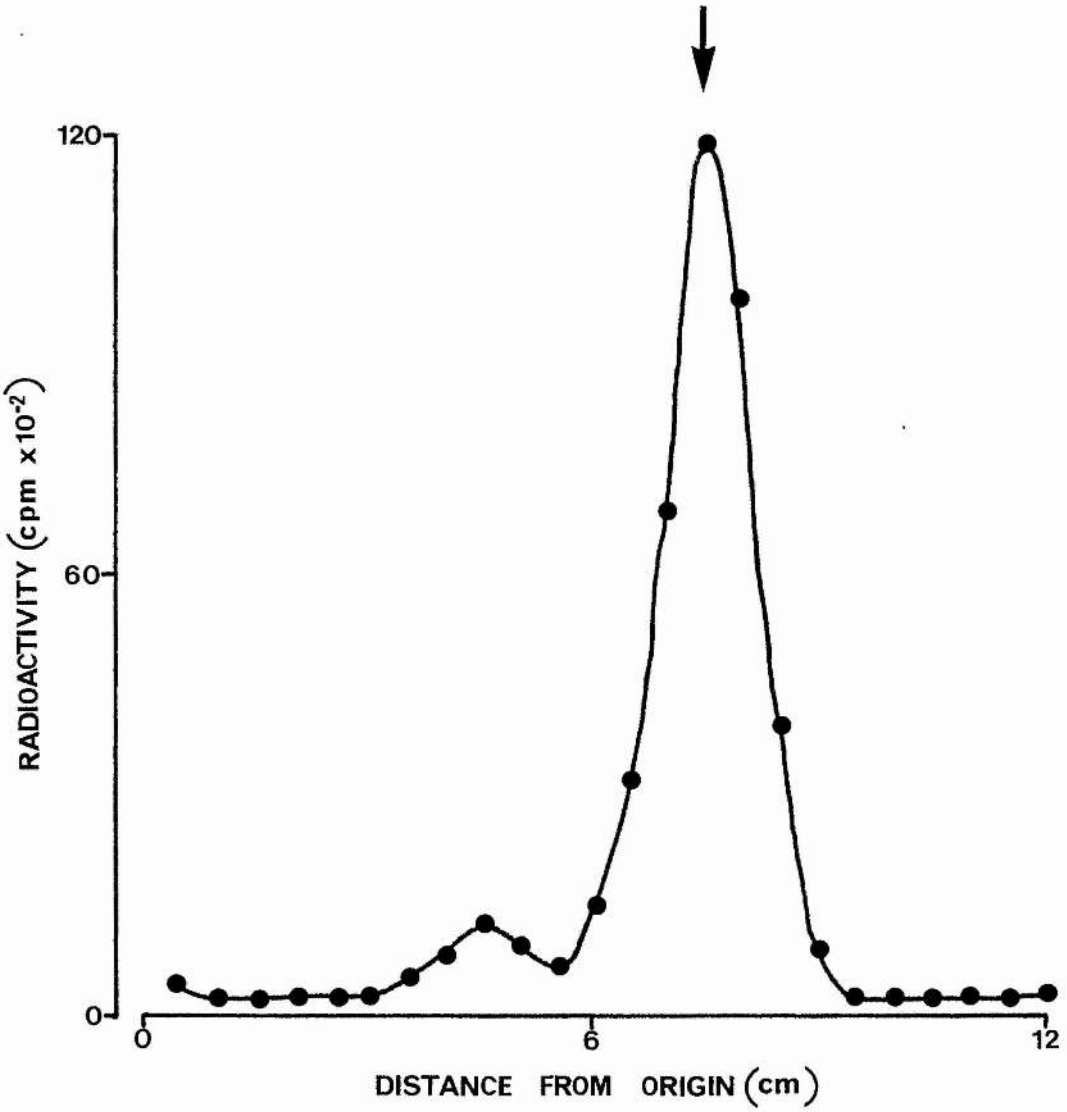


FIG. 7 Paper Chromatography of ^{3}H-Orotic Acid :

Ascending chromatography was carried out on Whatman No. 33 filter paper for 7 h at 20°C. Moving liquid phase was an isopropanol/water/HCl mixture (130:37:33). Strips were cut out and counted for radioactivity as described on page 98 . The arrow indicates the position of non-radioactive orotic acid applied on the same horizontal level as the radioactive sample and developed in parallel with the radioactive sample.

[5, 6-^{3}H] Uridine 5 -triphosphate (^{3}H-UTP)

^{3}H-UTP was obtained as the ammonium salt in 50% (v/v) aqueous ethanol. The specific activity of the material ranged from 40 - 60 Ci per mmol. It was stored at -15°C.

Treatment of Animals

Male Wister rats of body weight ranging between 140 and 190g were used in this study. They were housed individually in wire mesh cages in a windowless room in which lighting was regulated to provide a 12-h light and 12-h dark cycle (light on from 07.00 h). Temperature in this room was maintained at $70^{\circ}F \pm 1^{\circ}F$. As a pretreatment before commencement of any experiments, the animals were fasted overnight. Thereafter each animal was fed daily, at 16.00 h, 12g 'Diet 41B' containing 14% (w/w) dried skimmed milk (North Eastern Agricultural Co-operative Ltd., Burksburn, U.K.). The animals consumed their daily allowance of food throughout the experimental period and were allowed water ad libitum. Experimental animals and their controls were paired on the basis of initial fasted body weight.

Preparation of rat liver cell sap

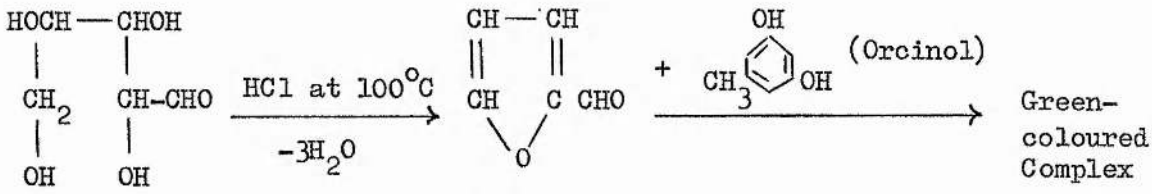
Liver, freshly excised from the animal, was washed in 0.85% (w/v) saline to remove blood. Thereafter it was chopped into fine pieces with scissors and suspended in 20 vol 0.05M Tris-HCl buffer, pH 7.5 (at $20^{\circ}C$), containing 0.001M $MgCl_2 \cdot 6H_2O$, 0.025M KCl and 0.25M sucrose. The suspension was homogenised using a Potter-Elvehjem (1936) homogeniser with a close fitting pestle and was centrifuged at 40,000g ave. for 90 min. at $3^{\circ}C$ in the MSE superspeed 65 centrifuge. The supernatant fluid was carefully withdrawn using a pasteur pipette to avoid contamination from the white lipid layer which formed a plug at the top of the supernatant layer. This supernatant fluid was taken as the cell sap. It was stored frozen in small portions and, for use, was thawed out and diluted ten-fold

with distilled water.

Methods employed for the estimation of ribonucleic acid (RNA)

RNA was estimated either by the orcinol method or by the ultraviolet absorbance of the nucleic acid at 260nm.

The orcinol method (Schneider, 1957) depends on the reaction of pentose sugars with hot strong acid to form the heterocyclic furfural which condenses with orcinol to yield a green-coloured product the absorbance of which is measured at 660nm.



Pentose Furfural

The conversion of the ribose units of RNA to furfural depends on the prior removal of the associated base. Purine bases are effectively removed by the acid used in the orcinol reagent but pyrimidine bases persist to some extent and render pyrimidine-bound ribose unreactive. This could lead to serious errors where the purine/pyrimidine ratio differs from that of the RNA used as standard. The molar proportions of these bases in yeast RNA (Crestfield et al, 1955) are however closely equivalent to those in rat liver RNA (Adams et al, 1976) and any errors arising from purine/pyrimidine ratios are therefore minimal. The orcinol method was used to measure RNA where ultraviolet measurements were liable to interference from cellular materials (e.g. protein and DNA) or reagents (e.g. trichloracetic acid).

Orcinol reagent was prepared by dissolving 0.5g $FeCl_3$ in 100ml

of conc HCl and adding orcinol to a final concentration of 1% (w/v). For assay, 3ml of a suitable dilution of the sample in 1.0M perchloric acid was mixed with 3ml of the orcinol reagent. This mixture was heated in a boiling water for exactly 20 min. Thereafter it was cooled in ice and its absorbance measured at 660nm against a reagent blank prepared by subjecting 3ml 0.5M perchloric acid to the assay procedure. A standard calibration plot (Fig. 8) was obtained by assaying serial dilutions of yeast RNA.

The green colour developed in the orcinol reaction was proportional to RNA concentration only when the final RNA concentration in the assay mixture was less than 300µg. Care was taken to use exact quantities of $FeCl_3$ (to keep blank readings constant) and HCl (which governs the rate of furfural release from ribose) in the preparation of the orcinol reagent. Heating time and temperature were also found to be critical factors in the development of the green colour.

The second method employed to estimate RNA utilised the characteristic absorption of ultraviolet light by purine and pyrimidine bases in the region of 260nm. This method was used in situations where the presence of traces of sucrose would interfere with the orcinol assay method. In extracting RNA which was to be measured by this method, the period of alkali digestion was limited to 1 h. This was necessary to avoid errors arising from the release of proteins and peptides into solution as well as avoiding deamination of cytidylic acid to yield uridylic acid which absorbs light maximally around 260nm (Fleck and Munro, 1962).

Extracts containing RNA were suitably diluted with 0.2M

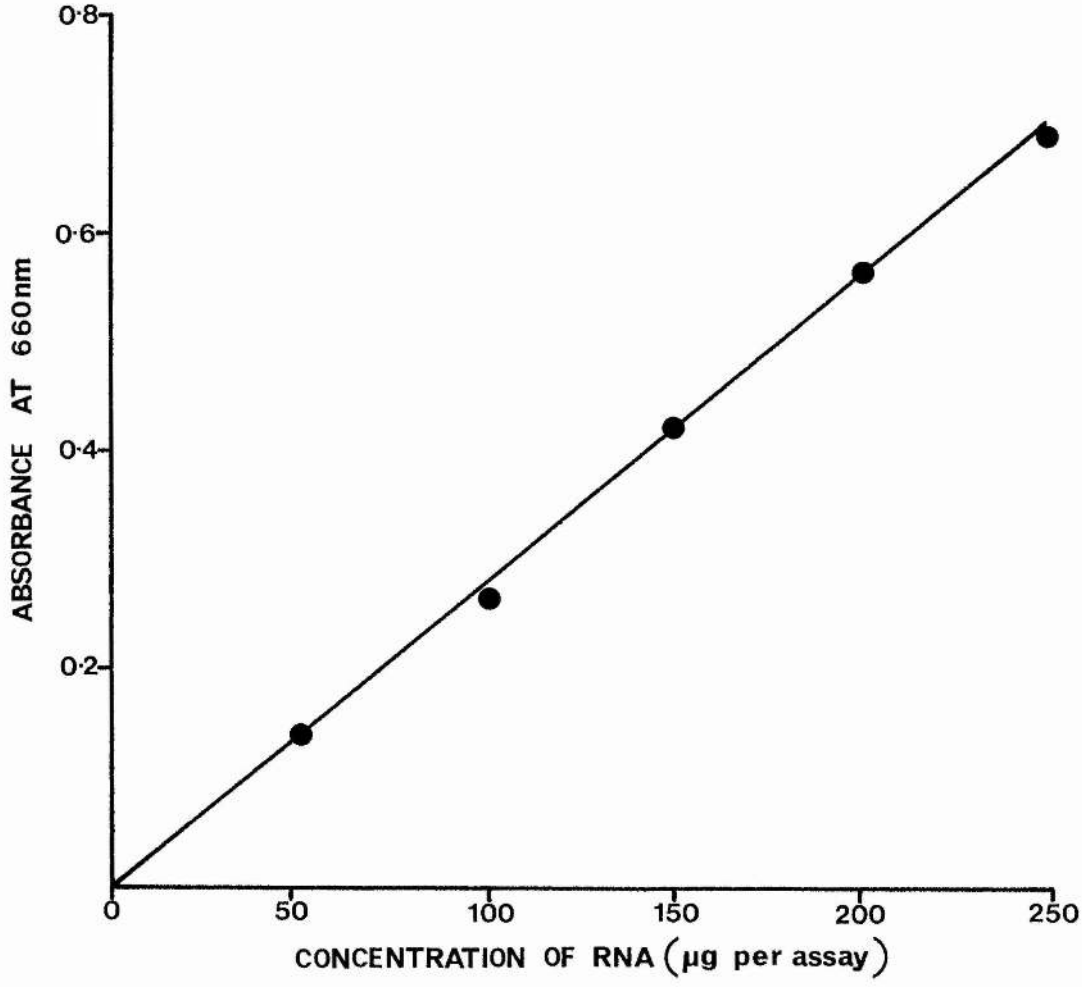


FIG. 8 Calibration Curve for RNA estimation by the orcinol method. Yeast RNA was used as the standard. Details of the assay procedure are described on page 43.

perchloric acid. The ultraviolet absorption of the solution was then measured at 260nm against a blank containing only 0.2M perchloric acid. A 1% (w/v) solution of yeast RNA in this acid was found to have an A_{260} of 230 and calculation of RNA measured by this method was based on this factor. The optical properties of a mixture of nucleotides is believed to be those of their component nucleotides (Munro and Fleck, 1966) and it is acknowledged that yeast RNA may have quite a different absorbance from that of similar amounts of rat liver or muscle RNA. However, any such difference would be expected to affect control and prednisolone-treated rats to a similar extent and, it was felt, would not invalidate these studies which were aimed solely at detecting the difference in RNA content between the two groups of animals.

Method employed for the estimation of deoxyribonucleic acid (DNA)

DNA was assayed as described by Burton (1955). Mild acid hydrolysis transforms sugar residues originally combined with purine bases in DNA into ω-hydroxy laevulinic aldehyde. This labile aldehyde and some of its derivatives are believed to be responsible for the blue colour formed with diphenylamine (Stacey et al, 1946). Acetaldehyde acts to potentiate this colour development presumably by also reacting with the aromatic amine and bringing about ring closure. The concentration of perchloric acid in the DNA sample is critical to linear colour development and a perchloric acid concentration of 0.5M is believed to be optimal (Burton, 1955).

The diphenylamine reagent was prepared by dissolving 1.5g diphenylamine in 100ml of glacial acetic acid and adding 1.5ml conc

H_2SO_4 to the solution. This reagent was stored in the dark. Before use, 0.1ml of an aqueous solution containing 16mg acetaldehyde per ml (stored in the dark) was added to every 20ml of the diphenylamine solution.

Samples, containing 20 - 40µg DNA, were made up to 2ml with 0.5M perchloric acid. 4ml of the diphenylamine reagent containing aldehyde were added and the blue colour was developed by incubating the solution for 16 - 20 h at 30°C. This colour was measured at 600nm against a blank prepared by subjecting 2ml 0.5M perchloric acid to the assay procedure. Calf thymus DNA served as standard and was used as an aqueous solution at a concentration of 40µg per ml. 1ml of the standard solution was mixed with 1ml 1.0M perchloric acid and, along with samples containing DNA, subjected to two 15-min. incubations at 70°C in a water bath. The mixture was cooled in an ice bucket, the diphenylamine reagent added, and colour allowed to develop as described above. The calibration curve is shown in Fig. 9.

Methods employed for the estimation of proteins

Three methods of protein estimation were used in the course of these studies.

(a) Biuret method

Samples containing protein at concentrations above 250µg per ml were assayed for protein using the 'biuret' method described by Gornal et al, (1949). This method measured the intensity of the violet colour produced by the reaction of cupric ions in the 'biuret' reagent and protein peptide bonds under the alkaline conditions of the assay.

To prepare the 'biuret' reagent 1.5g $CuSO_4 \cdot 5H_2O$ were dissolved

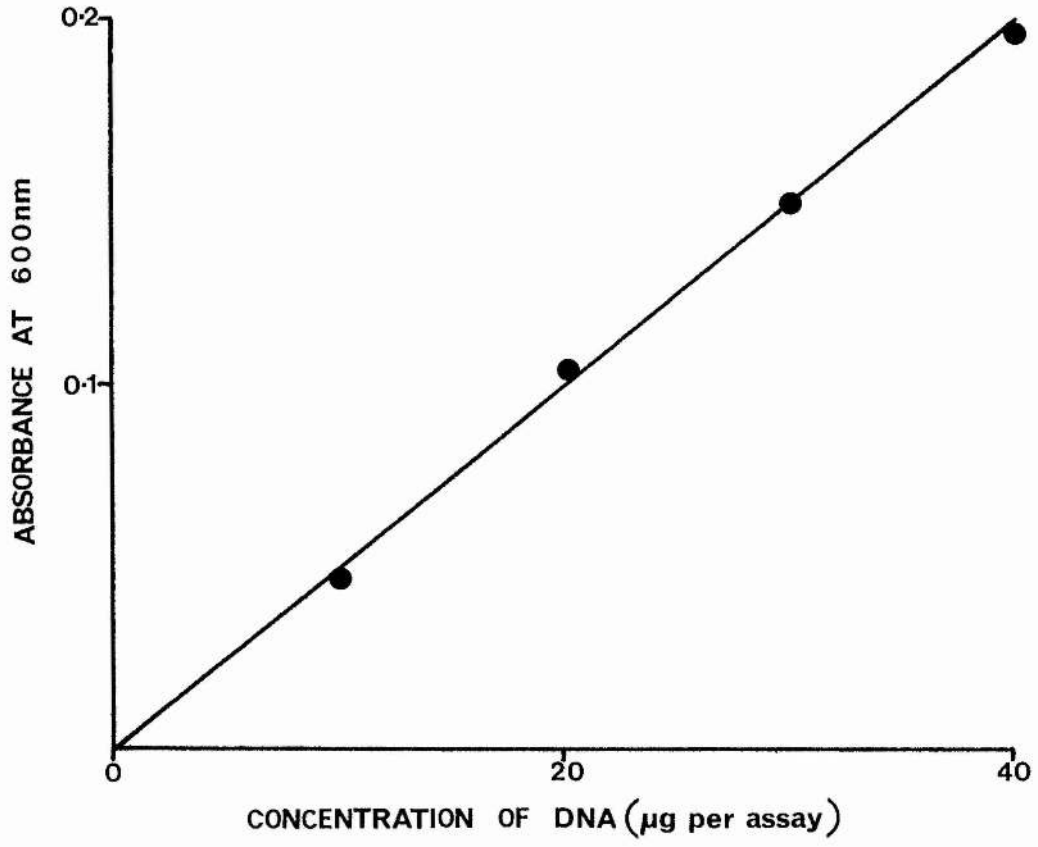


FIG. 9 Calibration Curve for DNA estimation by the diphenylamine method. Calf thymus DNA was used as the standard. Details of the assay procedure are described on page 47.

in 100vol distilled water and mixed with 6g potassium tartrate dissolved in water. This mixture was added, with swirling, to 300ml 10% (w/v) NaOH and the final volume was made up to 1.0l with distilled water. This reagent was stable and retained optical clarity for several weeks at 20°C.

For assay, 1.0ml of the test sample was mixed with 4.0ml of the 'biuret' reagent and allowed to stand for 30 min at 20°C. Thereafter the absorption of the complex was measured at 540nm against a reagent blank containing water instead of the sample. A standard calibration plot (Fig. 10A) was prepared by subjecting 1.0ml of serial dilutions of a 10mg per ml aqueous solution of bovine albumin to the assay procedure.

(b) Micro-Kjeldahl method

The micro-Kjeldahl method, as modified by Ma and Zuazago (1942) was used to measure the protein content of the commercial preparations of bovine albumin used as standards for protein assay. These preparations vary significantly in their water content, depending on storage conditions, and the Kjeldahl assay served to check and correct for any such errors.

1.5ml of an aqueous solution containing 1.0mg of the bovine albumin per ml were pipetted into a Kjeldahl digestion tube. 2.0ml nitrogen-free conc H_2SO_4 were added followed by a trace of finely-ground copper-selenium catalyst and a few anti-bump granules. The tube was then transferred to the gas burners and acid digestion was allowed to proceed for 16 h. The digest was cooled and the contents along with washings transferred to the micro-Kjeldahl distillation apparatus. Protein nitrogen, now present as $(NH_4)_2SO_4$

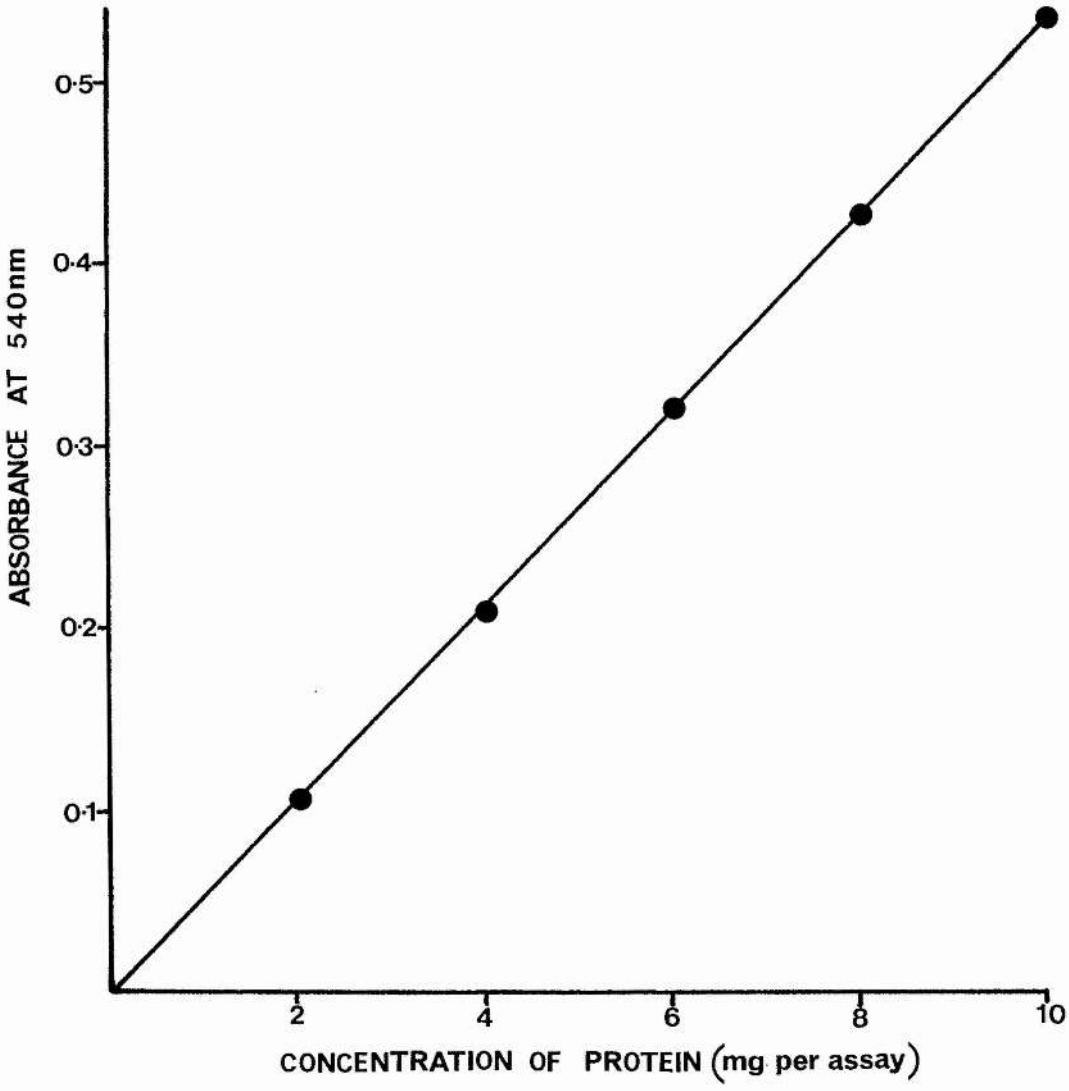


FIG. 10A Calibration Curve for protein estimation by the 'biuret' method. Bovine albumin was used as the standard. Details of the assay procedure are described on page 47.

was measured by adding 10ml 40% (w/v) NaOH to the digest and steam-distilling the liberated ammonia into a 5.0ml aqueous solution of 2% (w/v) H_3BO_3 cooled in ice. The H_3BO_3 solution contained 5 drops of a mixed indicator prepared by mixing 5vol 0.1% (w/v) bromocresol green in 95% (v/v) ethanol with 1vol 0.1% (w/v) methyl red in 95% (v/v) ethanol. Complete recovery of the ammonia was obtained in about 10.0ml of distillate which turned the mauve H_3BO_3 solution blue. $^{M}/_{175}$ HCl was then used to titrate this blue solution back to a pink end-point.

$$1.0\text{ml } 1.0\text{M HCl} \equiv 14\text{mg nitrogen}$$

$$\therefore\ 12.5\text{ml } {}^{M}/_{175}\text{ HCl} \equiv 1.0\text{mg nitrogen}$$

The nitrogen content was converted to protein content by multiplying the nitrogen content by 6.25, the assumption being made that 16% of the mass of protein is nitrogen. The result obtained was compared with the measured weight of the albumin and the ratio of these two values was used as a correction factor to allow for the water content of the albumin.

A comparative study in which protein extracts from rat liver and gastrocnemius muscle were analysed by both the 'biuret' and the Kjeldahl method was carried out. A close similarity was observed in the results obtained using the two methods (Table 1).

Table 1. A comparison of 'biuret' and Kjeldahl methods of protein analysis

Sample	Protein Concentration (mg/100ml)	
	Biuret assay	Kjeldahl assay
Liver	1796 ± 12	1809 ± 19
Muscle	453 ± 4	466 ± 10

The results are the means from 4 experiments $\pm$ S.E.M.

(c) Folin-Lowry method

Assays of proteins at concentrations below 250µg per assay were performed as described by Lowry et al (1951). This method is based on the copper-Folin reaction in which copper-treated protein reduces phosphotungstic/phosphomolybdic acid (the active ingredients in the Folin-Ciocalteau phenol reagent) in alkaline conditions to give dark greenish blue-coloured complex the absorbance of which is read at 500nm. Although the absorbance measured in this reaction has been reported to deviate slightly from linearity with protein concentration and its intensity varies with different proteins, the Folin-Lowry method is much more sensitive than the biuret method. It is therefore the preferred method for assays of small amounts of protein. Furthermore, the relative chromogenicity of mixed tissue proteins does not show too great a variability from tissue to tissue (Lowry et al, 1951).

For assay, 0.5ml of the sample was mixed with 5.0ml of an alkaline copper reagent prepared freshly each day by mixing 25ml of Reagent A (2% (w/v) Na_2CO_3 in 0.1M NaOH) with 0.5ml of Reagent B (prepared by dissolving 1.0g sodium potassium tartrate and 0.5g $CuSO_4 \cdot 5H_2O$ in 20ml distilled water, adding 10ml 0.1M NaOH and making the volume up to 100ml with water). The mixture was allowed to stand at 20°C for 10 min. Thereafter 0.5ml Folin and Ciocalteau phenol reagent, diluted before use with 1.36 vol distilled water to make it 1.0M with respect to acidity, was added very rapidly and mixed instantly. After 30 min. at 20°C the absorbance of the solution at 500nm was read against a blank in which 0.5ml distilled water replaced the sample. A calibration plot (Fig. 10B) was

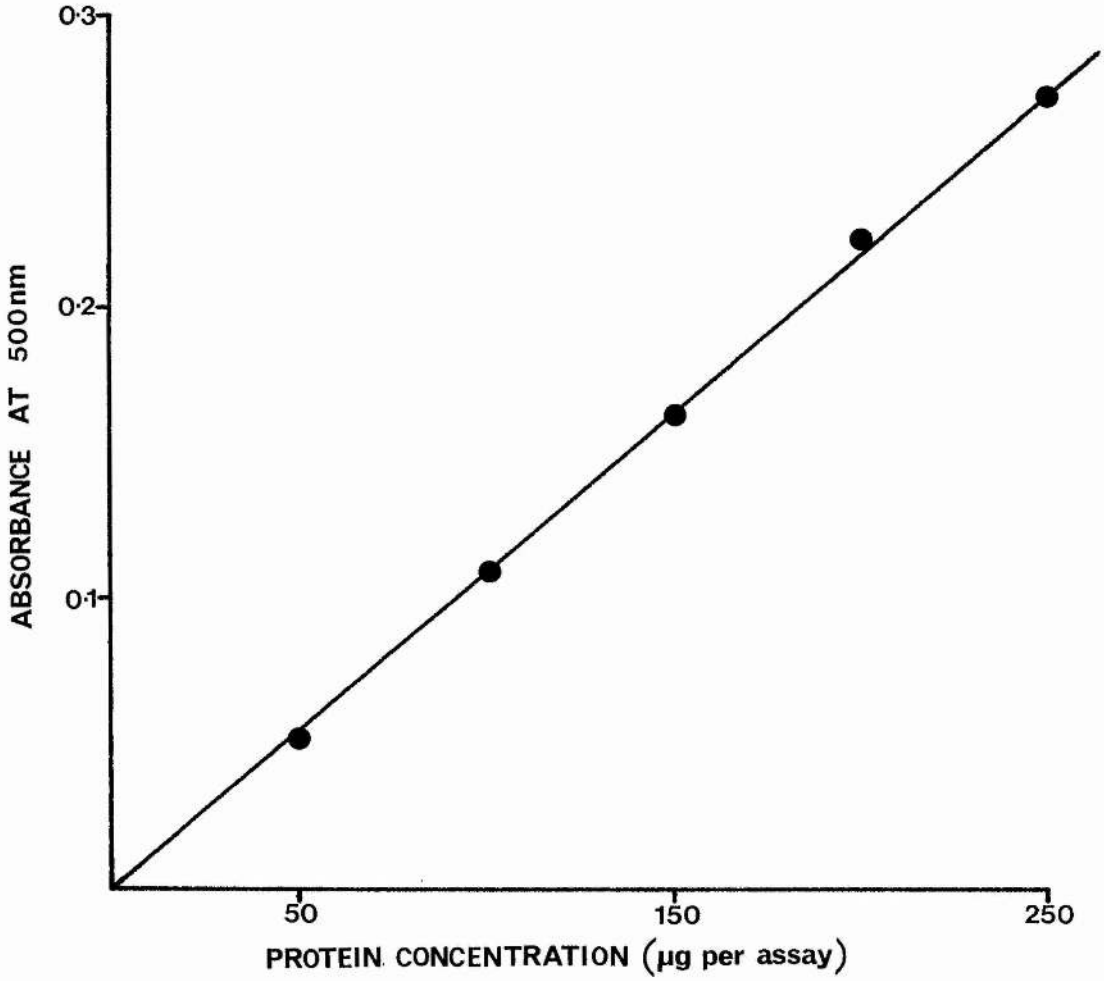


FIG. 10B Calibration Curve for the estimation of protein by the Folin-Lowry method. Bovine albumin was used as standard. Experimental details are as described on page 52.

obtained by subjecting dilutions of a solution of bovine albumin in 0.85% (w/v) NaCl to the assay procedure.

The concentration of NaOH used in this assay effectively neutralizes excess H_3PO_4, and Na_2CO_3 buffers the mixture at pH 10 where colour development is maximal. However, at this pH the reactivity of the phenol reagent is only short-lived, due to the dissociation of phosphate from molybdate, and it is essential, therefore, not to delay mixing once the reagent has been added.

Extraction of total RNA, DNA and protein from rat gastrocnemius muscle and liver

RNA, DNA and protein were extracted from muscle and liver tissues using a method based on that described by Schmidt and Thannhausser (1945).

Gastrocnemius muscles were removed rapidly from the animal, weighed frozen in liquid nitrogen, and then pulverized into fine fragments in a stainless steel mortar cooled with liquid nitrogen. The muscle fragments were suspended in 10 vol ice-cold 0.3M KCl and homogenised using the Silverson homogeniser (Silverson Machines Ltd. Bucks, England) driven at full speed. This homogenisation was carried out in two bursts each lasting 30 s. The homogenate was immersed in ice throughout and a 2-min. interval was interposed between each burst in order to maintain the temperature of the homogenate between 0° and $4^{\circ}C$. The homogenate was mixed with an equal volume of 20% (w/v) trichloroacetic acid and the mixture was kept at $0^{\circ}C$ for 20 min.

The liver was also removed rapidly from the animal, washed free of blood with ice-cold 0.85% (w/v) NaCl, weighed and stored in a frozen state until required. For extraction it was chopped into fine pieces and suspended in 3 vol distilled water. Homogenisation was performed as described above for muscle and 2 vol distilled water were subsequently added to the homogenate. An equal volume of 20% (w/v) trichloroacetic acid was added to this diluted homogenate which was then allowed to stand at $0^{\circ}C$ for 20 min.

Subsequent steps in the extraction process were the same for

both muscle and liver homogenates.

Acid-insoluble material was collected by centrifugation at 2000g ave. for 15 min. at 4°C. The precipitate was washed 3 times with 20ml ice-cold 10% (w/v) trichloroacetic acid to ensure complete removal of free nucleotides and other low molecular weight contaminants which might interfere with the subsequent estimation of nucleic acid content.

The preliminary removal of phospholipids with lipid solvents such as ethanol and ethanol/chloroform mixtures was considered unnecessary. Furthermore Munro and Downie (1964) have reported loss of RNA and protein into cold ethanol following trichloroacetic acid precipitation.

The trichloroacetic acid precipitate was treated with 20ml 0.3M KOH and incubated at 37°C for 18 h during which time RNA was completely dissolved and hydrolysed to mononucleotides. Protein estimations were performed on aliquots of this alkaline digest. DNA is not hydrolysed under these conditions because of the absence of an hydroxyl group on C_2, resulting in a failure to form cyclic nucleoside phosphates on exposure to alkali. In RNA it is this obligatory cyclic intermediate that is spontaneously hydrolysed to nucleoside 2′- or nucleoside 3′ -phosphates (Adams et al, 1976) as illustrated in Fig. 11.

The alkaline digest was cooled to 0°C and treated with 0.06ml ice-cold 10M perchloric acid per ml of digest to neutralise the alkali and acidify the medium sufficiently to cause maximal precipitation of protein and DNA. After standing in ice for 5 min. the precipitate was sedimented by centrifugation at 2000g ave. for

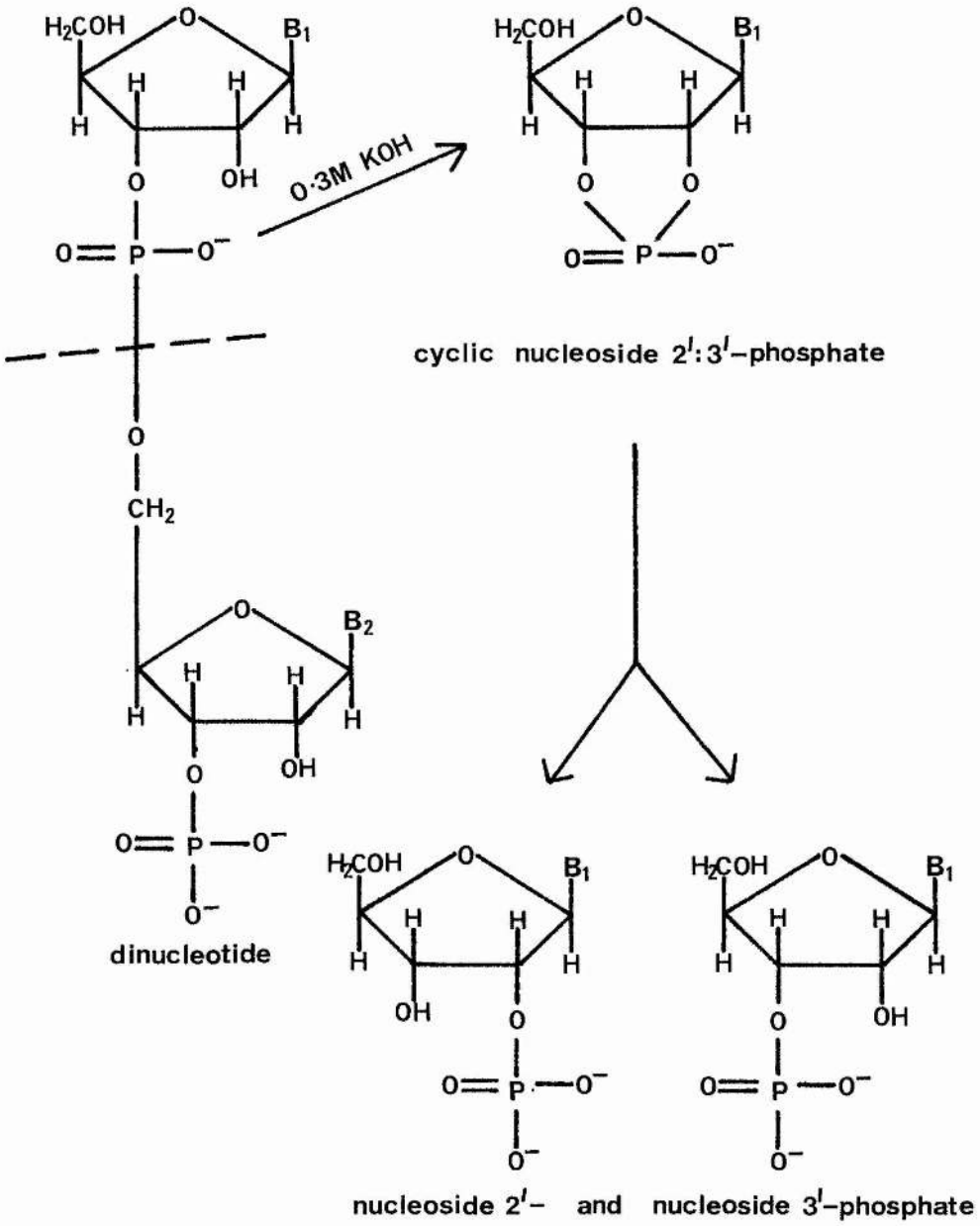


FIG. 11 Hydrolysis of a ribo-dinucleotide by alkali (after Adams et al, 1976).

B represents a purine or pyrimidine base.

10 min. and washed 2 times with 5ml 0.5M perchloric acid. The supernatant and washings were pooled and retained as the RNA fraction from which suitable aliquots were taken for the orcinol test.

The DNA of the precipitate was extracted by hot acid treatment. The precipitate was suspended in 10ml 1.0M perchloric acid and shaken at 70°C for 20 min. The suspension was cooled to 2°C and non-nucleic acid material was sedimented by centrifugation at 2000g ave. for 10 min. The hot acid treatment was repeated using 5ml 1.0M perchloric acid. The supernatants were pooled and aliquots taken for DNA assay by the diphenylamine method.

Isolation of ribosomes from rat gastrocnemius muscle and liver

Ribosomes were isolated from gastrocnemius muscle and liver using methods adopted from the procedures described by Chen and Young (1968) for muscle ribosomes and by Hirsch and Hiatt (1966) for liver ribosomes.

Gastrocnemius muscles from both hind legs of the rats were weighed and then frozen in liquid nitrogen to facilitate subsequent pulverization. They were pulverised into fine fragments in a stainless steel mortar cooled with liquid nitrogen. The fragments were suspended in 5 vol medium C (0.001M $MgCl_2$, 0.3M KCl, 0.05M Tris-HCl, pH 7.5 (at 20°C) and 1 drop of 1% (w/v) aqueous suspension of bentonite was added to protect RNA from enzymic degradation (Brownhill et al, 1959; Littauer and Sela, 1962).

Liver tissues were weighed fresh following extensive washings in ice-cold 0.85% (w/v) NaCl to remove any traces of blood. Thereafter, the liver was chopped into fine pieces and suspended

in 2 vol ice-cold medium C from which KCl had been omitted and to which 2 drops of 1% bentonite had been added.

Subsequent steps in the isolation procedure were identical for both muscle and liver and were carried out at 0°C unless reported otherwise. Homogenates of both tissues were prepared using the Silverson homogeniser driven at top speed. All of the muscle homogenate and 6.0ml of the liver homogenate were centrifuged at 27,000g ave. for 15 min. to sediment nuclei, mitochondria and cell debris. The resulting supernatants were treated first with 0.2ml 10% (w/v) solution of cirassol in medium C, with gentle stirring, and then with 1.0ml 10% (w/v) solution of sodium deoxycholate (Sigma Chemicals Ltd., London, England) in water, both detergents serving to dissociate ribosomes from the microsomes and other membranous structures. The preparation was allowed to stand for 1 h before 6.0ml medium C were added and a crude ribosomal pellet was sedimented by centrifugation at 105,000g ave. for 90 min. in an MSE super 65 centrifuge. The ribosomal pellet was rinsed with medium D (0.001M $MgCl_2$, 0.025M KCl, 0.05M Tris-HCl, pH 7.5 at 20°C) and allowed to soften overnight in 4.0ml medium D. Thereafter the ribosomes were resuspended in this medium using a Potter-Elvehjem homogeniser with a loose-fitting teflon pestle. The suspension was centrifuged at 600g ave. for 10 min. and an equal volume of medium E (0.1M $MgCl_2$, 0.05M Tris-HCl, pH 7.5 at 20°C) was added to the supernatant to raise the Mg^{++} concentration to above 50mM and thus cause an aggregation of the ribosomes. The precipitated ribosomes were collected after 1 h by centrifugation at 15,000g ave. for 15 min. This treatment with Mg^{++}

frees ribosomes from considerable protein material which remains in the supernatant following centrifugation (Hirsch and Hiatt, 1966). Further purification was achieved by resuspending the ribosomes in 4.0ml 0.1M KOH, using the Potter-Elvehjem homogeniser and centrifuging for 5 min. at 10,000g ave. to clarify the suspension and sediment any denatured proteins.

The quantity of ribosomes isolated by this procedure was measured by taking optical density readings of the suspension at 260nm on the basis that a suspension containing 90μg of ribosomes per ml has an A_{260} of 1.0 (Manchester, 1974). Yield ranged from 94 - 118μg ribosomes per g of muscle and 2,400 - 3,200μg ribosomes per g of liver.

The purity of the preparation was determined by measuring the A_{260}/A_{280} ratios. This was found to range between 1.51 and 1.67 for muscle preparations and between 1.85 and 1.99 for liver preparations. Although an A_{260}/A_{280} ratio of 1.51 is considered indicative of some protein contamination (Dr. G. A. Goodlad, personal communication), ribosomal RNA measurements were carried out on perchloric acid extracts of the ribosomal suspension (page 61) which were essentially protein-free. Also it is unlikely that any protein present in the ribosomal suspension contributed significally to the specific radioactivities obtained from these ribosomes since 93% of the tritium label resided in the RNA component of the ribosomes (Table 2).

KCl was used at a concentration of 0.3M in medium C to assist the solubilisation of muscle myosin, a major protein in this tissue, and increase the yield of muscle ribosomes normally closely

associated with myosin. Ribosomes have been shown to co-precipitate with myosin in this tissue (Heywood et al, 1967). This concentration of KCl, however, produced gelling of the post-mitachondrial supernatant of rat liver more especially in rats given prednisolone. This salt was therefore omitted in the preparation of the rat liver post-mitochondrial supernatant.

Sucrose has been used extensively in the isolation of ribosomes from rat liver since, at a concentration of 0.25M, it conferred upon the medium the isotonicity essential for the preservation of the morphology of intracellular organelles. However this reagent, in the presence of 0.3M KCl and at 0°C, formed a viscous muscle homogenate from which ribosomes could not be extracted. Sucrose was therefore omitted from medium C.

The protocol for the isolation of ribosomes from rat muscle and liver homogenates is presented in Fig. 12.

Estimation of RNA content of rat gastrocnemius muscle and liver ribosomes

The procedure for extracting RNA from rat muscle and liver ribosomes depended on the alkali lability of ribonucleic acids. The degraded RNA is soluble in perchloric acid which precipitates the protein components of these ribosomes.

1.0ml of a suspension of ribosomes in 0.1M KOH was mixed with 1.0ml 0.5M KOH and incubated at 37°C for 1 h. The alkaline digest (Fraction A) was cooled to 2°C and acidified with 0.06ml 10.0M perchloric acid per ml of digest. The protein precipitate (Fraction B) was sedimented by centrifugation at 600g ave. for 15 min. at 0°C and washed with 1.0ml 0.5M perchloric acid. The supernatant

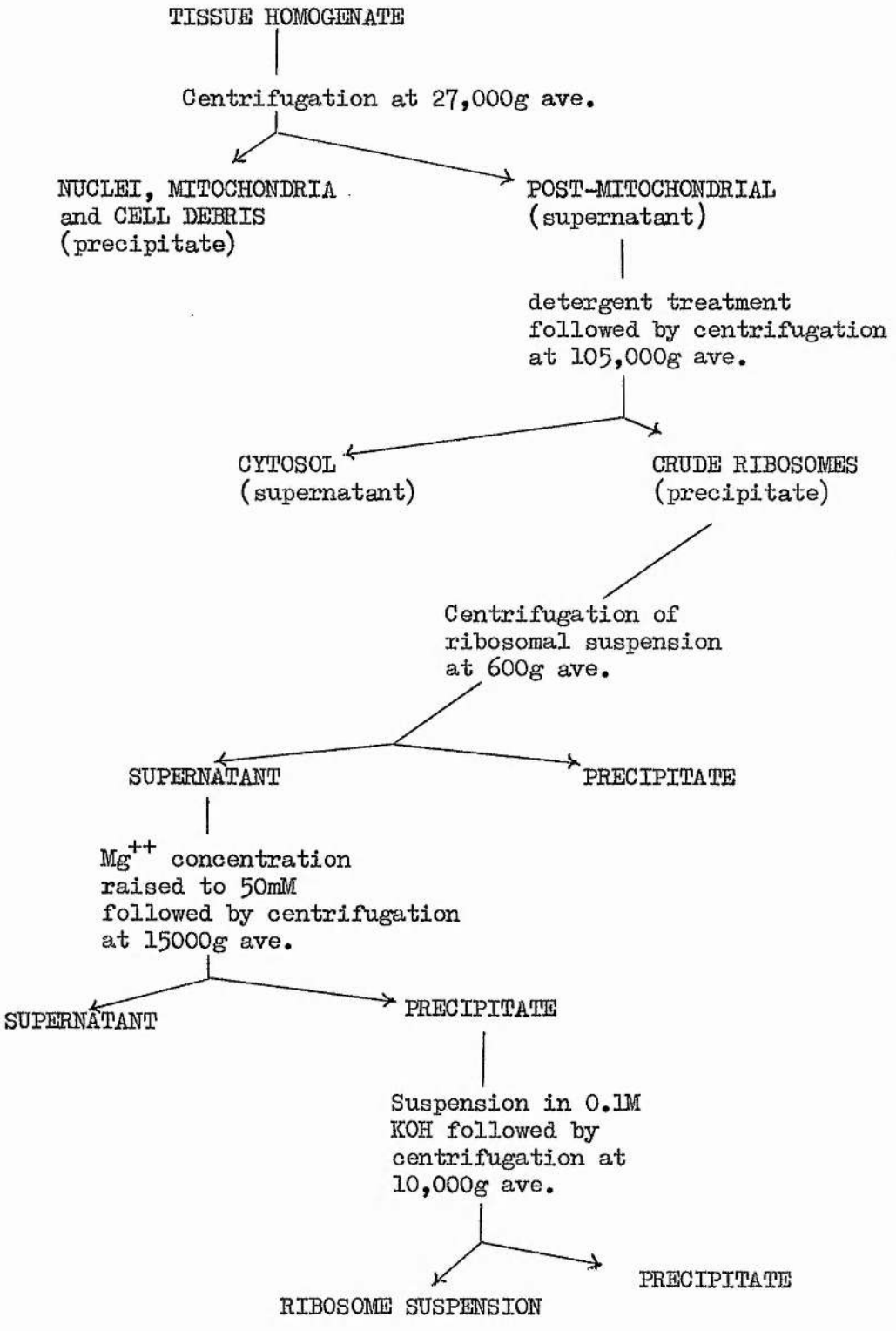


Fig. 12 Protocol followed for the isolation of ribosomes from rat gastrocnemius muscle and liver. Experimental details are described on page 58 . All procedures were carried out at 0° - $4^{\circ}C$.

and washing, which contained ribosomal RNA present as acid soluble nucleotides, were pooled (Fraction C). The RNA content was estimated by measuring the absorbance of a suitable dilution of Fraction C with 0.5M perchloric acid at 260nm. Quantitation was made on the basis that yeast RNA treated in a similar fashion was found to have an $A^{1\%}_{260}$ of 230 when measured through a light path of 1.0cm.

Measurement of radioactivity in muscle and liver ribosomes

The radioactivity in the muscle and liver ribosomes were measured on the Intertechnique Liquid Scintillation Spectrometer (Model SL30). Samples dissolved in 0.1M KOH were layered directly onto glass fibre discs (Type GF/A, diameter 2.5cm, Whatman Ltd., England) and dried under an infra-red lamp. It was not necessary to neutralise the KOH which, in the absence of water, produces no chemiluminescence in toluene (Davies and Hall, 1969).

In initial experiments, the precise location of radioactivity in muscle ribosomes was studied by performing radioactivity counts of the alkali digest, the protein precipitate, and the perchloric acid extract (Fractions A, B and C respectively on page 61 . The results obtained are presented on Table 2 and show that 93% of the radioactivity was located in the RNA of the ribosomes.

TABLE 2. The radioactivity of RNA and protein components of rat gastrocnemius muscle ribosomes

Fraction	Radioactivity (cpm/assay)	
	(A)	(B)
Alkaline digest	162.4	63.9
*Protein precipitate	7.8	6.0
Perchloric acid extract	155.1	58.1

* The protein precipitate was suspended in 1.0ml distilled water, neutralised with a few drops of 2.0M KOH, mixed with 1.0ml 0.3M KOH and incubated at 37°C for 2 h. Any precipitate of potassium perchlorate was removed by centrifugation at 1000g for 15 min. The supernatant was counted for radioactivity as described on page 98.

(A) refers to fractions extracted from rat muscle 2 days after the injection of ^{3}H-Orotic acid into the animals and (B) were fractions extracted 10 days after the injection.

Attempts to isolate undegraded RNA from rat gastrocnemius muscle

Most of the current methods for the isolation of RNA from mammalian tissues rely on the two-phase partition system introduced by Kirby (1956) in which the RNA extracted with phenol/water mixtures is re-extracted with 2-methoxyethanol, the nucleic acid remaining in the organic layer in the latter extraction. However, although this procedure was effective in eliminating water-soluble contaminants from phenol-prepared RNA, it does not completely free the RNA from ribonucleases (Huppert and Pelmont, 1962). The RNA isolation attempts described below are modifications of the phenol extraction system and were aimed at obtaining uncontaminated and undegraded RNA from rat muscle. All RNA isolated by these procedures were analysed by sucrose gradient centrifugation as described below.

(a) Hot phenol extraction

This method was similar to that used by Manchester (1967) to isolate RNA from rat diaphragm muscle.

Gastrocnemius muscle from one hind leg of the rat was excised quickly from the animal, frozen in liquid nitrogen and pulverised. The pulverised fragments were mixed with 10 vol of medium F (0.3M KCl and 0.3M trichloroacetic acid titrated to pH 7.5 with 2.0M NaOH). The mixture was homogenised using a Silverson homogeniser and solid SDS was added to the homogenate to a concentration of 1% (w/v), this addition being withheld until after the homogenisation step to prevent foaming. The mixture was stirred at 0°C for 10 min. and then was treated with 1 vol water-saturated phenol reagent. The mixture was shaken at 65°C for 20 min. and then cooled to 0°C

by immersion in an ice bucket. The aqueous and phenol phases were separated by centrifugation at 1000g ave. for 20 min. at 4°C. The upper, RNA-containing layer, was carefully withdrawn and the phenol layer containing DNA and protein material was re-extracted with ½ vol medium F. The aqueous layers were pooled and made 2.0M with respect to NaCl, by addition of the solid salt. RNA was precipitated by mixing the solution with 2 vol absolute ethanol and standing overnight at -15°C. The precipitate was collected by centrifugation at 1000g ave. for 15 min. at 4°C and washed 6 times with 80% (w/v) ethanol. The material was finally washed in diethyl ether and dried over anhydrous $CaCl_2$ in a desiccator after a brief incubation at 37°C to remove traces of the ether.

(b) Cold phenol extraction

This method was a slight modification of the procedure described by Wool and Munro (1963).

Gastrocnemius muscle, excised, frozen and pulverised was mixed with 10 vol ice-cold aqueous 0.3M KCl solution buffered with 0.05M Tris-HCl, pH 7.5 at 20°C. 0.5ml of an aqueous suspension of bentonite (50mg/ml) in 0.05M Tris-HCl buffer, pH 7.5 (at 20°C) was added quickly to this mixture to inhibit nucleases and the slurry was homogenised using a Silverson homogeniser. 0.1 vol SDS (10% (w/v) suspension of the solid in distilled water) was added and the mixture shaken at 20°C for 10 min. An equal volume of freshly-prepared water-saturated phenol reagent was added to this mixture and the shaking was continued at 4°C for a further 10 min. The phases were separated by centrifugation at 1000g ave. for 20 min. at 4°C. The aqueous layer was retained and the phenol

layer was re-extracted with ½ vol fresh phenol reagent. The pooled aqueous phases were treated with 1/9 vol 20% (w/v) sodium acetate and 3 vol absolute ethanol. RNA was allowed to precipitate from this solution overnight at -15°C and was collected by centrifugation at 1000g ave. for 20 min. at 0°C. The product was washed extensively 6 times with 80% (w/v) ethanol and finally with diethyl ether before drying over $CaCl_2$ in a vacuum dessicator.

(c) Cetyltrimethyl ammonium bromide extraction

This method was first described by Ralph and Bellamy (1964) and combined Kirby's 1956 two-phase system with a precipitation step where RNA was recovered as the insoluble cetyltrimethyl ammonium (CTA) salt.

Gastrocnemius muscle, excised, frozen and pulverised was mixed with 10 vol of an aqueous mixture containing 0.5% (w/v) napthalene 1,5-disulphonate (sodium salt) and 0.5% (w/v) bentonite. An equal volume of water-saturated phenol reagent was added to this mixture and the slurry was homogenised using the Silverson homogeniser. The homogenate was stirred at 20°C for 30 min. and the phases separated by centrifugation at 2000g ave. for 20 min. at 4°C. The aqueous phase was carefully withdrawn, treated with ½ vol fresh phenol reagent, shaken at 20°C for 10 min. and centrifuged again. The aqueous layer was removed and mixed with 1/9 vol 20% (w/v) sodium acetate followed by 2 vol absolute ethanol. RNA was precipitated from this solution within 90 min at -15°C and was recovered by centrifugation at 2,500g ave. for 20 min. at 0°C. The precipitate was dissolved in 7.0ml 0.01M sodium acetate buffered at pH 5.1 with acetic acid. 7.0ml potassium phosphate buffer, pH

8.1, were added followed by 7.0ml redistilled 2-methoxyethanol. The mixture was shaken vigorously for 2 min. and centrifuged at 1000g ave. to separate a clear upper phase which was carefully withdrawn. This supernatant fraction was mixed with 1 vol 0.2M sodium acetate. 0.5ml of 1% (w/v) cetyltrimethyl ammonium bromide was added for every ml of the supernatant fraction and the mixture was chilled at 0°C for 10 min. to precipitate the CTA-RNA complex which was collected by centrifugation at 1000g ave. for 10 min. at 2°C. The complex was washed 3 times with ice-cold 70% (w/v) ethanol containing 0.1M sodium acetate to precipitate RNA as its sodium salt and remove CTA in the supernatant as CTA-acetate. The RNA was collected by centrifugation at 1000g ave. for 10 min. at 2°C washed 4 times with 80% (w/v) ethanol, then with diethyl ether and finally dried over anhydrous $CaCl_2$ in a vacuum dessicator.

(d) Phenol/chloroform extraction

This method was a modification of the procedure described by Perry et al (1972).

Gastrocnemius muscle, excised, frozen and pulverised was mixed with 10 vol of medium G (0.1M NaCl, 0.001M EDTA in 0.01M sodium acetate buffer, pH 6.0). To inhibit any ribonuclease activity which might be present 0.2ml of a 5mg/ml solution of polyvinyl sulphate (Sigma Chemical Co. Ltd., London, England) in medium G was added for every 10ml of medium G and the mixture homogenised using the Silverson homogeniser. As further protection against enzymic degradation and to dissociate RNA-protein complexes, 0.1 vol of 5% (w/v) SDS in medium G was added to the homogenate which was then shaken at 20°C for 10 min. Protein and cellular debris were

precipitated from the homogenate by the addition of 1 vol of a mixture of water-saturated phenol reagent and redistilled chloroform (in a 1:1 volume ratio) and shaking for a further 25 min. at 20°C. The mixture was cooled to 0°C and RNA was recovered in the aqueous phase following centrifugation at 1,500g ave. for 20 min. at 2°C. The aqueous phase was re-extracted with 1 vol fresh phenol/chloroform mixture, made 0.2M with respect to NaCl and treated with 2 vol cold absolute ethanol to precipitate RNA. The phenol phase from the first extraction was re-extracted with 1 vol of fresh medium G and the post-centrifugation aqueous phase from this extraction was used to re-extract the phenol phase from the second extraction. RNA was precipitated from the final aqueous phase with 2 vol cold ethanol and the combined ethanol precipitates were kept at -15°C until required. RNA was recovered from the ethanol medium by centrifugation at 2000g ave. for 15 min. at 2°C and was washed twice with 67% (w/v) ethanol.

Isolation of poly-adenylated RNA from rat gastrocnemius muscle

The methods employed to obtain poly-adenylated RNA from rat gastrocnemius muscle involved, as a first step, phenol extraction to isolate all cytoplasmic RNA species from the tissue. These RNA species were then fractionated on a column of oligo(dT)-cellulose (Sigma Chemicals Co. Ltd., London, England) to isolate polyadenylated RNA from other RNA species.

(a) The phenol extraction step

Extraction of RNA by phenol procedures suffers the disadvantage that some RNA species, notably poly-adenylate containing RNAs are lost to the phenol layer depending on the pH and the cationic

concentration of the aqueous phase as well as on the nature of the phenol phase itself (Lee et al, 1971; Perry et al, 1972). To overcome this loss various modifications of the phenol procedure, as described below, were tried out.

(i) Procedure A

This method was adapted from that described by Neal and Florini (1975). 0.1M Tris buffer, pH 9.0 at 20°C was used as the aqueous phase and water-saturated phenol reagent as the phenol phase. The freshly excised muscle was frozen and pulverised before it was homogenised in 9 vol of the Tris buffer using a Silverson homogeniser. Prior to homogenisation 0.2ml of a 5mg/ml solution of polyvinyl sulphate in the Tris buffer was added for every 10ml of the homogenisation medium to inhibit any ribonuclease. The homogenate was made 0.1% (w/v) with respect to SDS and shaken vigorously for 10 min. at 20°C. Thereafter it was treated with 1 vol phenol reagent and shaken at 20°C for a further 25 min. After cooling to 0°C the phases were separated by centrifugation at 2000g ave. for 15 min. at 4°C. The aqueous phase was carefully withdrawn and re-extracted with 1 vol freshly-prepared phenol reagent. The two phenol phases were re-extracted with 1 vol of the Tris buffer. The combined aqueous phases were made 0.2M with respect to NaCl, treated with 2 vol cold ethanol and maintained at -15°C overnight to precipitate RNA.

(ii) Procedure B

This method was adapted from the procedure described by Mainwaring et al (1974). The aqueous phase was a solution containing 0.15M NaCl and 0.025M EDTA and the extraction with water-saturated

phenol was performed at 55°C in a heated water-bath. Other details of this procedure were the same as for procedure A above.

(iii) Procedure C

This method used a phenol/chloroform mixture (1:1 volume ratio) in place of water-saturated phenol alone, as recommended by Perry et al (1972). The aqueous homogenisation medium contained 0.1M NaCl and 0.001M EDTA in 0.01M Tris-HCl buffer, pH 6.0 at 20°C. Procedure C was the same as procedure A in all other aspects.

(b) Oligo(dT)-Cellulose Column Chromatography

This procedure fractionates a mixture of polynucleotides according to the ability of the individual components to form base-pairing complexes with complementary oligo deoxythymidylate, (Oligo(dT)), immobilised in the solid state by attachment to cellulose. In a medium of high ionic strength the additional structural flexibility conferred on poly-adenylate-containing nucleotides encourages the RNA species to bind selectively to Oligo(dT)-cellulose. Upon lowering the ionic strength, the contribution of electrostatic forces to this binding is reduced and at the slightly alkaline pH (7.5) identical charges on adjacent nucleotides repel each other enabling elution of any poly-adenylated RNA.

RNA from the phenol extraction step was collected by centrifugation at 2000g ave. for 20 min. at 2°C and dissolved in 5ml 0.01M Tris-HCl buffer, pH 7.5 at 20°C. This solution was freed of any DNA contaminant by incubating it first for 15 min. at 4°C and thereafter for 5 min. at 37°C with an equal volume of a 40µg/ml solution of ribonuclease-free DNAse (Sigma Chemical Co. Ltd., London,

England) in the same buffer. The reaction was stopped by adding 0.1 vol SDS (1% (w/v) in 1.0M Tris-HCl buffer, pH 9.0 (at 20°C)) to the medium followed by 1 vol water-saturated phenol reagent. The mixture was stirred at 20°C for 10 min. before centrifugation to separate the phases. The aqueous phase was withdrawn carefully and re-extracted with 1 vol fresh phenol reagent. The aqueous phase was then made 0.2M with respect to NaCl and treated with 2 vol cold ethanol to precipitate RNA overnight at -15°C. The RNA was collected by centrifugation and dissolved in 0.01M Tris-HCl buffer, pH 7.5 at 20°C, containing 0.001M EDTA to remove any traces of Mg^{++} which may cause aggregation of the RNA. The RNA solution was made 0.5M with respect to KCl and loaded on the column of oligo(dT) cellulose. This column contained 0.4mg of the cellulose prepared as a suspension in the application buffer (0.01M Tris-HCl buffer, pH 7.5 at 20°C, containing 0.5M KCl) and packed under gravity in a column made from a 2ml-capacity disposable syringe plugged with cotton wool. The column was then washed with 20ml of the application buffer and effluent and washings were collected. Material retained on the column was eluted with 10ml 0.01M Tris-HCl buffer, pH 7.5 at 20°C and collected as the polyadenylated RNA. Throughout the operation flow-rate of fluid through the column was maintained at approximately 2ml per min. and, at the end, the column was regenerated by washing it with 40ml 0.1M KOH followed by 20ml of the application buffer. The RNA contents of the different fractions were measured from their ultraviolet absorbance at 260nm. Where radioactivity measurements were required the RNA was precipitated from the solution with 2 vol cold ethanol. The

polyadenylated RNA fraction required the addition of 1.0mg yeast RNA as carrier to aid precipitation. The precipitated material was dissolved in 1.0ml of 0.1M KOH before its radioactivity was counted.

Separation of RNA species by sucrose gradient centrifugation

The separation of different classes of RNA by sucrose density gradient centrifugation relies on the difference in the rates with which these macromolecules sediment through centrifugal field in a supporting and stabilising medium provided by the sucrose gradient.

Linear sucrose gradients were always prepared with 'Analar' grade sucrose and, in the initial experiments, these gradients were made 15 - 30% (w/w) with respect to sucrose. However, contrary to the experience of McConkey (1967), the result obtained using a gradient with these sucrose concentrations showed a poor separation of RNA species even after 17 hours of centrifugation at 60,000g ave. The use of this gradient was, therefore, discontinued in preference to a 5 - 20% (w/w) sucrose gradients prepared as follows.

The day before the gradients were required, six aqueous solutions containing 5, 8, 11, 14, 17 and 20% (w/w) sucrose respectively were prepared in acetate buffer pH 5.1 containing 0.01M sodium acetate and 0.15M NaCl. The low pH of this medium minimises nuclease activity while the presence of the Na^+ ions at this concentration counters the charge on the nucleotide phosphate groups conferring a more compact structure on the RNA molecules and thus, ensuring a better separation of RNA types (McConkey, 1967). Gradients were prepared in polypropylene centrifuge tubes by underlayering equal volumes of all six sucrose solutions sequentially, starting with

2.5ml of the least concentrated solution and using a syringe fitted with a flat-nosed, medium-bore needle to reach the bottom of the tube. The gradients prepared in this manner were maintained at $4^{o}C$ for 14 - 18 h before use to permit diffusion and smoothening out of any local irregularities of concentration. The linearity of this gradient was tested by fractionating it as described below and measuring the refractive index of each fraction using a degree scale ABBE Type Refractometer (Bellingham and Stanley Ltd., London, England). This instrument measured the angle of refraction of light from a yellow sodium source passing into the sucrose solution and the readings obtained on the degrees scale were converted to refractive indices using a refractive index table. Since the refractive index of a solution at a constant temperature is linearly related to concentration, a plot of these indices against fraction number should give a straight line if the gradient were linear. The results presented on Fig. 13 show that except for minor deviations at the top and bottom of the column the gradient is essentially linear throughout the system.

The sample to be analysed was dissolved in 2.0ml of the same acetate buffer used to prepare the sucrose gradients and the solution layered over the sucrose gradient. The concentration of sample used never exceeded 0.5mg RNA per ml of the solvent buffer to ensure that the sample floated on the 5% (w/w) sucrose solution at the top of the gradient. Centrifugation was commenced as quickly as possible after sample loading to avoid any tailing of the sample.

For centrifugation the gradient bearing the sample was carefully loaded into the buckets of a pre-cooled 3 x 23ml swing-out

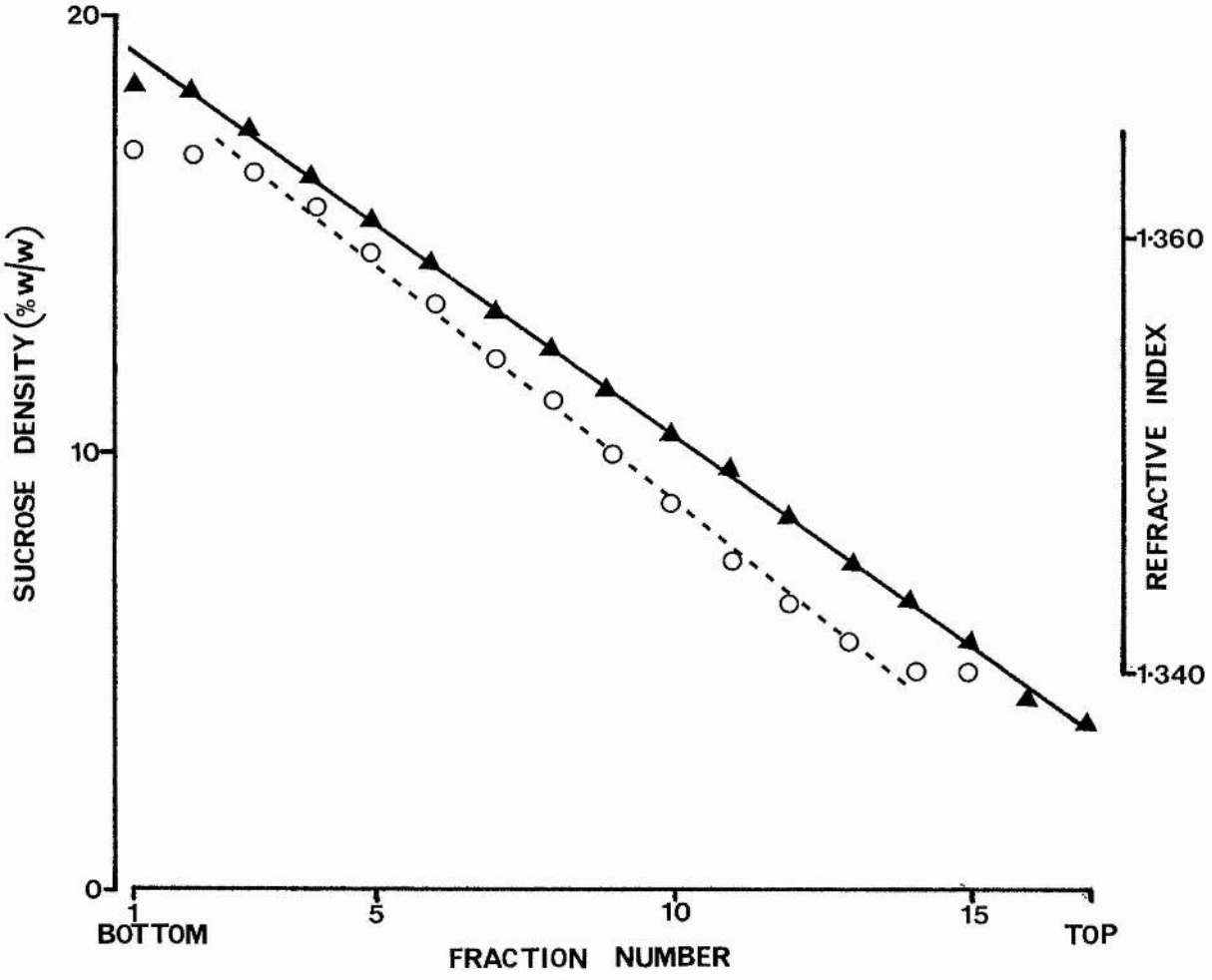


FIG. 13 The linearity of a manually formed 5 - 20% Sucrose Density Gradient:
Gradients were formed manually as described on page 73. Broken lines show the refractive indices of 1.0ml fractions of the gradient after allowing 14 - 18 h at 4°C for dispersion of local irregularities of concentration.
The post-centrifugation sucrose density distribution is shown in unbroken lines. It was obtained by layering the gradient with 2.0ml 0.05M sodium acetate buffer pH 5.1 containing 0.1M NaCl and centrifuging for 7 hours at 60,000g ave. at 5°C. Refractive indices of 1.0ml fractions were measured as described on page 74 and converted to sucrose densities from tables (Weast 1974).

aluminium rotor (MSE Ltd., England) which was then positioned inside an MSE superspeed 65 centrifuge and centrifuged at the appropriate speed and temperature for the time desired.

Following centrifugation, 1.0ml fractions of the gradient were collected sequentially from the bottom of the tube at 4°C using a tube piercer (MSE Ltd., England). The RNA content of each fraction was estimated by measuring the absorbance of the solution at 260nm. To overcome the problem of decreasing radioactivity counting efficiency with increasing sucrose concentration (McDowell and Copeland, 1971), RNA was re-precipitated from each fraction overnight at -15°C with 2 vol cold ethanol. To aid this precipitation, 1.0mg yeast RNA in an aqueous solution was added to each fraction before the addition of ethanol. The precipitate was dissolved in 0.1M KOH before radioactivity counts were measured.

Calculation of 'S' values of RNA species from data obtained by sucrose gradient centrifugation

The sedimentation coefficients ('S' values) associated with the various RNA peaks following sucrose density gradient centrifugation were calculated as described by McEwen (1967). This calculation assumes that the sucrose concentration distribution does not change appreciably during centrifugation and that the sedimentation velocity of an RNA particle varies directly with density differences between particle and solution and inversely with solution viscosity.

The first step in the computation of an 'S' value was the estimation of Z_o, the sucrose concentration corresponding to the extrapolation of the linear sucrose concentration distribution to

zero radius

$$Z_o = \frac{Z_1 r_2 - Z_2 r_1}{r_2 - r_1}$$

where Z_1 and Z_2 are the sucrose concentrations at the top and bottom of the gradient respectively. Both these expressions are dimensionless and, in practice $Z_1 = 4.0$ and $Z_2 = 19.3$ (McEwen, 1967). Although the starting sucrose concentration was 5% (w/w), this concentration is lowered somewhat by the diffusion of the sucrose into the sample layer above the gradient. Post-centrifugation refractive index measurements of 1.0ml fractions of a sucrose gradient layered with 2.0ml of the acetate buffer but containing no sample showed that 4.0 and 19.3 were indeed the closest approximations of the sucrose concentrations at the top and bottom of the gradient (refractive index was converted to sucrose concentration from tables compiled by Weast (1974).

Z_o generally has a negative value.

r is the distance (cm) in the direction of centrifugal force field from zero radius to the top (r_1) and bottom (r_2) of the gradient. In practice r_2 was sum of the radius of the rotor and the length of the centrifuge tube and r_1 was the difference between this sum and the height of the gradient.

Using the Z_o value obtained, and with due regard to the temperature of centrifugation (5°C), sedimentation intergrals (I) were computed from the appropriate tables (McEwen, 1967). I_1 is the sedimentation intergral for the RNA particle for the starting sucrose concentration (4.0% (w/w) due to the diffusion effect explained above) and I_2 is the sedimentation intergral for the

sucrose concentration at the position of the RNA peak. The sucrose concentration at the position of the RNA peak was read off a calibration plot obtained by measuring the refractive indices of post-centrifugation 1.0ml fractions of the sucrose gradient (Fig. 13).

Finally the 'S' value was calculated from the equation

$$S \times 10^{-13} = \frac{(I_2 - I_1)}{w^2 t}$$

where w is the angular velocity in radians per second (1 rpm = 2π radians and at 60,000g ave. (29,000 rpm) $w = 29000 \times 2 \times \frac{22}{7} \times \frac{1}{60}$) and t is the duration of centrifugation in seconds.

Attempts to investigate the effect of prednisolone on rates of synthesis and degradation of RNA in liver and muscle in rats given a single injection of ^{3}H-Orotic Acid

It has been shown by several groups of workers that the rate of degradation of liver RNA and of ribosomal RNA in particular follows first order kinetics (Ottolenghi and Barnabei, 1970; Enwonwu and Munro, 1970; Goodlad and Ma, 1975). The rate constant for this process can therefore be estimated by labelling the RNA by injecting the animal with a suitable radioactive precursor and plotting the logarithm of the total radioactivity associated with the RNA against a linear time scale. The slope of the resultant straight line is equal to kd/2.303 where kd is the RNA degradation constant (Fig. 14).

In applying this mathematical formulation to the experimental data, it was necessary to assume that during the experimental period, a steady-state situation existed in which (a) The rate at which labelled precursors appear on the RNA is the rate at which they disappear from the macromolecule. Thus a measure of the latter will be a measure of the RNA turnover rate; (b) There was a large RNA precursor pool available in the tissue which did not change significantly during the experimental period. Thus re-utilisation of RNA breakdown products was minimal and insignificant; (c) The rate of the RNA metabolic process was constant and was not complicated by the formation of new cells.

In the present studies, each 'test' and 'control' animal received 20μCi ^{3}H-Orotic Acid intraperitoneally. Prednisolone treatment was started 2 or 4 days after the injection of the orotic

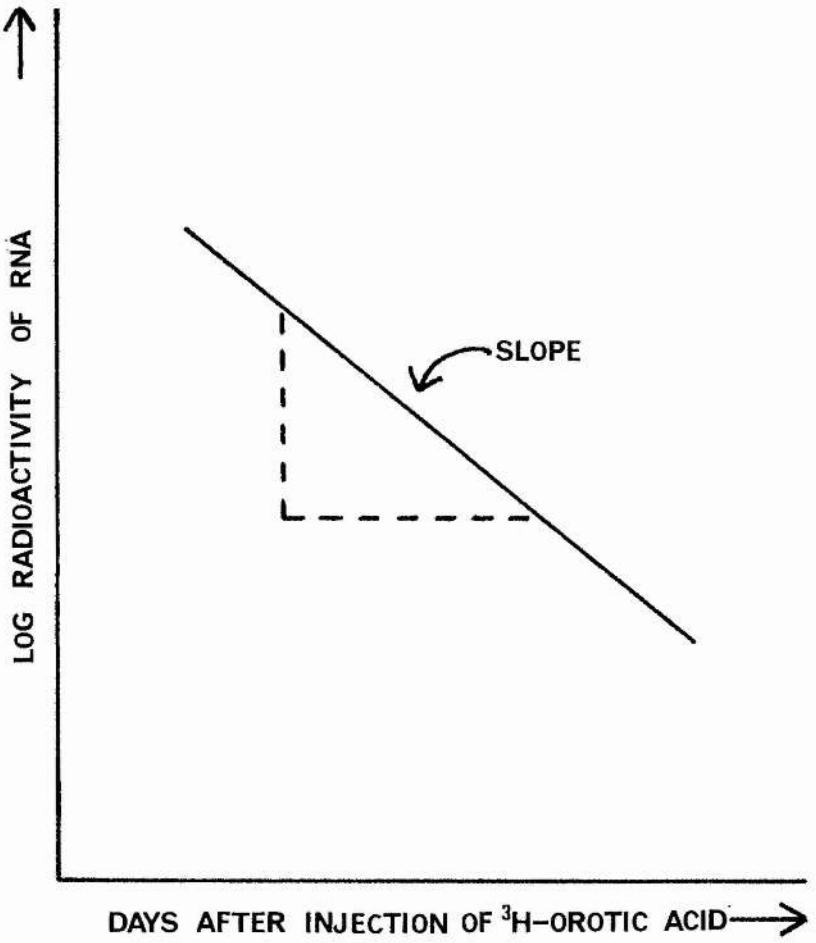


FIG. 14 Loss of radioactivity from RNA with time

Kinetics of first Order Reaction:

$$\frac{dx}{dt} = kd \cdot x$$

$$\text{Thus } \log x = \frac{kd\ t}{2.303} + \text{constant}$$

$$\text{Therefore, Slope} = \frac{kd}{2.303}$$

acid and the effect of the steroid on RNA turnover was monitored by following the loss of radioactive label from RNA isolated from the animals. The biological half-life of the RNA was calculated from the value of the degradation constant using the relationship:

$$t_{\frac{1}{2}} = \frac{0.693}{kd}$$

Turnover times for RNA and rates of RNA synthesis were calculated as described by Enwonwu et al (1971).

Estimation of 'Total' acid-soluble and acid-soluble nucleotide pools in rat gastrocnemius muscle and liver

Each of 9 male rats received 20.0μCi ^{3}H-orotic acid in 0.85% (w/v) saline by intraperitoneal injection. Starting 2 days later, each of the prednisolone-treated animals received daily subcutaneous injections of 1.0mg prednisolone acetate per 100g initial body weight while the 'control' partner received the same volume of saline. One animal was sacrificed 2 days after being injected with ^{3}H-orotic acid and served as a basal control. A pair of animals (one prednisolone-treated and the other its control) were sacrificed on the 4th, 6th, 8th and 10th day after the injection of ^{3}H-orotic acid.

A homogenate of the gastrocnemius muscle was prepared in 5 vol aqueous 0.3M KCl solution using the Silverson homogeniser. The liver homogenate was prepared in 1 vol of this solution and was then diluted 100-fold with distilled water. 2.0ml of each homogenate was treated with an equal volume of 0.4M perchloric acid and, after stirring the mixture for 20 min. at 0°C, centrifuged at 1000g ave. for 15 min. at 4°C. The precipitate was washed 2 times with 1.0ml 0.2M perchloric acid. Each washing was recovered by centrifugation and pooled with the initial supernatant to make the 'Total' acid soluble pool. This pool was titrated to neutral pH with 0.6M KOH. $KClO_4$ was precipitated from the solution by allowing it to stand overnight at 4°C and was removed by centrifugation at 1000g ave. for 15 min. at 4°C.

The nucleotide components of the total acid-soluble pool were obtained by passing a 2.0ml aliquot of the perchlorate-free solution

through a column (9cm by .5cm) of Amberlite resin (CG400 Type II, 200 mesh) as described by Miller and Baggett (1972). This resin served as an anion exchanger retaining nucleotides via the negatively charged phosphate groups in exchange for chloride ions. Unadsorbed material was washed from the column with 20ml distilled water at a flow rate of 2ml per min. and the adsorbed nucleotides were eluted with 20ml 1.0M HCl. The eluent was collected and freed of HCl by repeated (5 times) vacuum distillation in a water bath maintained at 50°C. The dry residue was dissolved in 2.0ml 0.1M KOH and constituted the acid-soluble nucleotide pool.

In a preliminary experiment 0.5mg cytidine 2′, 3′ - monophosphoric acid dissolved in 1.0ml distilled water was loaded on the resin and eluted as described above. A comparison of the absorbances at 260nm of the eluent with that of sample before the chromatography showed that the resin effectively retained and, upon the HCl treatment, released 95-97% of the cytidine nucleotide.

Isolation of enzymically active nuclei from rat gastrocnemius muscle and liver for assay of RNA polymerase activity

Nuclei were isolated from gastrocnemius muscle using a slight modification of the procedure described by Breuer and Florini (1966). To obtain sufficient nuclei it was necessary to use, in addition to the gastrocnemius muscle, the anterior tibial group of muscles and quadriceps femoris muscles from both hind limbs. The weight of this pool of muscles ranged between 10 and 15g per animal. The chilled tissue was cut into fine pieces and minced by forcing it 3 times through a pre-cooled mechanical tissue press/mincer. Thereafter the tissue was suspended in 2 vol ice-cold medium H (0.375M sucrose, 0.025M KCl, 0.01M $MgCl_2$ in 0.05M Tris-HCl buffer, pH 7.6 (at 20°C)) and homogenised in 5 bursts, each lasting 30 s, using the Silverson homogeniser. The tough and fibrous nature of the muscle permits such vigorous homogenisation to release nuclei without damaging them (Held, 1977). Throughout the homogenisation procedure, the sample was immersed in an ice-bath and 1.0 min. was interposed between each burst to prevent the temperature of the homogenate rising. The homogenate was filtered by passing through a plastic screen of narrow mesh fitted to the end of a 20ml-capacity disposable syringe. This filtration step was repeated, after cleaning out the apparatus, to ensure the removal of the larger bundles of muscle fibres which would otherwise trap nuclei during high-speed centrifugation. The filtrate was divided into two aliquots each of which was under-layered with an equal volume of aqueous 0.375M sucrose containing 0.001M $MgCl_2$. This suspension was centrifuged at 600g ave. for 7 min. at 4°C to

sediment a crude myofibrillar-nuclear fraction. The sediment was resuspended in 16ml of aqueous 2.4M sucrose containing 0.001M $MgCl_2$ and homogenised gently using a Potter-Elvehjem homogeniser with a loose-fitting pestle. Thereafter the mixture was centrifuged for 1 h at 29,000g ave. The intense viscosity of the medium at lower temperatures necessitated the centrifugation being performed at 15°C as recommended by Baieve and Florini (1970). The subsequent fraction was discarded along with myofibrils, whole cells and other non-nuclear subcellular components which formed a plug at the top of the tube. The sides of the tubes were wiped clean with paper tissue and the nuclear pellet was re-suspended in 16ml of aqueous 2.2M sucrose containing 0.001M $MgCl_2$. Thereafter the pellet was homogenised and centrifuged again at 29,000g ave. for 1 h at 15°C. The final pellet was suspended in 2ml of 0.25M sucrose containing 0.001M $MgCl_2$.

Rat liver nuclei were isolated using a modification of the method described by Widnell and Tata (1964). The tissue was weighed, minced and homogenised in 3 vol of a solution containing 0.32M sucrose and 0.003M $MgCl_2$. 25 - 30 upward and downward strokes of a tighting-fitting pestle in a Potter-Elvehjem homogeniser were sufficient to ensure good cell breakage without causing extensive nuclei damage. The homogenate was freed of unbroken cells and connective tissue by filtration through 2 layers of cheese cloth. The concentration of sucrose in the medium was then raised to 0.25M by adding solid sucrose and the mixture rehomogenised. An equal volume of 0.375M sucrose solution containing 0.003M $MgCl_2$ was layered under the mixture and a crude

nuclear fraction was sedimented by centrifuging the suspension for 6 min. at 500g at 4°C. The sediment was suspended in 16ml of a 2.4M sucrose solution containing 0.001M $MgCl_2$ and homogenised with the Potter homogeniser using a loose-fitting pestle. The mixture was centrifuged at 29,000g ave. for 1 h at 4°C. Mg^{++} was used at a concentration of 0.001M to prevent agglutination of nuclei (Widnell and Tata, 1964). Contamination of the nuclear pellet with whole cells was found to occur at higher Mg^{++} concentration. Centrifugation of the nuclear preparation through 2.4M sucrose was carried out to remove contaminating erythrocytes.

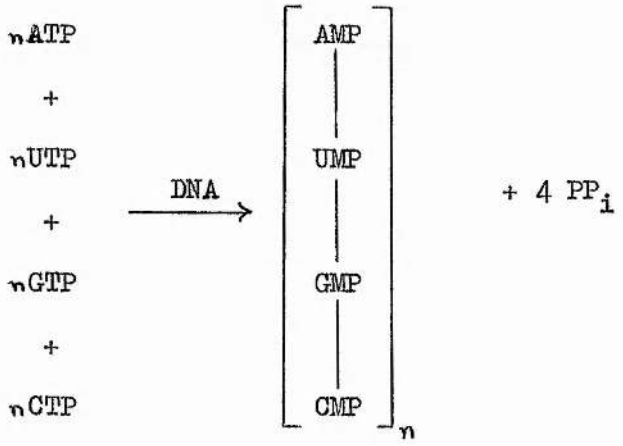
After centrifugation the supernatant was discarded, as was the plug of whole cells, blood corpuscles and mitochondria at the top of the tube. The sides of the tubes were wiped clean with paper tissue and the nuclear pellet re-suspended in 5ml 0.25M sucrose containing 0.001M $MgCl_2$.

Both muscle and liver nuclei preparations were examined by phase contrast microscopy. Muscle nuclei although undamaged were contaminated to a slight extent by myofibrils. Neither the inclusion of additional filtration steps nor the adoption of less severe homogenisation techniques solved this problem. However the myofibrils were observed to settle at the bottom of the tube on standing for a few minutes at 4°C and the top portion of the preparation was relatively free of the contaminants. This portion was retained. Nuclei were counted using a haemocytometer with a Thoma ruling. The protein contents of the preparations were measured by the Folin-Lowry method described on page 52. RNA and DNA were precipitated with 1 vol 0.4M perchloric acid and assayed

as described on pages 43 and 46 respectively, starting with 0.5ml of the nuclear preparation. The mean DNA content of muscle nuclei was 7.24pg, the RNA/DNA ratio was 0.23 and the protein/DNA was 8.12. Corresponding values for liver nuclei were 12.7pg, 0.19 and 4.9 respectively. The results of an analysis of protein RNA and DNA contents of the nuclear fractions at various stages in the isolation of muscle nuclei are presented on Table 3.

Assay of RNA polymerase activity of isolated nuclei

RNA polymerase catalyses the polymerisation of ribonucleotides to RNA as illustrated below:



The nucleotide composition of the RNA produced depends on the nature of the DNA template which in the present studies was derived endogeneously from the isolated nuclei. The enzymic activity was measured by determining the quantity of acid-soluble ^{3}H-uridine incorporated into the acid insoluble polyribonucleotide from ^{3}H-UTP. The procedure, adapted from Davies and Griffiths (1973), requires dithiothreitol in the incubation mixture to stabilise the enzyme during the assay and KCl to stimulate enzymic activity.

For the Mg^{++}- activated enzyme assay the incubation mixture consisted of 0.1ml of solution J (0.025M $MgCl_2 \cdot 6H_2O$, 0.003M NaF,

TABLE 3. The isolation of nuclei from rat muscle: purity of nuclear fractions at key fractionation steps

Fraction	Protein content (mg/g muscle)	RNA content (mg/g muscle)	DNA content (mg/g muscle)	protein/ DNA	RNA/ DNA
Tissue homogenate	204	2.1	0.76	269	2.7
Filtered homogenate	171	1.8	0.65	264	2.7
Myofibrillar -nuclear sediment	28	0.25	0.18	159	1.4
Final nuclear suspension	0.07	0.002	0.01	8.1	0.23

Experimental details appear on page 84.

0.002M dithiothreitol and 0.15M KCl in 0.6M Tris-HCl buffer pH 8.1 (at 20°C)); 0.05ml of the mixture of nucleotides (prepared by mixing prior to use 0.2ml aqueous 0.0005M UTP with 0.8ml aqueous 0.0075M ATP, 0.0075M GTP and 0.0075M CTP); and 0.1ml aqueous ^{3}H-UTP (pmols).

For the assay of the Mn^{++}- activated enzyme the incubation mixture was the same as above except that solution J was replaced by solution K (0.015M $MnCl_2$, 2.0M$(NH_4)_2SO_4$, 0.003M NaF, 0.002M dithiothreitol and 0.15M KCl all in 0.6M Tris-HCl buffer, pH 8.1 (at 20°C)).

All solutions were prepared not more than 2 h before use and maintained at 0 - 4°C.

The assay medium was pre-incubated at 37°C for 3 min. and the enzyme reaction was initiated by the addition of 0.2ml of the nuclear suspension. The DNA content in this amount of muscle nuclear suspension was 8.0µg and the corresponding value in the liver nuclear suspension, diluted 10-fold before use, was 40µg. The reaction was allowed to proceed for 5 min. at 37°C and then terminated by the addition, with vigorous shaking, of 2.0ml of aqueous 10% (w/v) trichloroacetic acid containing 0.001M $Na_4P_2O_7$. A 'blank' assay in which the nuclei preparation was added after the trichloroacetic acid was carried through with each test. Test and 'blank' samples were chilled in an ice-bath and 0.1ml of liver cell sap was added to each sample to provide carrier protein required to assist the precipitation of acid-insoluble polynucleotides. This precipitation was allowed to proceed for 2 h at 4°C and the sediment was collected by centrifugation at 2000g ave. for 10 min. at 4°C.

The precipitate was washed twice with 5.0ml 5% (w/v) trichloroacetic acid, twice with 5.0ml of a mixture of n-propanol diethyl ether (1:1) and finally with 5.0ml diethyl ether. The precipitate was dried at 37°C and dissolved in 0.5ml 0.1M KOH by incubating at 37°C for 1 h. The amount of ^{3}H-uridine incorporated into RNA was determined by counting the radioactivity in a suitable aliquot of the alkaline hydrolysates of the test and blank samples and subtracting 'blank' counts from 'test' counts. The DNA in 0.5ml of the nuclear preparation was measured and the specific radioactivity of the RNA polymerase was expressed as nmols uridine incorporated into RNA per mg DNA.

Assay of Ribonuclease Activity in Rat Liver, Muscle and Pancreas

In these experiments prednisolone-treated animals received, subcutaneously, 1.0mg prednisolone acetate per 100g body weight daily and control animals received the same volume of 0.85% (w/v) saline. After 8 days of such treatment animals were sacrificed by stunning and exsanguition. Liver, gastrocnemius muscle from both hind limbs and pancreas were excised quickly, washed free of any blood with ice-cold saline solution and weighed.

Muscle was frozen in liquid nitrogen and pulverised. Thereafter it was suspended in 5.0ml of 0.44M sucrose solution and homogenised using a Silverson homogeniser. Liver and pancreas were minced finely with scissors and each organ was homogenised in 10.0ml of 0.44M sucrose.

1.0ml of each homogenate was diluted 5-fold with distilled water to render the medium hypotonic in order to disrupt subcellular granules containing enzymes. The mixture was then subjected to freezing in liquid nitrogen and thawing to ensure maximum release of ribonuclease activities from subcellular organelles (Shortman, 1961). Finally the mixture was centrifuged at 1000g ave. for 10 min. at 4°C to sediment cell debris and nuclei. The supernatant fraction was used for the assay of ribonuclease activity.

The ribonuclease assay technique was adapted from that described by Shortman (1961) and depended on the measurement of the release of acid ethanol-soluble nucleotides by the ribonuclease-catalysed hydrolysis of yeast RNA. 'Free' ribonuclease activity refers to the enzymic activity measured in the absence of p-chloromercuribenzoate and 'Total' ribonuclease activity refers to activity measured in

the presence of p-chloromercuribenzoate (PCMB).

(a) Acid ribonuclease assays

For 'free' acid ribonuclease assays the 'test' incubation mixture contained 0.2ml of 0.03M veronal acetate buffer, pH 5.8, 0.1ml distilled water and 0.2ml 1% (w/v) aqueous solution of yeast RNA. A 'tissue/reagent' blank in which 0.2ml distilled water replaced the RNA was set up with each test, to correct for any absorption due to the reagents and for any ribonuclease activity in the absence of added substrate.

For the estimation of 'total' acid ribonuclease the procedure was the same as for the free enzyme assay except that 0.1ml of 0.003M p-chloromercuribenzoate replaced 0.1ml distilled water both in the test and in the tissue/reagent blank. The p-chloromercuribenzoate suspension was prepared by mixing the material in 0.03M veronal acetate buffer, pH 5.8, and titrating the solution to pH 5.8 with acetic acid before making up to the desired volume with the veronal buffer.

For both 'free' and 'total' ribonuclease assays, the reaction was started by adding 0.1ml of the tissue supernatant to each tube and incubating the tubes at 37°C for 30 min. At the end of the incubation period the tubes were cooled in ice for 1.0 min. after which the reaction was stopped by the addition of 0.6ml of an acid-ethanol reagent prepared by adding 12.0ml HCl to 8.0ml distilled water and making the volume up to 100ml with 96% (v/v) ethanol. Undegraded RNA and protein were removed by centrifugation at 1000g ave. for 15 min. at 2°C and the absorbance of the supernatant at 260nm was measured against distilled water.

For each batch of assays two substrate blanks each containing 0.2ml of the yeast RNA solution and 0.4ml of the veronal acetate buffer were set up. One blank was treated with the acid-ethanol reagent without prior incubation and the other was treated with the acid-ethanol reagent after a 30 min. incubation at 37°C. By comparing the A_{260} of the supernatants of these blanks after centrifugation it was confirmed that there was no breakdown of the yeast RNA in the absence of tissue extract.

To calculate the ribonuclease activity in each 'test' sample the sum of the absorbance at 260nm of the tissue/reagent blank and the substrate blank was subtracted from the absorbance of the 'test' at the same wavelength. One unit of ribonuclease activity was the enzyme activity which caused an increase of 0.1 in the absorbance at 260nm of the acid-ethanol soluble fraction under the experimental conditions described above.

(b) Alkaline ribonuclease assays

Experimental conditions for 'free' and 'total' alkaline ribonuclease assays were the same as for the acid enzyme assays except that the pH of the veronal acetate buffer was 7.8 and that p-chloromercuribenzoate was used at a concentration of 0.01M for the 'total' alkaline ribonuclease assays as recommended by Shortman (1961).

Preparation and Assay of prednisolone-receptor complexes from cytosol of rat muscle and liver

The methods employed to prepare cytosols from rat gastrocnemius muscle and liver and to assay the prednisolone-receptor complexes from these preparations were adapted from techniques described by several workers (Baxter and Tomkins, 1971; Petrovic and Markovic, 1975; Mayer and Rosen, 1975; Agawaral, 1977).

Gastrocnemius muscle, perfused *in situ* for 2 min. with ice-cold 0.85% (w/v) saline was removed quickly from the animal and minced finely. The tissue was suspended in 4 vol ice-cold homogenisation buffer (0.001M sodium phosphate buffer, pH 7.4, containing 0.5% (v/v) thiodiglycol to protect sulphydryl groups essential to the stability of the receptors), homogenised using a Silverson homogeniser at $0^{\circ}C$ and centrifuged at 60,000g ave. for 2 h at $2^{\circ}C$. The 'cytosol fraction' was carefully withdrawn with a syringe and stored at $2^{\circ}C$. It was used for prednisolone-binding studies within 24 h of preparation and without further purification.

Liver cytosol was prepared as described for muscle cytosol after perfusing the tissue *in situ* for 2 min. with cold 0.85% (w/v) saline through the portal vein.

The protein concentration of each preparation was measured by the biuret method and appropriate dilutions with the homogenisation buffer were made to give a cytosol preparation containing 4.0mg protein per ml. 1.0ml of this preparation was further diluted to 4.0ml with the same buffer and used for the prednisolone-binding assays. Prednisolone receptors were exposed to the steroid by incubating the dilute cytosol with a solution of ^{3}H-prednisolone for

90 min. at 0°C. Any unadsorbed steroid was removed by adsorption on activated charcoal. A determination of the radioactivity remaining in supernatant was taken as a measure of prednisolone-receptor complexes in the preparation. The steroid solution was prepared by mixing 0.1ml of a toluene/ethanol (9:1) solution containing 0.36mg unlabelled prednisolone per ml with 0.4ml of a solution of ^{3}H-prednisolone to give a final specific activity of 173μCi per mg prednisolone. The final concentration of the steroid in the assay mixture was 9.6μM. For use the steroid mixture was evaporated to dryness under nitrogen at 20°C and redissolved in 0.5ml absolute ethanol. 0.2ml of this solution was incubated with 4.0ml of the cytosol preparation at 0°C.

At the end of the incubation period any unbound prednisolone was separated from prednisolone-receptor complex by adsorption on activated charcoal. 0.5ml of a suspension of the activated charcoal (300mg/ml in 0.001M sodium phosphate buffer, pH 7.4) was added to the incubation mixture which was vigorously agitated on a vortex mixer for 1.0 min. The mixture was allowed to stand at 0°C for 5 min., centrifuged for 5 min. at 1000g ave. at 2°C, and the supernatant filtered through glass wool to remove any traces of charcoal.

Fractionation of prednisolone-receptor complexes in rat liver and muscle by ion-exchange chromatography

In order to obtain information on possible ionic differences in prednisolone-receptor subpopulations, the final filtrate from the preceding section was adsorbed on DE-52 cellulose ion-exchange column and eluted with a linear sodium phosphate gradient. The

cellulose was packed under gravity in a glass tube to give a column 25cm high and 1.0cm in diameter and used at 4°C. The column was washed with 100ml of the 'initial' buffer (0.001M sodium phosphate buffer, pH 7.4) before the sample (3.5mg protein in 3.5ml) was applied. Sodium phosphate of increasing ionic strength was the mobile phase and the fractionation of the receptor subpopulations depended on the fact that differences in the chemical nature of these macromolecules give rise to differences in their ionic charges and behaviour. These differences alter the readiness with which the macromolecules are held by the stationary phase and the ease with which they are subsequently eluted and released from the column by sodium phosphate. As the eluting buffer passes through the band containing the mixture of ions to be separated, bands of this mixture containing separate components are formed and, during their progress down the column, the degree of separation between the bands increases making efficient fractionation possible.

First, the sample on the column was washed with 70ml of the 'initial' buffer and 5.0ml fractions of the eluate were collected. The column was then eluted with a linear sodium phosphate gradient prepared by mixing 60ml each of 0.001M and 0.2M sodium phosphate buffer, pH 7.4 in a gradient former and 5.0ml fractions of the eluate were again collected. A flow rate of eluant of 1.0ml per min. was maintained throughout this procedure with the aid of peristaltic pump. Each fraction was assayed for protein by measuring its absorbance at 280nm and radioactivity was counted on the Intertechnique liquid scintillation spectrometer using NE 250 liquid scintillator.

Estimation of molecular sizes of prednisolone-receptor complexes in rat liver and muscle cytoplasm

Some information was sought about the molecular sizes of prednisolone-receptor complexes in the rat liver and muscle by studying the sedimentation behaviour of these complexes in a 5 - 20% (w/w) linear sucrose gradient. The gradient was prepared in 0.01M Tris buffer, pH 7.4 at 20°C, containing 0.001M EDTA according to the procedure described on page 73 . 1.0ml of the sample (the final filtrate obtained as described on page 94) was layered carefully over the gradient which was then centrifuged at 60,000g ave. for 24 h at 2°C. Thereafter 25-drop fractions of the gradient were collected sequentially from the bottom of the tube using a tube piercer. These fractions were counted for radioactivity. As the standard marker, 1.0ml of a solution of serum bovine albumin containing 2.0mg albumin per ml in 0.001M sodium phosphate buffer, pH 7.4, containing 0.5% (v/v) thiodiglycol was centrifuged concomitantly in an identical gradient. The sedimentation profile of this standard was obtained from the ultraviolet absorbance at 280nm of the 25-drop fractions collected at the end of the centrifugation. Approximate sedimentation coefficients and molecular weights were calculated for the prednisolone-receptor complexes by comparison with the standard (Martin and Ames, 1961).

RADIOACTIVITY MEASUREMENTS

Radioactivity was measured in the present studies by liquid scintillation counting on the Intertechnique liquid scintillation spectrometer Model SL30. In the scintillation process the disintergration of the radioactive sample results in the emission of electrons (β particles) the energy of which raises the surrounding solvent molecules to an excited state. The energy of the electron is thus transferred via the solvent molecules to a scintillator molecule which in turn becomes excited and emits its energy in the form of photons of light. The emitted light is then transmitted through the counter vial to a photomultiplier tube inducing an electrical signal which is amplified and counted.

Where glass fibre discs were employed, 0.15ml of the solution containing the radioactive sample was layered on the glass microfibre disc (Type GF/A, diameter 2.5cm, Whatman Ltd., England) which was then dried under an infra-red lamp for 30 min. The disc was transferred into a counting vial containing 5.0ml, of the toluene-based scintillation fluid (NE 233). For a 'blank' count, the same volume of the solvent in which the sample was dissolved was applied to the disc, dried and counted alongside the sample. The difference between test and blank counts was the true sample count. The duration of counting depended on the radioactivity of the sample under assay. Normally at least 4000 counts per sample were recorded. Where necessary, more than one and up to three sample-containing discs were loaded into a vial to increase the count rate with no significant loss of counting efficiency (Fig. 15).

The efficiency with which the samples were counted was determined

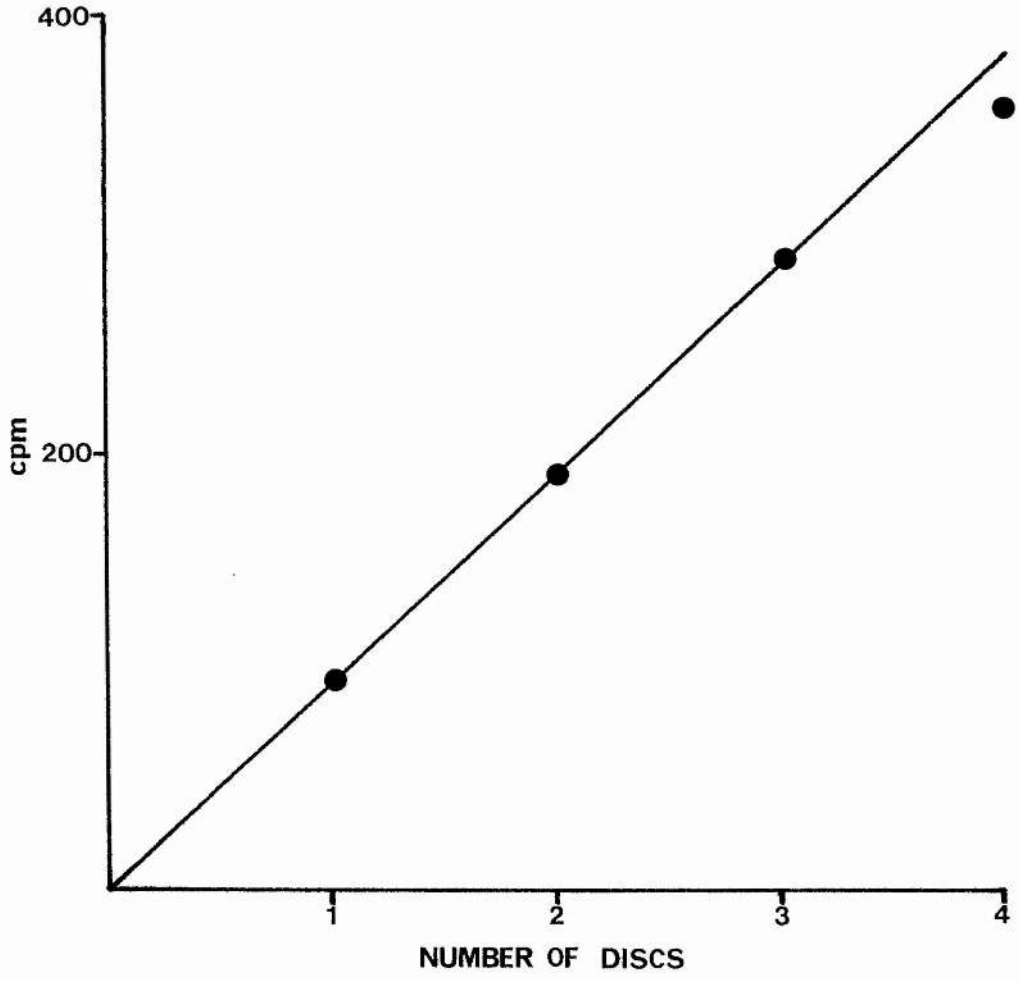


FIG. 15 The count rate of tritium-labelled samples as a function of the number of discs present in counting vials:
0.15ml of radioactive rat liver ribosomal nucleotides in 0.1M KOH was carried on each disc. Counting was performed on the pre-set ^{3}H Channel and background counts were obtained for each sample by subjecting the same number of discs, each carrying 0.15ml of 0.1M KOH to the assay procedure.
The cpm is the sample count less the background count.

by internal standardization. The sample on the disc was first counted. Then a known amount of radioactivity from the same isotope (internal standard) was added to the sample on the disc and another count made. The count rate (cpm) of the internal standard was the difference (D) between these two counts. The efficiency with which it was counted was calculated by dividing (D) by the known activity (dpm) of the standard. Since the standard is assumed to have counted with the same efficiency as the sample, the result of this calculation is also the counting efficiency of the sample. ^{3}H-labelled samples dissolved in 0.1M KOH counted with an efficiency averaging 15.6% and no significant variation between samples was observed (Table 4).

In some cases, liquid samples were counted directly in a dioxane-based scintillation fluid (NE 250). Up to 0.3ml of the sample was dissolved in 5ml scintillation fluid and the counting efficiency of these samples was obtained from an efficiency calibration curve (Fig. 16) which was plotted from data obtained by the external standardization technique. This technique makes use of the spectral shift towards lower energies as the counting efficiency of a sample is reduced by a quenching agent. The external standard is a gamma source (^{137}Cs) which generates electrons in the scintillator similar to in energy spectrum to a β emitter. When this external standard is placed in proximity to the sample it is counted along with sample and is subject to the same quench effect as the sample. These quench effects take the form of spectral shifts which are observed as changes in the ratios of counts in the lower and upper counting windows (channel ratios).

TABLE 4. The counting efficiency of nucleotide samples on the Intertechnique spectrometer (Model SL 30)

		cpm	dpm	Efficiency (%)
A	Sample X	19.2	-	-
B	Sample X + Orotic Acid (11190 dpm)	1794.4	-	-
	(B - A)	1775.2	11190	15.86
C	Sample Y	921.5	-	-
D	Sample Y + Orotic Acid (11190 dpm)	2644.3	-	-
	(D - C)	1722.8	11190	15.40
E	Sample Z	284.5	-	-
F	Sample Z + Orotic Acid (11190 dpm)	2024.5	-	-
	(F - E)	1740	11190	15.55

Sample X was the acid-soluble extract from the alkaline digest of rat gastrocnemius muscle homogenate.

Sample Y was the acid-soluble extract from the alkaline digest of rat liver homogenate.

Sample Z was the acid-soluble nucleotide pool from rat liver.

Details of the preparation of acid soluble extracts from alkaline digests and the preparation of Total acid soluble pool were described on pages 55 and 82.

All samples were assayed in duplicate and the values presented above are the calculated means.

Thus the external standard channel ratio can be related to the counting efficiency of the sample isotope.

To prepare the calibration curve, 0.3ml of an aqueous solution of ^{3}H-Orotic acid (11190 dpm) was introduced into each of 7 vials containing 5.0ml of the scintillation fluid (NE 250). Increasing quantities of a quenching agent (0.2 ——→ 3.0ml methanol) were added to each vial and the vials were loaded into the spectrometer. Channel C on the spectrometer was switched to the factory-set level for ^{3}H and the external standardization mode was switched to automatic. On the commencement of counting the following sequence of events took place automatically: The radioactivity in each vial was counted in channel C to give a count rate (C_c); The external standard source was positioned close to the detection chamber and counted in channels A and B to give count rates AI_a and BI_a respectively; finally, with the external standard source removed, the sample alone was counted in channels A and B to give count rates AI_b and BI_b respectively.

Dividing C_c by the activity of the sample (11190 dpm) gave the efficiency with which each sample was counted. The channel ratio ($^{A}/B$) for each sample was calculated from the relationship

$$\frac{A}{B} = \frac{AI_a - AI_b}{BI_a - BI_b}$$

where A is the channel with the greater count rate and B is the channel with the lesser count rate. The efficiency curve was then plotted with efficiency values against the corresponding channel ratios. The counting efficiency of a test sample was read off from

this curve using channel ratios obtained by subjecting the test sample (without methanol) to the procedure described above. Samples dissolved in 0.1M KOH were counted with an efficiency of 37.5% under these conditions.

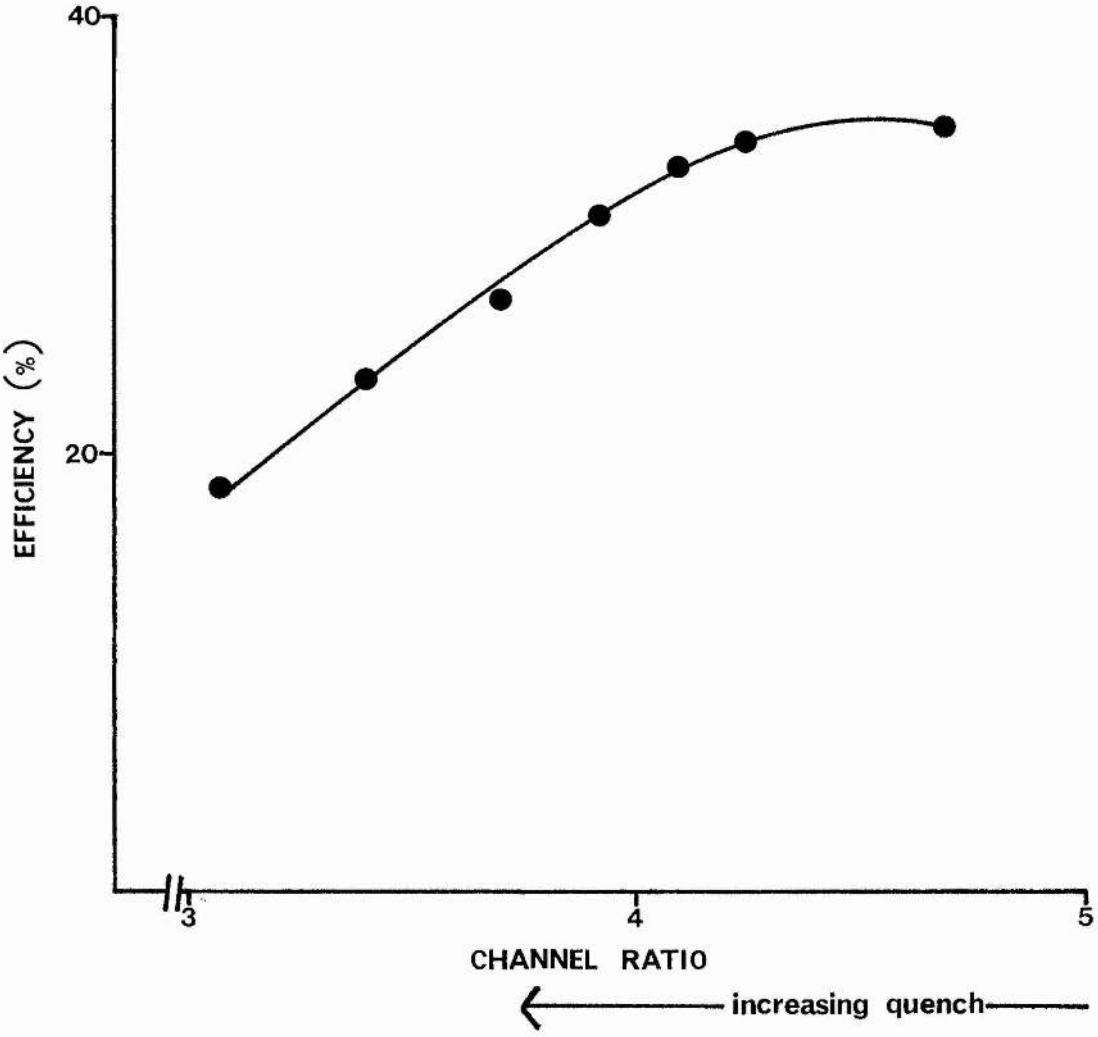


FIG. 16 The quench curve for ^{3}H-Orotic Acid (11190 dpm) using the external standard counting mode. Experimental details are described on page 102.

MATHEMATICAL TREATMENT OF RESULTS

Derivation of standard deviation and standard error of mean, and tests of statistical significance

The standard deviation and the standard error of mean of a set of results were calculated following the procedure described by Spiegel (1961). To determine the level of statistical significance of data obtained from 'test' and 'control' experiments a group or paired 't' test was performed as appropriate as described by Spiegel (1961).

Calculation of slopes by method of least squares

If n is the number of points on a graph in which values on the x axis are plotted against values on the y axis then the slope, m, of these points is given by the equation

$$m = \frac{\sum x . \sum y - n \sum xy}{\left[\sum x\right]^2 - n \sum x^2}$$

and the y intercept (c) is given by the relationship

$$c = \frac{\sum x . \sum xy - \sum x^2 . \sum y}{\left[\sum x\right]^2 - n \sum x^2}$$

These values of m and c fit into the equation for a straight line ($y = mx + c$) and a point for x at a fixed value of y can be calculated from this equation. The calculated slope is the line joining this point to the y intercept (c).

3. RESULTS

EFFECT OF PREDNISOLONE TREATMENT ON PROTEIN AND NUCLEIC ACID CONCENTRATIONS IN RAT LIVER AND MUSCLE

This group of experiments investigated the effect of daily injections of prednisolone acetate on RNA, DNA and protein concentrations in rat liver and gastrocnemius muscle. The changes in the body weight of the animal during the experimental period were also measured. Prednisolone acetate was administered daily over an eight-day period to male rats by subcutaneous injections into the hind limbs at a dose level of 1.0mg per 100g initial body weight.

The results presented on Table 5 and Fig. 17 show the changes in the overall body weight of the animal over the experimental period. There did not appear to be any alteration in body weight in prednisolone-treated rats sacrificed on the day after the first prednisolone injection (Day 2). During the same period, control rats injected with saline gained weight. On subsequent days, control animals exhibited a progressive rise in body weight while prednisolone-treated rats showed a marked loss of weight.

Over the experimental period, the gastrocnemius muscle of prednisolone-treated animals showed a loss of weight (Table 6 and Fig. 18). This loss was statistically significant from the fourth day, averaging about 20% when compared with gastrocnemius from the control animal and became more pronounced as prednisolone treatment progressed. In contrast the liver of prednisolone-treated animals gained weight (Table 8 and Fig. 18). The liver of control animals treated with saline did not exhibit the same rapid increase in weight. This difference was evident as early as two days after the

initial prednisolone injection and persisted all through the experimental period with increases averaging between 15% and 24% of the liver weight of control rats.

The protein content of the gastrocnemius muscle of prednisolone-treated animals showed a decrease during the experimental period. This decrease was statistically significant from the fourth day when protein content of prednisolone-treated rats was 75% of that of the control animals (Table 6). This loss of protein from gastrocnemius muscle was still evident when the result was expressed per unit weight of the tissue (Table 7 and Fig. 19), indicating that the protein loss was disproportionate to the overall loss of muscle mass. Total liver, on the other hand, increased in prednisolone-treated rats, averaging 25% more than the control levels on the second day and rising to approximately 30% by Day 8 (Table 8). These changes in the protein concentrations in the liver of prednisolone-treated animals parallel the changes in the wet weight of the organ as indicated by data on Table 9 and Fig. 20.

There was also a decrease in the total RNA content of the gastrocnemius muscle of prednisolone-treated animals when it was compared with the RNA content of the saline-treated control animals. This change was apparent two days after the initial prednisolone injection and had become statistically significant by Day 4. The RNA loss averaged 11% on the second day and reached almost 50% on Day 8 (Table 6). This RNA loss was disproportionate to the overall loss of muscle mass (Table 7). The RNA content in the liver of prednisolone-treated animals increased, compared with

that of controls (Table 8). This increase attained statistical significance ($P < 0.05$) from the second day and by Day 8 the RNA concentration in the prednisolone-treated animal was 26% more than the concentration in the liver of the control animal.

Throughout the experimental period, the DNA concentration in the gastrocnemius muscle of both prednisolone-treated animals and their controls remained constant (Table 6). There was also no significant change in liver DNA content.

TABLE 5 EFFECT OF PREDNISOLONE ACETATE TREATMENT ON RAT BODY WEIGHT

	Day 2		Day 4		Day 6		Day 8	
	Prednisolone-treated	Control	Prednisolone-treated	Control	Prednisolone-treated	Control	Prednisolone-treated	Control
Initial Body weight (g)	157 ±4.2	153 ±3.8	150 ±3.9	151 ±3.6	150 ±3.7	148 ±3.8	152 ±3.1	154 ±4.4
Body weight on sacrifice (g)	156 ±4.5	161 ±3.6	142 ±4.1	163 ±3.1	142 ±4.5	173 ±2.9	140 ±4.8	178 ±3.3
% loss or gain in body weight	-0.89 ±2.0	+5.0 ±2.9	-5.4 ±3.0	+8.4 ±2.9	-5.5 ±4.0	+16.7 ±3.4	-7.9 ±3.4	+16.1 ±4.1
	(<0.005)		(<0.005)		(<0.005)		(<0.005)	

Prednisolone-treated animals received daily subcutaneous injections of 0.06ml prednisolone acetate (25mg/ml in 0.85% saline) per 150g initial body weight into their hind limbs. Control animals received the same volume of 0.85% (w/v saline).
Results are mean values from 6 rats ± standard deviation.
Figures in parentheses indicate statistical significance level (Student's group 't' test) between prednisolone-treated and control animals.

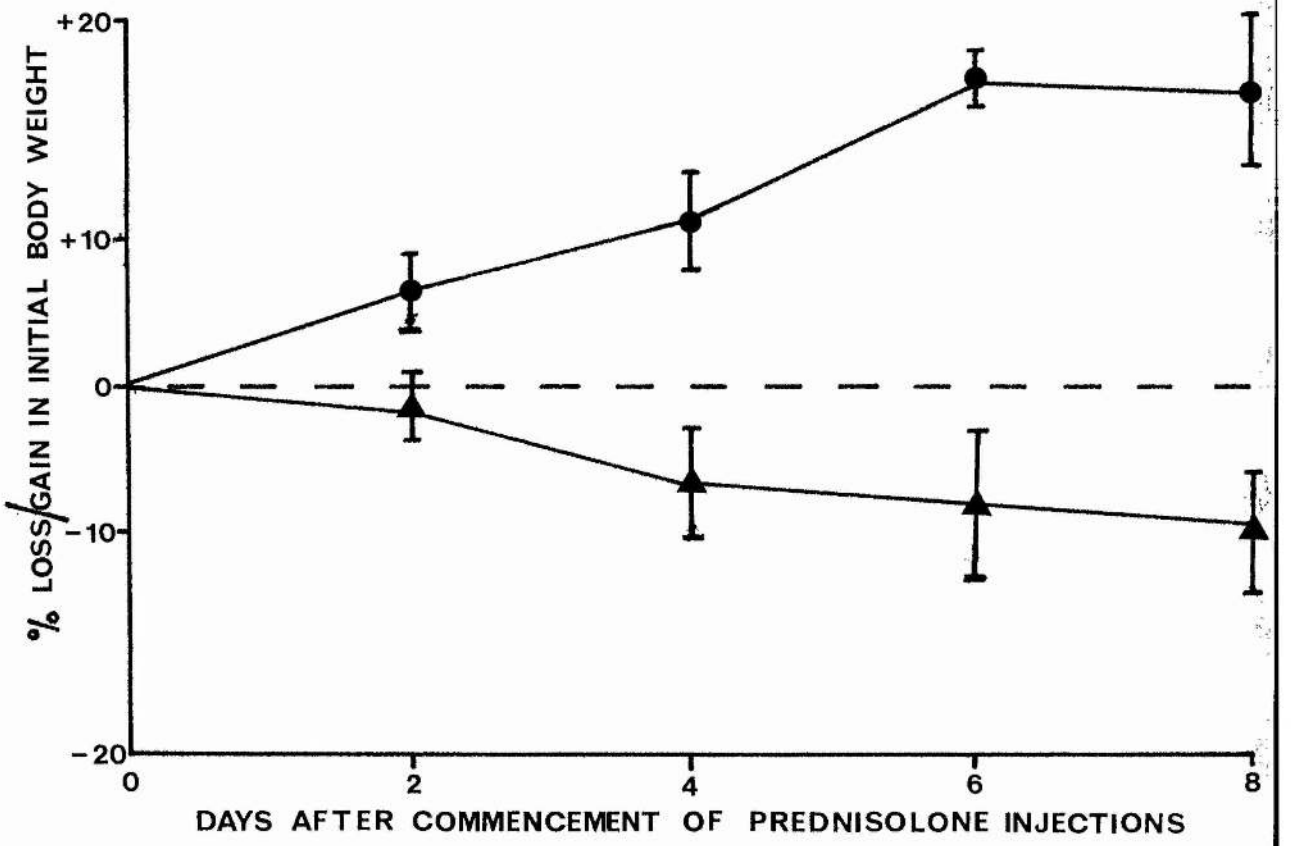


FIG. 17 The effect of prednisolone acetate treatment on rat body weight: Prednisolone-treated animals received 0.06ml prednisolone acetate (25mg/ml) per 150g initial body weight daily while control animals received the same volume of 0.85%(w/v) saline. Results are mean values from 6 animals ± standard deviation.
Triangles indicate results obtained with prednisolone-treated animals and circles indicate results from control animals.

TABLE 6 EFFECT OF PREDNISOLONE ACETATE TREATMENT ON PROTEIN AND NUCLEIC ACID CONCENTRATIONS IN RAT GASTROCNEMIUS MUSCLE

	Day 2		Day 4		Day 6		Day 8	
	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control
Muscle wet weight (g/100g initial body weight)	0.49 ±0.01	0.52 ±0.04	0.45 ±0.03	0.56 ±0.03	0.42 ±0.02	0.58 ±0.04	0.40 ±0.01	0.61 ±0.06
	(N.S)		(<0.005)		(<0.005)		(<0.005)	
Total protein control (mg/muscle/ 100g initial body weight)	82.5 ±12.1	91.9 ±12.6	74.4 ±8.0	99.8 ±17.1	68.9 ±3.1	109 ±6.4	62.5 ±8.4	108 ±6.7
	(N.S)		(<0.01)		(<0.005)		(<0.005)	
Total RNA control (mg/muscle/100g initial body weight)	0.93 ±0.16	1.05 ±0.15	0.72 ±0.06	1.11 ±0.12	0.62 ±0.08	1.12 ±0.06	0.57 ±0.08	1.13 ±0.12
	(N.S)		(<0.005)		(<0.005)		(<0.005)	
DNA content (mg/ muscle/100g initial body weight)	0.49 ±0.02	0.50 ±0.03	0.50 ±0.02	0.50 ±0.02	0.50 ±0.02	0.51 ±0.02	0.50 ±0.03	0.51 ±0.03
	(N.S)		(N.S)		(N.S)		(N.S)	

Experimental details are as described in Table 5. Results are mean values from 6 animals ± Standard Deviation. Figures in parentheses indicate least of statistical significance between prednisolone-treated and control animals (Student's group 't' test). Only gastrocnemius muscle from the right hind limb was used in these studies.

TABLE 7 EFFECT OF PREDNISOLONE ACETATE TREATMENT ON PROTEIN AND RNA CONCENTRATIONS IN RAT GASTROCNEMIUS MUSCLE

	Day 2		Day 4		Day 6		Day 8	
	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control
Protein Concentration (mg/g Muscle)	168 ±20.7	176 ±25.4	165 ±18.4	178 ±20.8	162 ±11.0	191 ±15.9	152 ±14.5	188 ±8.9
	(<0.025)		(<0.005)		(<0.005)		(<0.005)	
RNA concentration (mg/g Muscle)	1.9 ±0.16	2.0 ±0.14	1.6 ±0.15	2.0 ±0.18	1.6 ±0.09	2.0 ±0.10	1.5 ±0.10	1.9 ±0.11
	(N.S)		(N.S)		(<0.05)		(<0.05)	

This table was compiled from data used for Table 6 but here results are expressed as mean values per gram muscle ± standard deviation.

TABLE 8 EFFECT OF PREDNISOLONE ACETATE TREATMENT ON PROTEIN AND RNA CONCENTRATIONS IN RAT LIVER

	Day 2		Day 4		Day 6		Day 8	
	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control
Liver weight (g/100g initial body weight)	5.1 ±0.41	4.1 ±0.42	5.7 ±0.41	4.6 ±0.31	5.6 ±0.34	4.8 ±0.08	5.6 ±0.21	4.8 ±0.06
	(< 0.025)		(< 0.01)		(< 0.025)		(< 0.025)	
Total protein content (mg/liver/ 100g initial body weight)	722 ±826	576 ±78.1	800 ±31.2	620 ±30.0	963 ±67.1	759 ±60.5	979 60.3	760 ±47.4
	(< 0.05)		(< 0.005)		(< 0.025)		(< 0.01)	
Total RNA content (mg/liver/100g initial body weight)	44.5 ±5.4	37.6 ±5.2	47.8 ±4.7	37.7 4.3	46.2 ±2.6	38.3 ±3.8	47.2 ±2.8	37.6 ±1.6
	(<0.05)		(< 0.025)		(< 0.025)		(< 0.01)	
DNA content (mg/ liver/100g initial body weight)	12.5 ±0.35	12.5 ±0.34	12.6 ±0.40	12.6 ±0.45	12.6 ±0.21	12.9 ±0.50	12.4 ±0.50	12.7 ±0.50
	(N.S)		(N.S)		(N.S)		(N.S)	

Experimental details are as described on Table 5.
Results are mean values from 4 animals ± standard deviation.
Figures in parentheses indicate level of statistical significance between controls and prednisolone-treated animals (Student's group 't' test)

TABLE 9 EFFECT OF PREDNISOLONE ACETATE TREATMENT ON PROTEIN AND RNA CONCENTRATIONS IN RAT LIVER

	Day 2		Day 4		Day 6		Day 8	
	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control	Prednisolone -treated	Control
Total Protein Concentration (mg/g liver)	144 ±19.7 (N.S)	138 ±15.6	141 ±16.4 (N.S)	139 ±18.7	154 ±16.1 (N.S)	150 ±13.7	175 ±20.3 (<0.01)	155 ±19.9
RNA Concentration (mg/g liver)	8.8 ±0.93 (N.S)	8.8 ±0.99	8.4 ±0.96 (N.S)	8.6 ±0.87	8.0 ±0.83 (N.S)	7.9 ±0.82	8.2 ±0.81 (N.S)	7.9 ±0.78

This table was compiled from data used on Table 8.

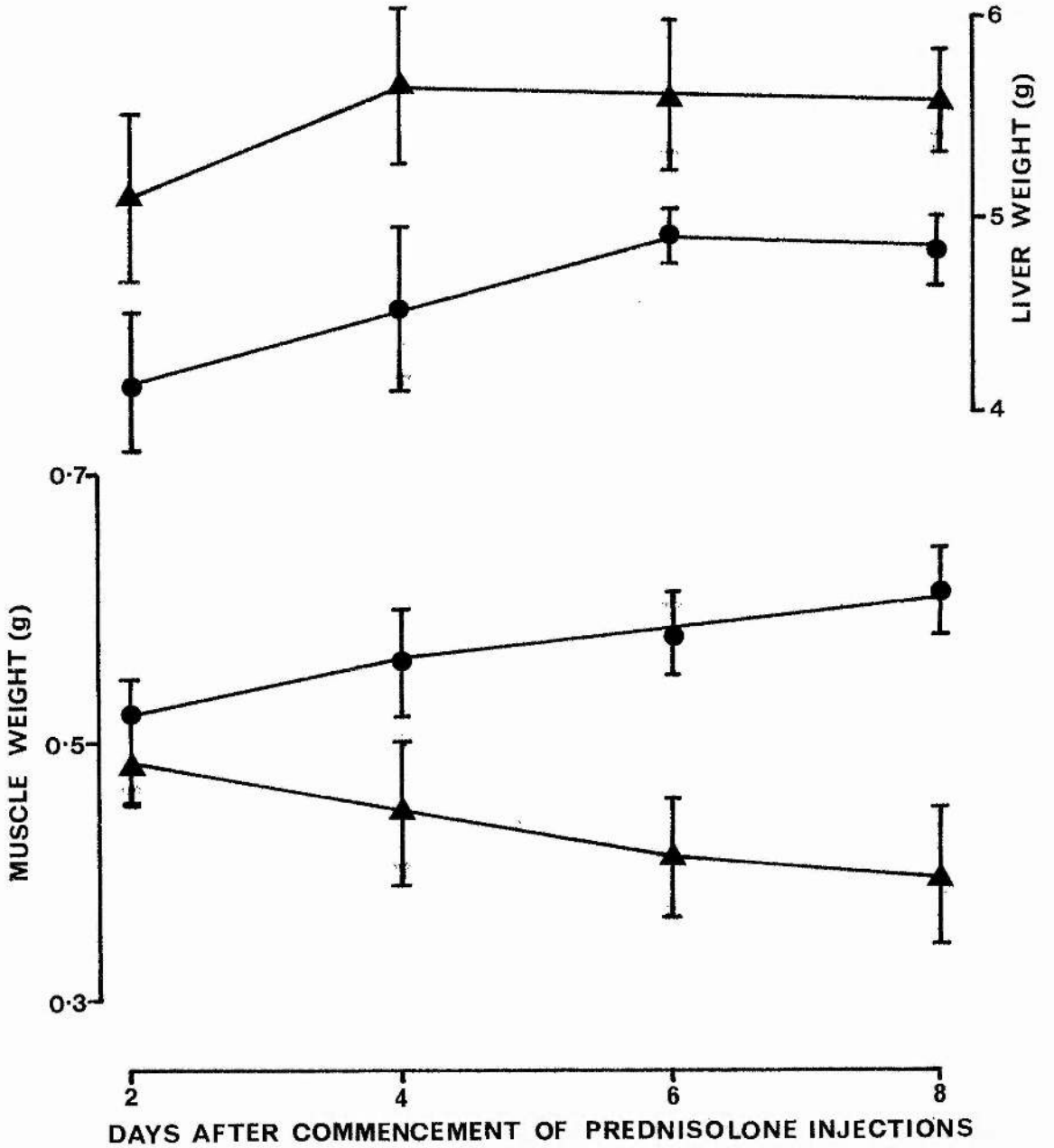


FIG. 18 The effect of prednisolone acetate treatment on rat gastrocnemius muscle and liver weights: Experimental details were as described on Table 5 Results are mean values from 6 rats $\pm$ standard deviation. Circles indicate controls and triangles indicate results obtained with prednisolone-treated animals.

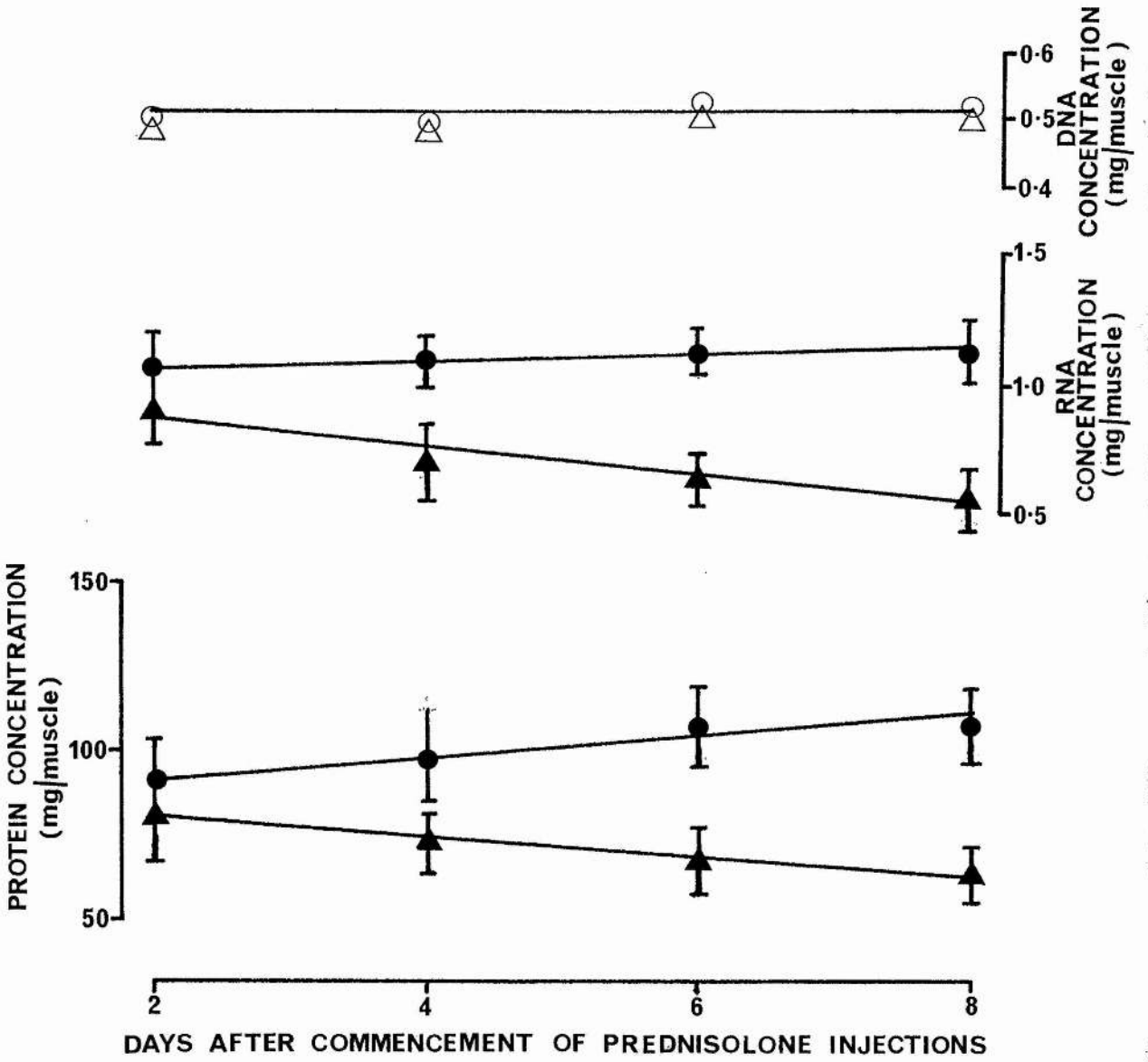


FIG. 19 The effect of prednisolone treatment on protein and Nucleic Acid Concentrations in rat gastrocnemius muscle:
Experimental details were as described on Table 5.
Results are mean values from 6 rats ± standard deviation.
Circles indicate controls and triangles indicate results obtained with prednisolone-treated animals.

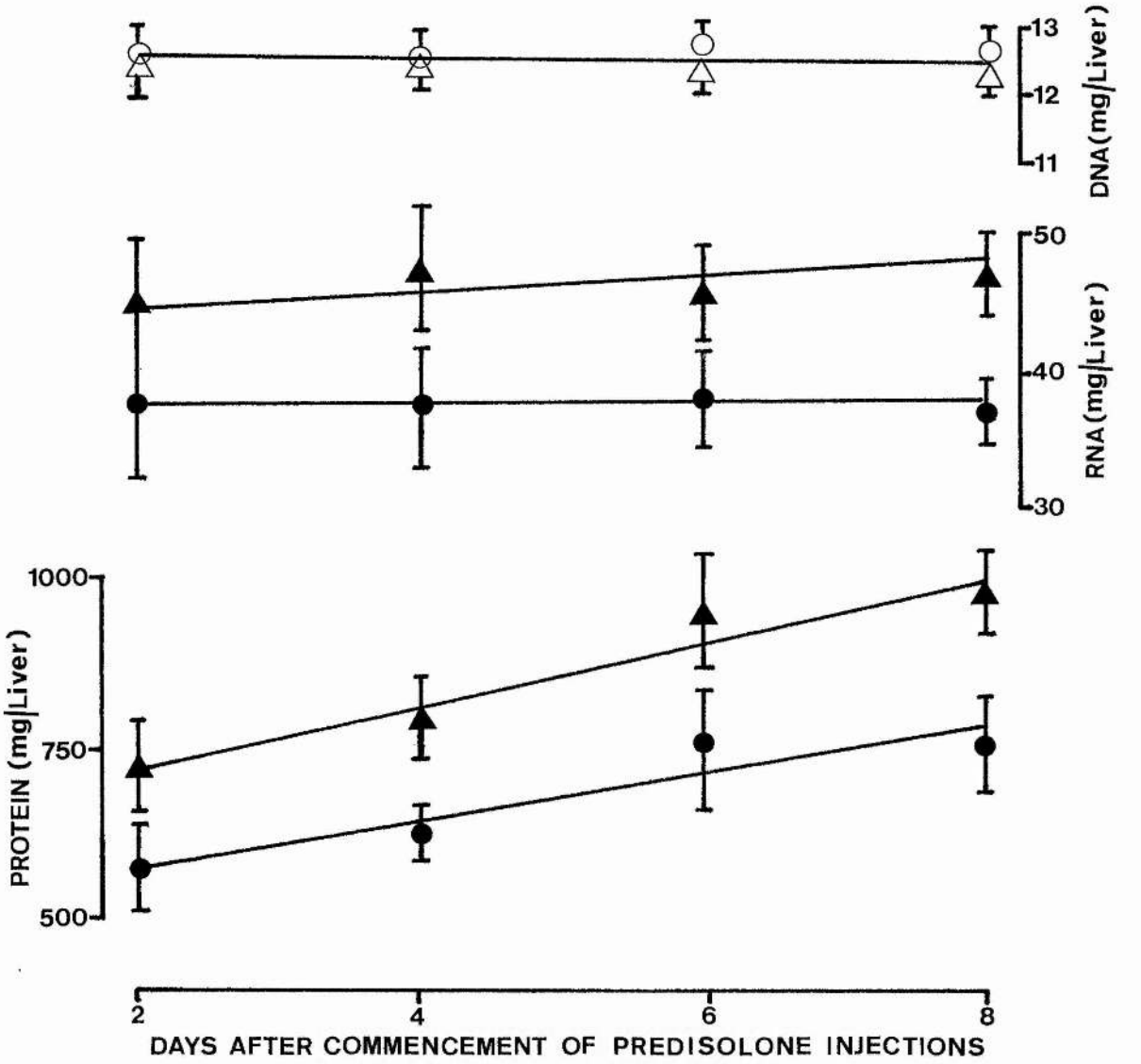


FIG. 20 The effect of prednisolone acetate treatment on Protein and RNA concentrations in rat liver. Experimental details are as described on Table 5. Results are mean values from 4 rats ± standard deviation.
Circles indicate controls and triangles indicate results obtained with prednisolone-treated animals.

STUDIES ON THE TURNOVER OF TOTAL RNA IN LIVER AND MUSCLE FOLLOWING PREDNISOLONE TREATMENT

As early as 48 h after daily injections of prednisolone acetate (i.e. on Day 2) alterations were observed in the amounts of RNA in rat liver and muscle (Figs. 19 and 20). Reports that responses to corticosteroids are prevented by actinomycin D (Greengard et al, 1963; Garren et al, 1964) and that these steroids alter the rates of RNA breakdown in rat liver (Ottolenghi and Barnabei, 1970) suggest that RNA synthesis or breakdown may be involved in these responses. In this section an investigation of the effect of prednisolone treatment on the rates of synthesis and breakdown of cytoplasmic RNA in the liver and gastrocnemius muscle of the rats was undertaken. Since it had been demonstrated in this laboratory that maximal labelling of rat liver RNA was achieved two days after the initial injection of ^{3}H- labelled Orotic acid (Ma, 1973), this time duration was allowed before the commencement of prednisolone injections. 2 days after injecting 20μCi of ^{3}H-Orotic acid intraperitoneally into the animals, daily subcutaneous injections of prednisolone acetate were administered to the 'test' group of animals while the control group received the same volume of saline. Every second day a test animal and its control partner were sacrificed, liver and muscle were excised and the radioactivity and RNA content of both tissues were measured using techniques described in the 'methods' section.

Table 10 shows the pattern of loss of specific radioactivity of the total RNA in the liver over the experimental period. Both the prednisolone-treated animals and their controls exhibited a

TABLE 10. EFFECT OF PREDNISOLONE ACETATE TREATMENT ON THE LOSS OF RADIOACTIVITY FROM ^{3}H-LABELLED LIVER RNA

Days after Orotic Acid Injection	Specific Activity cpm/mg RNA (x 10^{-3})		Total Activity (cpm/liver/100g body weight) x 10^{-3}	
	Prednisolone -treated	Control	Prednisolone -treated	Control
2	-	45.0 ±0.9	-	1701 ±29.2
4	29.0 ±0.9 (N/S)	30.3 ±1.0	1411 ±40.5 (<0.025)	1178 ±31.6
6	19.9 ±0.8 (N/S)	21.0 ±0.8	1021 ±49.6 (<0.005)	768 ±42.8
8	15.3 ±1.1 (N/S)	15.6 ±0.9	725 ±41.4 (<0.005)	601 ±38.5
10	11.3 ±1.1 (N/S)	9.6 ±0.8	532 ±36.2 (<0.005)	390 ±45.6

Each animal received a single dose of ^{3}H-Orotic acid (20.0μCi) intraperitonally. Commencing two days after this injection prednisolone-treated animals received 0.06ml prednisolone acetate (25mg/ml) daily subcutaneously. Controls received the same volume of 0.85% (w/v) saline.

Total RNA and radioactivity were measured as described on pages 55 and 98 respectively. Each result is the mean from 5 animals ± standard deviation. Figures in brackets indicate level of statistical significance between prednisolone-treated rats and controls as calculated by grouped 't' test.

progressive loss of specific radioactivity and although the rate of this loss was slightly slower in the prednisolone-treated animals than in controls, the difference was not statistically significant on any of the days studied. However, when the total radioactivity in each tissue was calculated taking into account the increase in liver RNA concentrations in the prednisolone-treated animals it was evident that the administration of prednisolone resulted in a slower decay of RNA radioactivity in the treated animals. Statistically significant differences between the total radioactivities of the prednisolone-treated animals and the control group were observed as early as two days after the onset of prednisolone administration. In the control animal, total liver RNA radioactivity, after reaching a peak, dropped to half of this peak radioactivity in approximately four and a half days (Fig. 21 A). At this time, the total liver RNA in the prednisolone-treated animal had lost only 40% of the peak radioactivity. When data from these experiments were plotted on a logarithmic scale against a linear time scale, a straight line relationship was observed in both control and prednisolone-treated animals (Fig. 21 B), an indication that total RNA degradation in rat liver followed first order kinetics in both groups of animals. From the slope of the lines on Fig. 21 B the biological half-lives of total RNA in the liver of the prednisolone-treated and control animals were calculated to be 5.85 and 4.26 days respectively. The corresponding rate constants of RNA degradation were 0.1185 and 0.1627 respectively. Since no significant alteration occurred in the amount of total RNA in the liver of the control animal

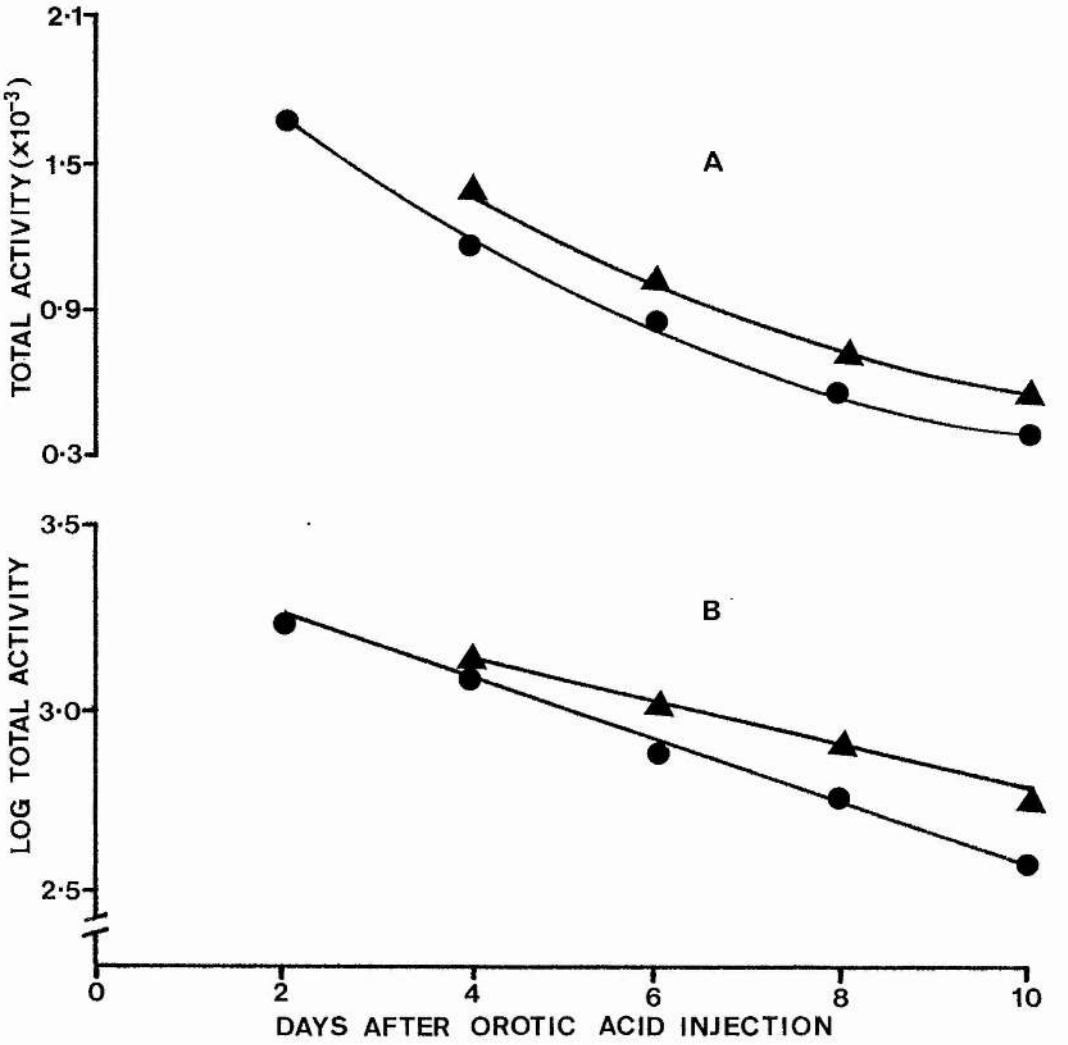


FIG. 21 Loss of radioactivity from total liver RNA following prednisolone treatment.
Experimental details are as described for Table 10.
Each point is the mean value from 5 animals. Triangles represent results from prednisolone-treated animals and circles represent controls. On graph B the slope of each line was plotted by method of 'least squares'.
Total activity was expressed as cpm/liver/100g body weight.

during the experimental period (Table 8), the amount of RNA synthesised per day was assumed to be the same as the amount broken down. Using a rate constant of RNA degradation of 0.1627, the amount of RNA synthesised per day in the control animal was calculated to be 6.13mg per liver per 100g initial body weight. In the prednisolone-treated animal there was a mean increase of 24% in the RNA levels during the 8-day experimental period. In this animal the amount of RNA synthesised per day will be equal to the amount broken down per day plus the daily change in total RNA concentration. This value was calculated to be 8.8mg RNA per liver per 100g initial body weight. From the biological half-lives obtained above, the turnover times for total RNA is prednisolone-treated and control rats were 8.5 and 6.15 days respectively.

The results of a similar investigation of total RNA radioactivities in rat gastrocnemius muscle are presented on Table 11. The progressive decrease in specific radioactivities observed in liver RNA was not evident in muscle where the levels of specific radioactivity remained unchanged or, sometimes, tended to show an increase (Fig. 22). This pattern was observed in both control and prednisolone-treated animals and is not consistent with the general concept of breakdown of RNA, its replacement by newly synthesised unlabelled RNA and a consequent drop in the specific radioactivity of the macromolecule. To investigate this apparent discrepancy, the radioactivities of the RNA precursor pools in both groups of animals were examined and the turnover of various RNA species in muscle was determined. The findings are described in subsequent sections.

TABLE 11. RADIOACTIVITY LEVELS IN THE TOTAL RNA OF GASTROCNEMIUS MUSCLE OF PREDNISOLONE-TREATED RATS

Days after Orotic Acid Injection	Specific Activity (cpm/mg RNA) x 10^{-3}		
	Prednisolone -treated		Control
2	-		0.88 ± 0.07
4	0.89 ± 0.06	(N/S)	0.99 ± 0.06
6	1.14 ± 0.05	(N/S)	1.11 ± 0.05
8	1.20 ± 0.06	(N/S)	1.25 ± 0.04
10	1.33 ± 0.05	(N/S)	1.29 ± 0.05

Each animal received a single dose of ^{3}H-Orotic acid (20.0 μCi) intraperitoneally. Commencing two days after this injection, each prednisolone-treated animal received 0.06ml prednisolone acetate (25mg/ml) daily subcutaneously. Controls received the same volume of 0.85% (w/v) saline.

RNA and radioactivity were measured as described on pages 55 and 98 respectively. Each result is the mean from 5 animals $\pm$ standard deviation.

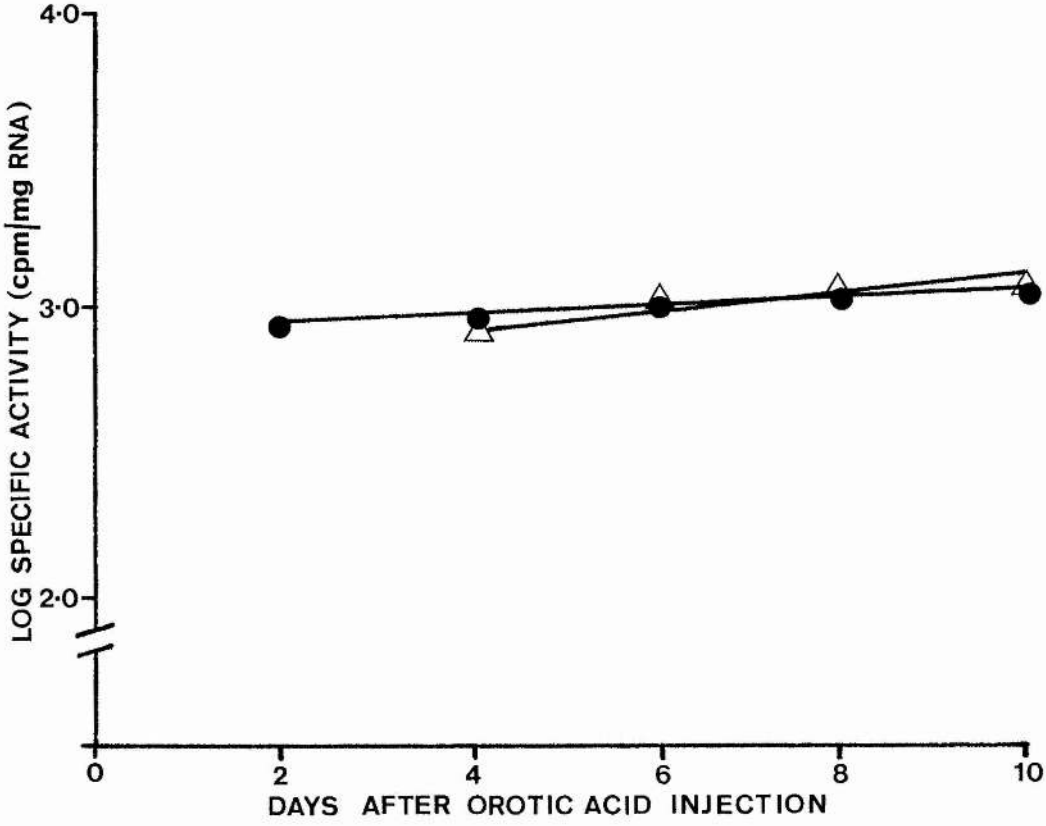


FIG. 22 Radioactivity of total RNA in rat gastrocnemius muscle following prednisolone treatment: Experimental details are as described for Table 11. Each point is the mean value from 5 animals. Triangles represent results obtained from prednisolone-treated animals and circles represent controls. The slope of each line was calculated by method of least squares.

TURNOVER OF RIBOSOMES IN LIVER AND MUSCLE OF PREDNISOLONE-TREATED RATS

The central role which ribosomes play in cytoplasmic protein synthesis is well established and it has been demonstrated that hormones can influence the *in vitro* activity of muscle (Florini and Breuer, 1966) as well as liver (Rancourt and Litwack, 1968) ribosomes. Ribosome turnover may therefore be a controlling factor in the biological responses observed following prednisolone treatment and the translation of genetic information and protein synthesis may be regulated by the glucocorticoid at this ribosomal level. This series of experiments investigated ribosomal turnover in the liver and gastrocnemius muscle of prednisolone-treated animals. The RNA component of the ribosome was labelled with 20 μCi of ^{3}H-Orotic Acid and the turnover of the ribosomal RNA was determined by following the rate of loss of radioactivity. Procedures for isolating ribosomes from the liver and muscle are described on page 58 RNA and radioactivity measurements were performed as described on pages 61 and 98 respectively.

With liver ribosomes, there was a progressive loss of specific radioactivities in both the prednisolone-treated animals and their controls (Table 12) over the experimental period. This loss of specific radioactivity was slightly slower in the prednisolone-treated animals than in the control group. The slower rate of loss of radioactivity in the prednisolone-treated group became more obvious when the total radioactivities in these ribosomes were calculated. Statistically significant differences between these total radioactivities in the two groups of animals were observed

TABLE 12. LOSS OF RADIOACTIVITY FROM RNA IN RAT LIVER RIBOSOMES FOLLOWING PREDNISOLONE TREATMENT

Days after Orotic Acid Injection	Specific Activity cpm/mg RNA (x 10^{-3})		Total Activity cpm/liver/100g body weight (x 10^{-3})	
	Prednisolone-treated	Control	Prednisolone-treated	Control
2	-	40.6 ± 3.1	-	1598 ± 50.3
4	29.8 ± 1.8 (N/S)	30.3 ± 2.6	1323 ± 39.6 (<0.025)	1142 ± 41.3
6	24.6 ± 1.3 (N/S)	26.0 ± 2.1	1176 ± 50.1 (<0.010)	982 ± 48.5
8	19.3 ± 2.0 (N/S)	19.4 ± 2.8	892 ± 34.5 (<0.010)	727 ± 57.8
10	13.3 ± 2.8 (N/S)	12.5 ± 2.6	631 ± 40.0 (<0.010)	471 ± 46.1

Each animal received a single dose of ^{3}H-Orotic acid (20.0 μCi) intraperitoneally. Commencing two days later, each prednisolone-treated animal received subcutaneous injections of 0.06ml prednisolone acetate (25mg/ml). Controls received the same volume of 0.85% (w/v) saline.

Ribosomes were isolated as described on page 58 and RNA and radioactivity measurements were performed as described on pages 61 and 98 respectively. Each result is the mean from 4 animals $\pm$ standard deviation. Figures in brackets indicate level of statistical significance between prednisolone-treated animals and their controls as calculated by Students' grouped 't' test.

from two days after the onset of prednisolone treatment (Table 12). The liver of the control animal lost 50% of its ribosomal radioactivity in 4.4 days when the prednisolone-treated animal still retained 62% of its radioactivity (Fig. 23A). When these data were plotted on a logarithmic scale against a linear time scale the rate of loss of radioactivity, for both animals, followed first order kinetics showing a straight line relationship between the total radioactivity and time (Fig. 23B). From the slope of these lines, the rate constants of RNA degradation in the prednisolone-treated group and in the control group were 0.1065 and 0.1612 respectively. These constants correspond to biological half-lives of 6.51 and 4.3 days for the prednisolone-treated and control animals respectively. Ottolenghi and Barnabei (1970), from studies of liver microsomal RNA turnover in cortisone-treated and control rats reported half-lives of 7.4 and 4.7 respectively. The amount of ribosomal RNA synthesised per day per liver per 100g initial body weight, in the control group was 6.06mg while the corresponding value in the prednisolone-treated group was 8.23mg. Turnover time for this RNA was 9.39 days for the prednisolone-treated animals and 6.20 days for the control animals.

The results of the investigation of ribosome turnover in the rat gastrocnemius muscle are presented on Table 13. Ribosomes from the muscle of the control group of animals showed a progressive loss of specific radioactivity over the experimental period. Such a pattern of loss of radioactivity was not evident in the prednisolone-treated animals. If synthesis were occurring during the period of prednisolone treatment, any new RNA formed would be

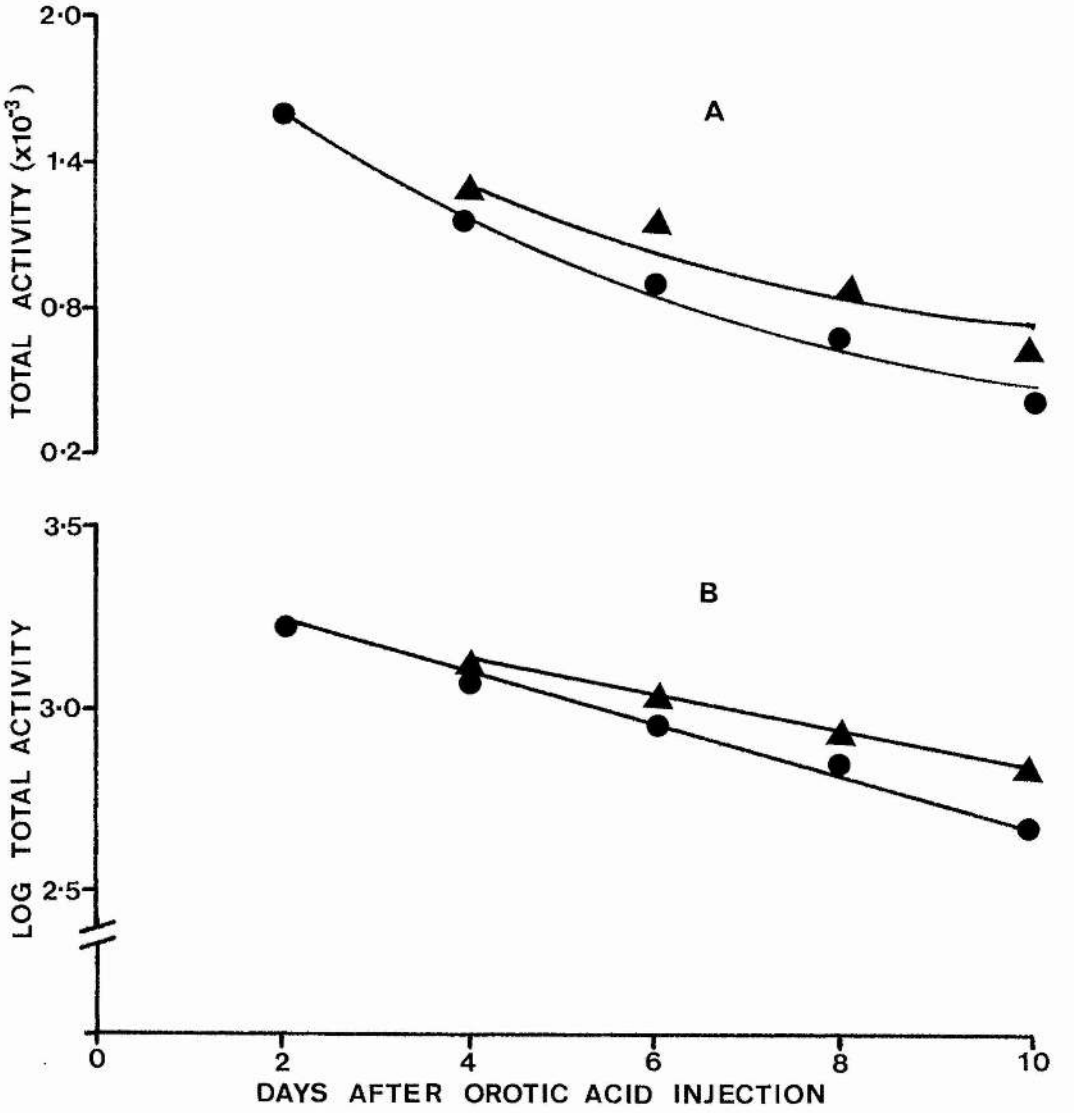


FIG. 23 Loss of radioactivity from RNA in liver ribosomes following prednisolone treatment:
Experimental details are as described on Table 12. Each point represents the mean result from four animals. Triangles indicate results from prednisolone-treated animals and circles represent the controls. On graph B the slope of each line was plotted by the 'least squares' method.

TABLE 13. RADIOACTIVITY IN RIBOSOMES OF GASTROCNEMIUS MUSCLE OF PREDNISOLONE-TREATED RATS

Days after Orotic Acid Injection	Specific Activity of Ribosomes (cpm/μg RNA)		Total Activity of Ribosomes (cpm/muscle/ 100g body weight) x 10^{-3}	
	Prednisolone -treated	Control	Prednisolone -treated	Control
2	-	2.7 ± 0.18	-	2.8 ± 0.11
4	1.9 ± 0.07 (N/S)	2.3 ± 0.16	1.8 ± 0.14 (< 0.025)	2.3 ± 0.24
6	2.0 ± 0.11 (N/S)	1.7 ± 0.14	1.5 ± 0.18 (N/S)	1.8 ± 0.15
8	1.9 ± 0.16 (< 0.025)	1.2 ± 0.11	1.3 ± 0.14 (N/S)	1.3 ± 0.16
10	1.8 ± 0.12 (< 0.01)	1.0 ± 0.17	1.0 ± 0.22 (N/S)	1.0 ± 0.13

Each animal received a single dose of ^{3}H-Orotic Acid (20.0μCi) intraperitoneally. Commencing two days later, each prednisolone-treated animal received daily subcutaneous injections of prednisolone acetate (25mg/ml). Control animals received the same volume of 0.85%(w/v) saline.

Ribosomes were isolated as described on page 58 ; RNA and radioactivity measurements were performed as described on pages 61 and 98 . Each result is the mean from 4 animals $\pm$ standard deviation. Figures in brackets indicate the level of statistical significance between prednisolone-treated animals and their controls, as determined by students grouped 't' test.

less radioactive due to loss of radioactivity from the precursor pool and this would be reflected in a drop of specific radioactivity throughout the experimental period. Since there was no change in specific radioactivity it would appear that synthesis of RNA associated with ribosomes in the prednisolone-treated rats was depressed. When the total radioactivity in the ribosomes was calculated, taking the total RNA content of the ~~liver~~ muscle as an estimate of the amount of ribosomal RNA, it was found that muscle ribosomes from the prednisolone-treated group suffered a progressive loss of radioactivity (Table 13). Over the 8-day experimental period, muscle ribosomes in both prednisolone-treated and the control group of animals lost approximately 62% of their respective total radioactivities (Table 13). Except on Day 4 (two days after the commencement of prednisolone injections) when the calculated radioactivity was significantly lower than in the control rats, there was no significant difference in the calculated radioactivity present in control and treated rats. One interpretation of these results is that there was a more rapid degradation of this RNA in the prednisolone-treated rats during the first 2 days of prednisolone administration but that thereafter the amount of RNA degraded was similar in both groups of animals.

It is acknowledged that ribosomes may bind to messenger RNA and be isolated as polysomes to which, depending on the translational state of the cell, various transfer RNAs may also be attached. RNA turnover measured from these ribosome preparations may, therefore, be reflective of turnover of these various RNA species rather than of just ribosomal RNA. A truer measure of

ribosomal RNA turnover may be obtained by isolating this RNA from the bulk of cytoplasmic RNA. Work aimed at achieving this objective will be reported in subsequent sections.

ISOLATION OF RNA FROM RAT GASTROCNEMIUS MUSCLE USING PHENOL EXTRACTION METHODS

In the preliminary experiments reported on page 118 muscle RNA was measured in the alkali digests of the tissue as described in 'Methods'. This RNA is composed of various RNA species whose turnover rates may be different. Furthermore, the tritium label from the orotic acid used in these experiments can be incorporated into other cellular constituents (e.g. glycogen). These constituents remain in the alkali digest whose radioactivity is measured and thus complicate an interpretation of results from the experiments. Such considerations prompted attempts to extract RNA for the tissue in a purer form and in good yield. Various modifications (page 65) of Kirby's 1956 method were tried and the purity and percentage recovery of RNA by each method were determined.

When RNA was extracted from the muscle by the Hot Phenol method, 61% of the total RNA present in the tissue was recovered (Table 14). This recovery was considerably less than that (75 - 90%) reported by Manchester (1967). Several repetitions of the experiment did not improve the result. It may be significant, however, that diagraphm muscle with which Manchester worked is more easily homogenised and processed than gastrocnemius muscle. The A_{260}/A_{280} ratio of the RNA isolated by this method was 1.55, a figure which is lower than the 1.7 reported by Manchester (1967).

Phenol extraction was then carried out at $0^{o}C$ as recommended by Wool and Munro (1963). The RNA recovered by this method was only 58% of the quantity estimated to be present in the tissue.

TABLE 14. YIELD AND PURITY OF RNA ISOLATED FROM MUSCLE BY VARIOUS PHENOL EXTRACTION TECHNIQUES

	mg RNA/g Muscle	% Recovery	A_{260}/A_{280}
RNA isolated by Hot Phenol Extraction	1.2 ±0.13	61.3 ±2.3	1.55
Total RNA Content	1.9 ±0.12	-	-
RNA isolated by Cold Phenol Extraction	1.1 ±0.06	57.9 ±3.8	1.80
Total RNA Content	1.9 ±0.11	-	-
RNA isolated by CTA extraction	0.80 ±0.06	43.6 ±6.1	1.95
Total RNA Content	1.81 ±0.07	-	-
RNA isolated by Phenol/Chloroform extraction	1.5 ±0.03	78.9 ±3.6	1.98
Total RNA Content	1.90 ±0.05	-	-

The RNA extracted from the right gastrocnemius muscle was dissolved in 3.0ml sodium acetate buffer, pH 5.1 and assayed by the Orcinal method.

'Total RNA content' refers to RNA from the left gastrocnemius muscle of the same animal solubilised by alkali digestion as described on page 55 and assayed by the Orcinal method.

$$\% \text{ Recovery is } \frac{\text{RNA concentration in extract}}{\text{Total RNA content}} \times 100$$

The results are mean values from 3 rats ± standard error of mean.

The A_{260}/A_{280} ratio of the material was 1.80, an improvement on the figure obtained using the Hot Phenol method.

The attempt to extract RNA with CTA involved the insolubilisation of an RNA-CTA complex as described on page 67. Only 44% of the total RNA present in the tissue was recovered using this method. However, the purity of the isolated RNA was an improvement on the two previous methods since the product gave an A_{260}/A_{280} ratio of 1.95.

In an attempt to improve the yield of RNA, KCl was included in the tissue homogenisation medium. This salt solubilises muscle proteins, particularly myosin, which are insoluble in cytoplasmic extracts when the ionic strength of the medium is lowered much below 0.3 (Heywood et al, 1967) and with which ribosomes will co-precipitate with a resultant dimunition of RNA yield. The results of these investigations are presented on Table 15. In the presence of 0.05M KCl the recovery of RNA using the Cold Phenol method was increased by 16% and further increases in KCl concentration did not improve this recovery significantly. With the CTA method, the highest yield of RNA was obtained in the absence of KCl. It was evident from these investigations that the effect of KCl on RNA yield depends on the RNA extraction method employed.

The fourth method used to isolate cytoplasmic RNA from gastrocnemius muscle required the use of phenol/chloroform mixtures as recommended by Perry et al, (1972). Details of this RNA extraction technique appear on page 68. As the results on Table 14 show, this method yields the most RNA (79% of the total

TABLE 15. INFLUENCE OF KCl ON THE YIELD OF MUSCLE RNA OBTAINED BY TWO EXTRACTION PROCEDURES

	No KCl		0.05M KCl		0.3M KCl		0.6M KCl	
	A	B	A	B	A	B	A	B
Total RNA Content (mg/g Muscle)	1.8 ±0.07	1.8 ±0.03	1.9 ±0.11	1.8 ±0.04	1.8 ±0.04	1.8 ±0.02	2.0 ±0.08	1.8 ±0.02
RNA Content of Extract (mg/g Muscle)	0.66 ±0.11	0.75 ±0.09	1.0 ±0.08	0.50 ±0.02	1.1 ±0.05	0.43 ±0.06	0.99 ±0.06	0.07 ±0.01
% Recovery of RNA	35.6 ±4.2	40.9 ±2.8	52.8 ±5.3	26.6 ±6.2	57.9 ±5.0	22.8 ±7.5	49.0 ±5.5	3.9 ±1.1

RNA was extracted from the right gastrocnemius muscle of the animal by the cold phenol method (A) and by the CTA method (B). 'Total RNA content' refers to RNA in the left gastrocnemius muscle as estimated by the alkali digestion method described (page 55). All RNA measurements were performed by the Orcinol method (page 43) and the results are the mean values from 2 experiments ± S.E.M.

RNA present in the tissue). The isolated material contained RNA in a purer form than had been achieved with any of the other phenol extraction methods ($A_{260}/A_{280} = 1.98$).

EXAMINATION OF RNA ISOLATED BY VARIOUS PHENOL EXTRACTION PROCEDURES BY DENSITY GRADIENT CENTRIFUGATION

To determine the integrity and the extent of possible degradation of the RNA species isolated by the various phenol extraction procedures reported on page 132, the RNA extracts were subjected to centrifugation on a linear sucrose gradient as described on page 73. The results of these investigations are presented in Figs. 24A to 24F and the experimental conditions for each experiment appear in the legend below the result.

A 15 - 30% sucrose density gradient, recommended by McConkey (1967), was used in the initial experiments. This gradient, as the result on Fig. 24A shows did not fractionate the RNA species satisfactorily. In the light of the results obtained by Dr. C.M. Goodlad (personal communication) with 5 - 20% sucrose gradients, subsequent RNA fractionations were carried out using this sucrose gradient. More satisfactory results were obtained.

The sucrose gradient profile of RNA isolated by the hot phenol method presented on Fig. 24B. The pattern observed differed markedly from those of undegraded cytoplasmic RNA reported by Ralph and Bellamy (1964). There was some loss of material from the presumptive 28S ribosomal RNA peak, possibly an indication of degradation of this RNA species. There was also a steep rise in the ultraviolet absorption, at 260nm near the top of the gradient presumably due to RNA degradation products (Ralph and Bellamy, 1964), DNA breakdown products (Hayashi et al, 1975), traces of sodium dodecyl sulphate or a combination of these factors.

The RNA extracted by the cold phenol method also gave a

profile which suggested that degradation of RNA had occurred during the isolation process. Intense absorption at 260 nm was again observed towards the top of the gradient (Fig. 24C).

The sucrose gradient profile of the RNA extracted by the CTA method is presented on Fig. 24D. There was no evidence of significant degradation of RNA isolated by this method. Three RNA peaks were observed. Based on the calculations described on page 76, these peaks had sedimentation coefficients of 29, 19 and 3.8S respectively, corresponding to the two ribosomal RNA components and transfer RNA. In an attempt to obtain a better separation of the RNA species the duration of the centrifugation was extended from 5 to 7 h. This modification, as the results on Fig. 24E show, gave a more satisfactory distribution of the RNA species throughout the gradient. The relative amounts of these three RNA species, based on the areas under the peaks were in the ratios 3.4 : 1.6 : 1. This result is in good agreement with accepted relative proportions of these RNA types in animal tissues (Ralph and Bellamy, 1964; Sirlin 1972; Adams et al, 1976). However these results could not be reproduced consistently and the CTA method was abandoned.

The results presented on Fig. 24F were obtained with RNA extracted using the phenol/chloroform technique described on page 68. Here, there was little evidence of significant RNA degradation and the sedimentation profile was reproducible with several preparations using this RNA extraction technique. The proportion of the three RNA species were in the ratios 3.8 : 1.6 : 1. Thus, approximately 84% of the RNA isolated from gastrocnemius

muscle appeared to be ribosomal RNA. This figure is in keeping with a value of 80% reported by Manchester (1967) and Young (1970). Since the method employed for the isolation of RNA from muscle gave only a 79% recovery (Table 14) the data obtained in the present work only permit the suggestion that at least 66% of gastrocnemius muscle RNA is ribosomal in origin.

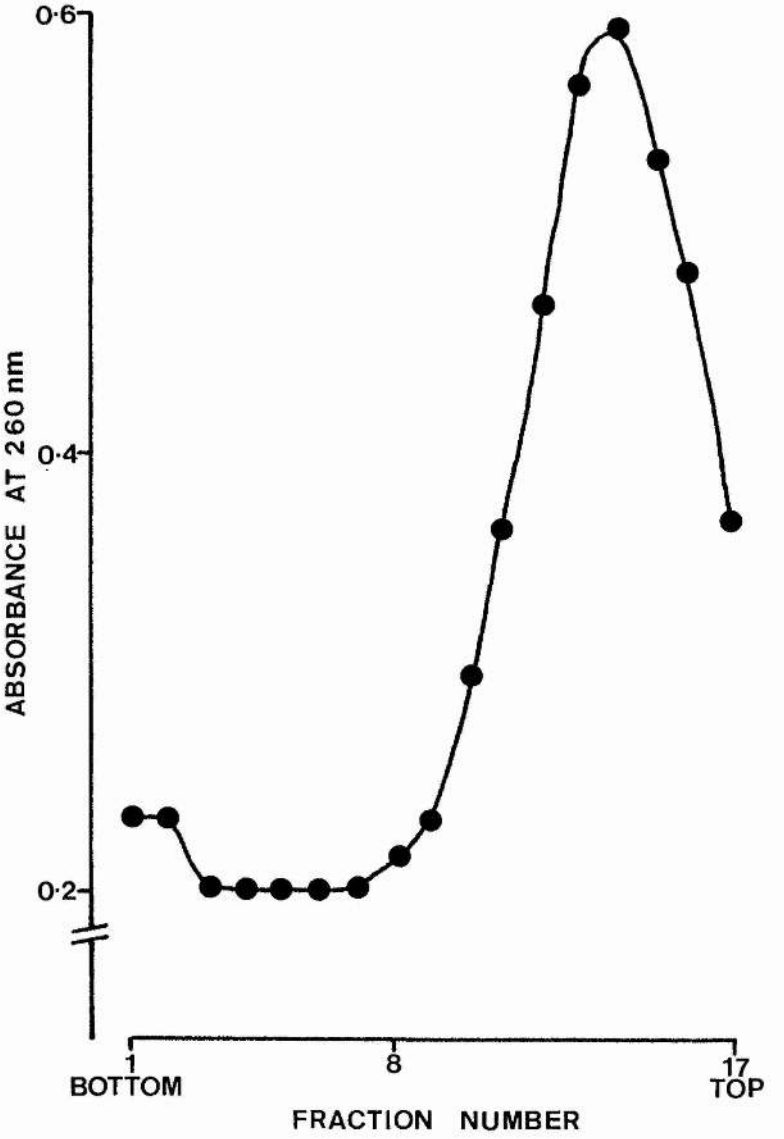


FIG. 24A Sucrose gradient profile of rat gastrocnemius muscle RNA isolated by the Hot Phenol Method: 1.0mg of RNA extract was dissolved in 2.0ml 0.05M sodium acetate buffer pH 5.1, containing 0.1M NaCl and layered over a 15 - 30% (w/w) sucrose gradient. Centrifugation was performed at 5°C for 17 h at 60,000g ave. using the 3 x 25ml swing-out rotor on the MSE superspeed 65 centrifuge.

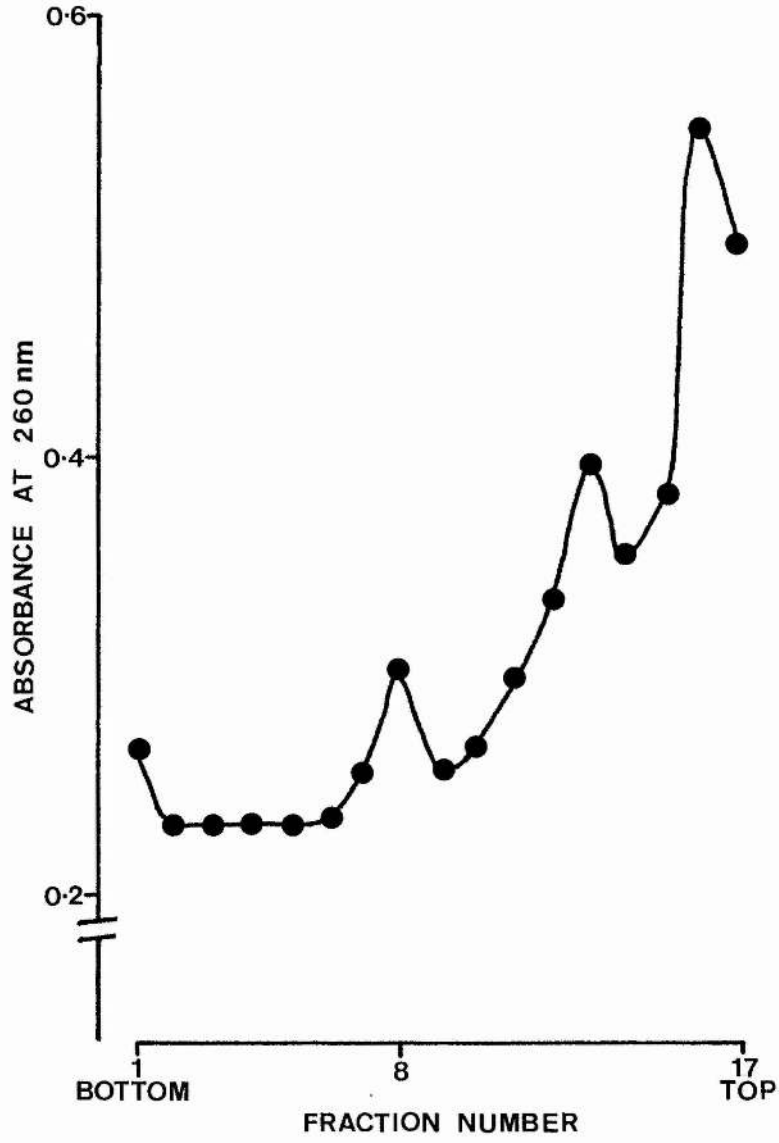


FIG. 24B Sucrose gradient profile of RNA isolated by the Hot Phenol Method:

Sample was layered over a 5 - 20% (w/w) sucrose gradient and centrifuged at 60,000g ave. for 7 h at 5°C. Other experimental details were as described for Fig. 24A.

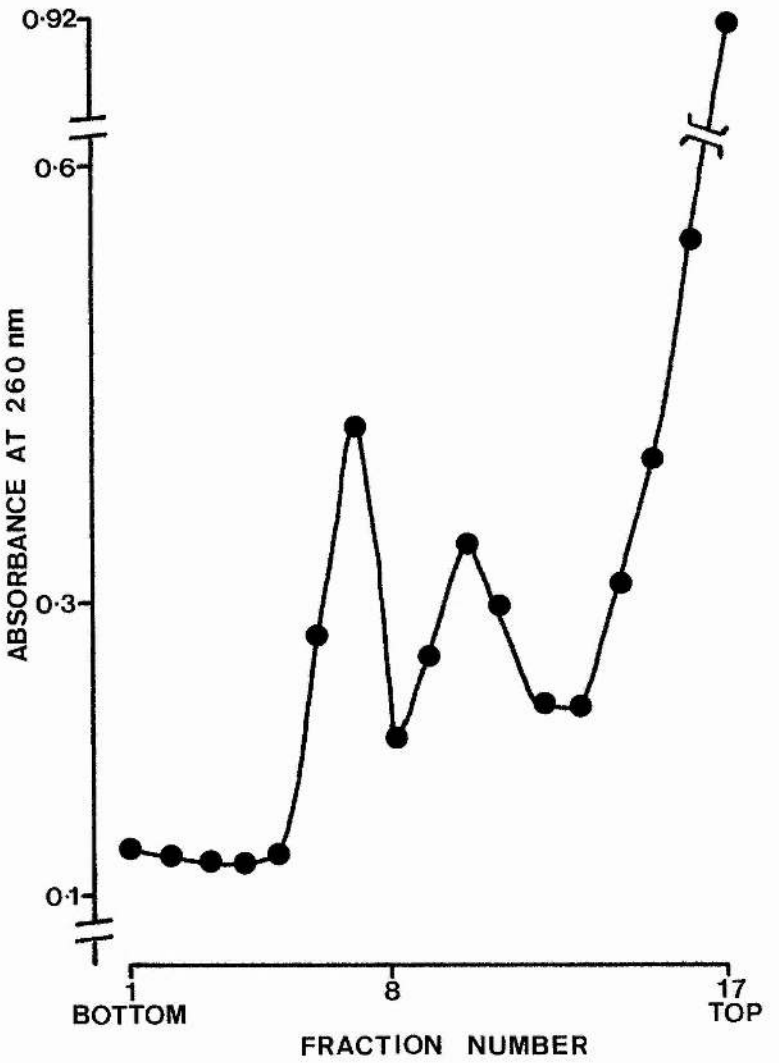


FIG. 24C Sucrose gradient profile of RNA isolated by the Cold Phenol Method.

Sample was layered over a 5 - 20% (w/w) sucrose gradient and centrifuged at 60,000g ave. for 7 h at 5°C. Other experimental details were as described for Fig. 24A.

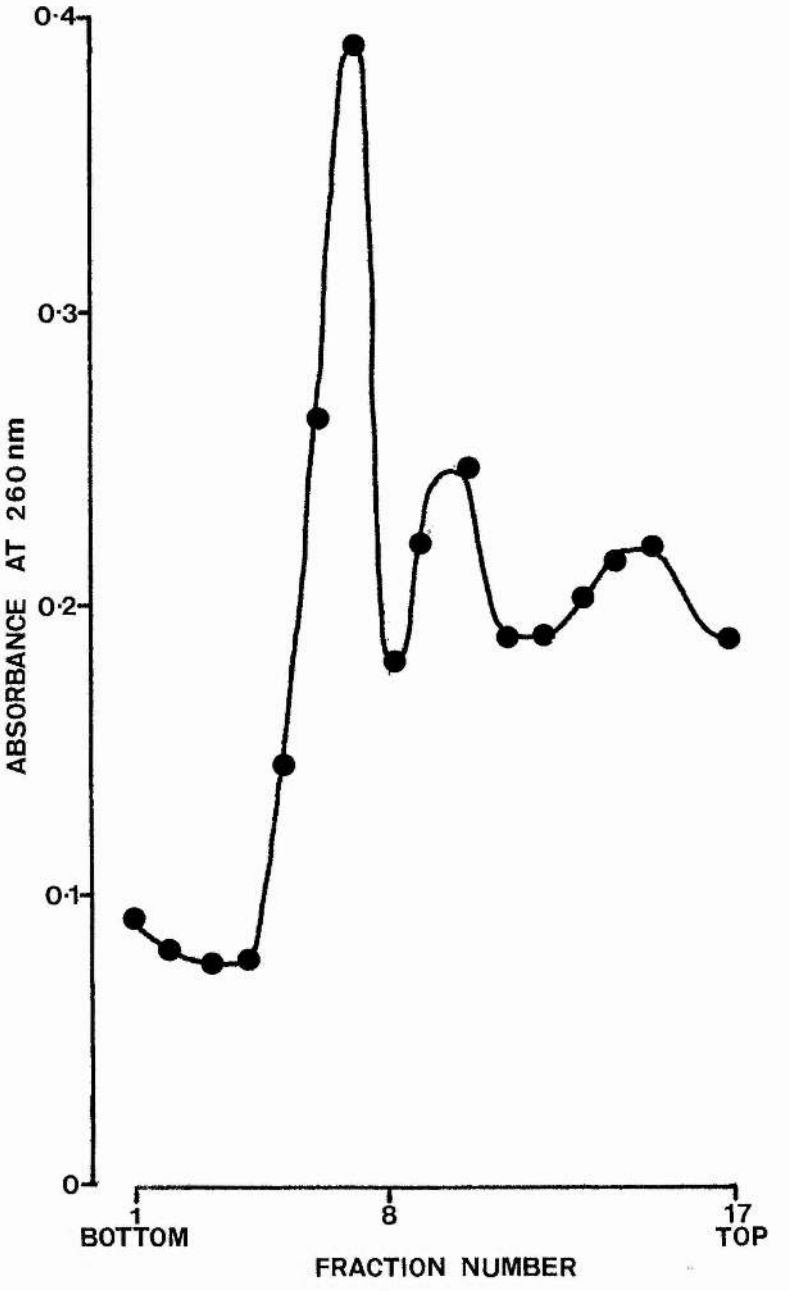


FIG. 24D Sucrose gradient profile of RNA isolated by the CTA Method.

Sample was layered on a 5 - 20% (w/w) sucrose gradient and centrifuged at 5°C for 5 h at 60,000g ave. Other experimental details were as described in Fig. 24A.

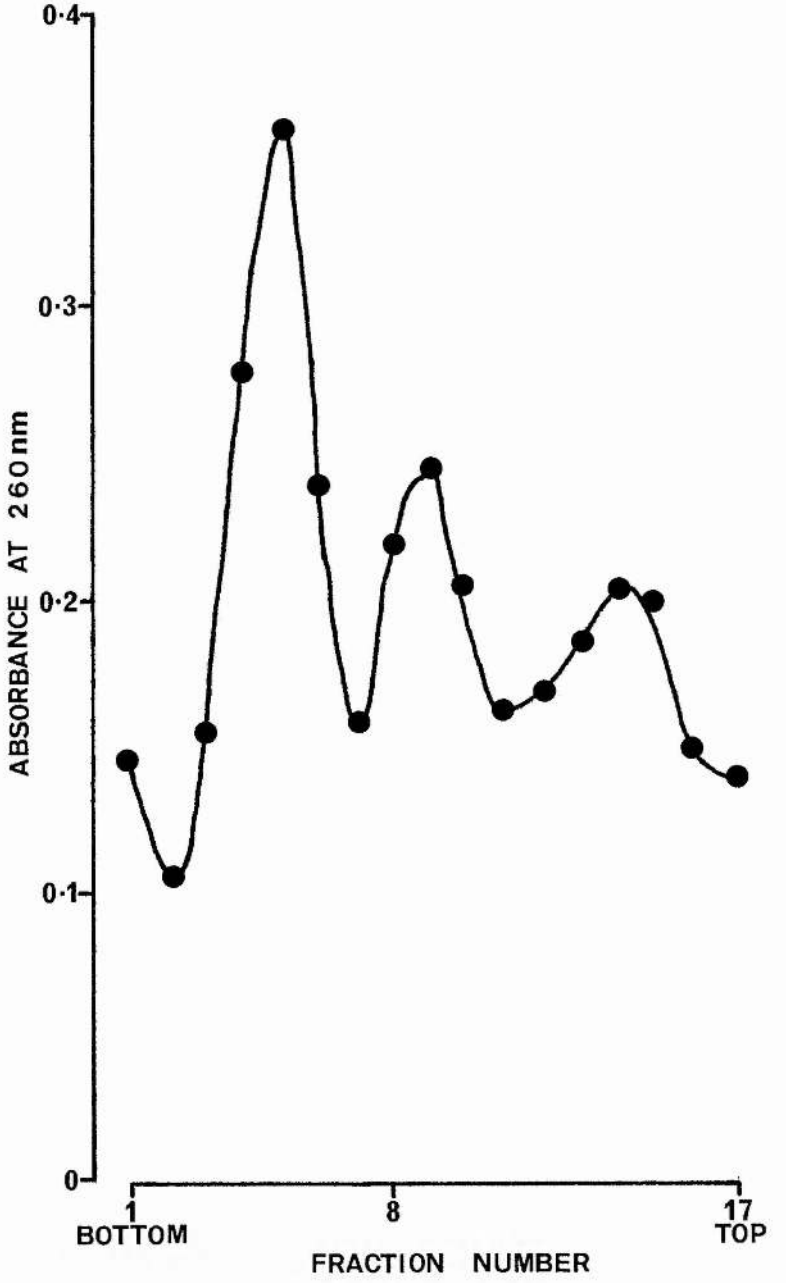


FIG. 24E Sucrose gradient profile of RNA isolated by the CTA Method.

Sample was layered on a 5 - 20% sucrose gradient and centrifuged at 5°C for 7 h at 60,000g ave. Other experimental details were as described on Fig. 24A.

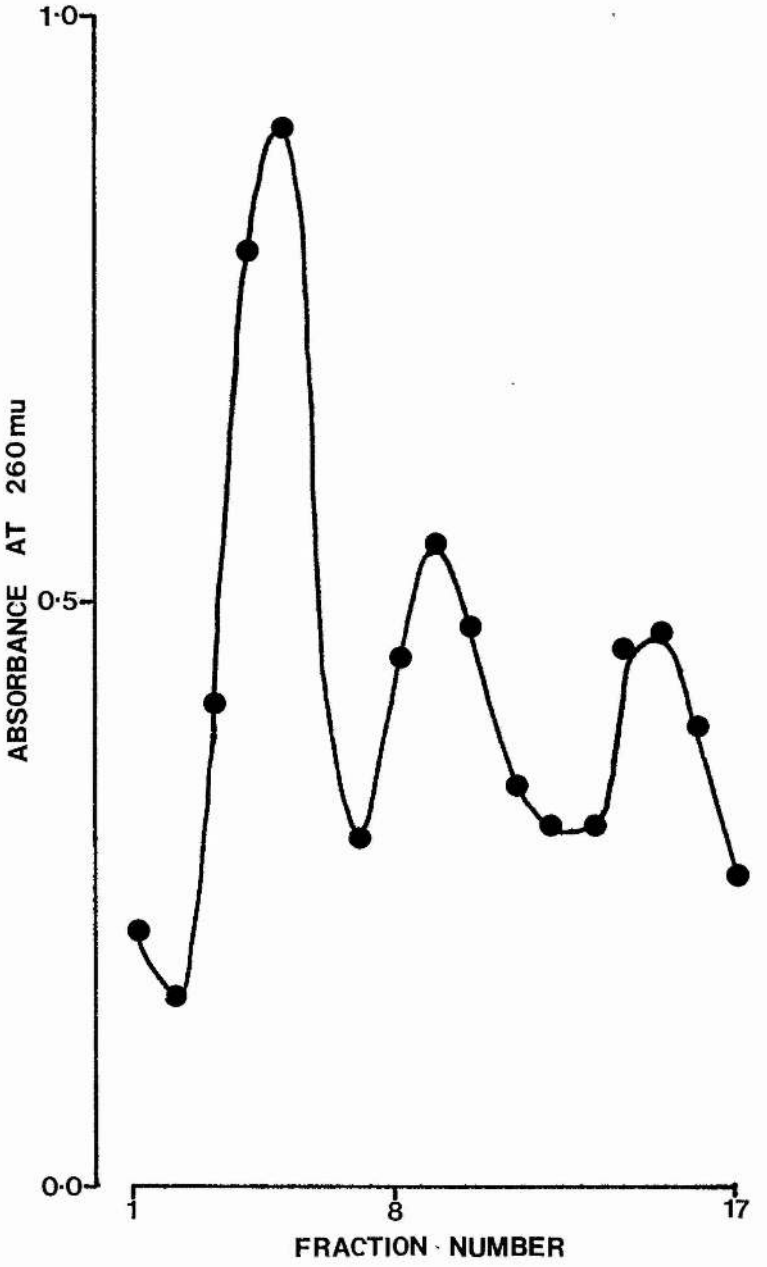


FIG. 24F Sucrose gradient profile of RNA isolated by the Phenol/Chloroform Method.

Sample was layered on a 5 - 20% sucrose gradient and centrifuged at 5°C for 7 h at 60,000g ave. Other experimental details were as described on Fig. 24A.

CONTRIBUTION BY POLYADENYLATED RNA TO MUSCLE CYTOPLASMIC RNA RADIOACTIVITY

Although analysis of the RNA extracted from muscle indicated that most of cytoplasmic RNA was ribosomal, the data on radioactive content of muscle RNA might be influenced to a considerable extent by small amounts of heavily labelled RNA species. Messenger RNA in particular might be expected to incorporate ^{3}H-Orotic acid rapidly (Brawerman 1974) and might also be present in ribosomal preparation. Most messenger RNA molecules in eukaryotic cells as well as some of the heterogeneous nuclear RNA have a polyadenylate 3′ terminus (Kates and Beeson, 1970; Sheldon et al, 1972). This species of RNA can be separated from the rest of the cellular RNA by adsorption on an oligo(dT)-cellulose column (Aviv and Leder, 1972). The present series of experiments was carried out with the aim of isolating polyadenylated RNA molecules and determining their radioactivity. It was also hoped to examine the radioactivity present in ribosomal RNA preparations freed from contamination with these molecules. The general approach was to (i) maximally extract cytoplasmic RNA using the phenol/chloroform procedure described on page 71; (ii) isolate the polyadenylated RNA from other RNA species using the oligo(dT)-cellulose column chromatographic technique described on page 71; (iii) estimate the specific radioactivity of the polyadenylated RNA and the effect of prednisolone treatment on this radioactivity; (iv) determine the influence of the polyadenylated RNA on the apparent radioactivity levels of other muscle RNA species. The results of these investigations are presented here. In these experiments administration of prednisolone

acetate was performed 2 days after the injection of ^{3}H-Orotic acid to ensure that maximal labelling of muscle RNA had occurred.

The effectiveness of three RNA isolation techniques, described on page 69, in extracting polyadenylated RNA along with other RNA species is evident from results presented on Table 16. The phenol/chloroform method (Procedure C) was the most efficient in extracting these RNAs from rat gastrocnemius muscle. RNA extracted by this technique was the purest, as determined by A_{260}/A_{280} ratios. It was evident too that although polyadenylated RNA constituted only about 3%(w/w) of the total RNA content of the extract, its specific radioactivity was more than three times the activity of other RNA species (Table 17). Approximately 10% of muscle RNA radioactivity could thus be attributed to this polyadenylated RNA fraction.

The effect of prednisolone treatment on the specific radioactivity of polyadenylated RNA is presented on Table 18, and shown graphically in Fig. 25. After 8 days of prednisolone treatment there was no statistically significant alteration in the amount of polyadenylated RNA present in the tissue. The specific radioactivity of this RNA was only slightly lower than control values (Table 18). The graphical representation of these results shows that the radioactivity associated with polyadenylated RNAs from both the prednisolone-treated animals and their controls did not decrease over the 10-day experimental period and in fact showed a slight rise. The pattern of this increase parallels the results obtained from studies of total RNA radioactivities in the gastrocnemius muscle (Fig. 22).

TABLE 16. EFFECTIVENESS OF VARIOUS PHENOL PROCEDURES IN THE EXTRACTION OF POLYADENYLATED RNA FROM RAT GASTROCNEMIUS MUSCLE

	Total RNA in tissue (mg/g tissue)	A_{260}/A_{280}	PolyA-RNA content (% w/w)
Procedure A	1.1	1.8	2.1
" B	0.8	1.6	1.7
" C	1.5	1.9	3.4

Procedures A, B and C are various modification of phenol extraction methods as described on page 69 . Isolation of polyA-RNA was performed as described on page 71. RNA was measured by the ultraviolet absorption technique (page 44).

TABLE 17. DISTRIBUTION OF RADIOACTIVITY IN POLY A-RNA AND OTHER RNA SPECIES FOLLOWING TREATMENT WITH ^{3}H-OROTIC ACID

	RNA content (mg/g tissue)	Specific activity (cpm/μg RNA)
Fraction retained by oligo (dT) column	0.04	0.74
Fraction not retained by column	1.38	0.23

The animal received a single intraperitoneal injection of 20 Ci ^{3}H-Orotic acid and was sacrificed 48 h later. RNA was extracted by Procedure C as described on page 71 and fractionated on an oligo(dT)-cellulose column using the technique described on page 71.

TABLE 18. EFFECT OF PREDNISOLONE TREATMENT ON OLIGO(dT)-CELLULOSE-BOUND RNA RADIOACTIVITY IN RAT GASTROCNEMIUS MUSCLE

Days after Orotic Acid Injection	PolyA-RNA content (mg/g tissue)		Specific Activity of PolyA-RNA (cpm/μg RNA)	
	Prednisolone -treated	Control	Prednisolone -treated	Control
2	-	0.05	-	0.93
4	0.05	0.05	0.81	1.08
6	0.05	0.05	0.97	1.14
8	0.05	0.05	0.88	1.23
10	0.05	0.05	1.12	1.24

Each animal received a single injection of ^{3}H-Orotic Acid (20μCi) intraperitoneally. Commencing two days after the Orotic Acid injection animals given prednisolone acetate received 0.06ml of a 25mg/ml suspension of the drug in saline whilst the 'controls' each received the same volume of saline, all by subcutaneous injections. PolyA-RNA was isolated and measured as described on page 71 using Procedure C. Each result is the mean from 2 experiments.

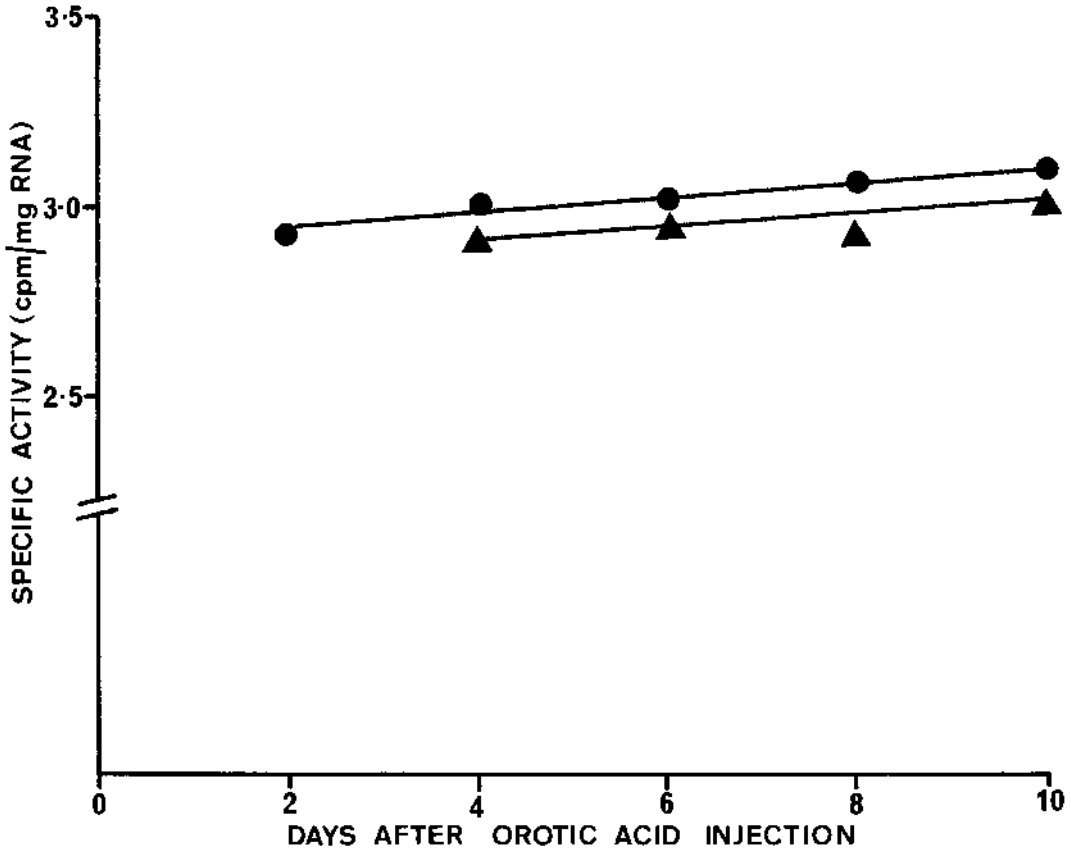


FIG. 25 Radioactivity in Polyadenylated RNA isolated from gastrocnemius muscle of rats given a single dose of ^{3}H-Orotic Acid.
Experimental details were as described on Table 18. Triangles represent results from prednisolone-treated animals and circles represent controls. Each point is the mean from two experiments. The slope of each line was calculated by the method of least squares.

The contribution of the radioactivity in polyadenylated RNA to the RNA fractions separated by sucrose density gradient centrifugation is evident from a comparison of the profiles on Figs. 26A and 26B. The removal of polyadenylated RNA did not significantly alter the relative ratios of the amounts of RNA in the 28S and 18S peaks, as determined by absorbance measurements at 260nm. There was however a pronounced change in the radioactivity profile of the sample with a general drop of radioactivity levels in all three RNA peaks and significant alterations in the relative ratios of the specific radioactivities in these peaks (Table 19). The 28S RNA fraction lost 18% of its specific radioactivity, the 18S fraction lost 6% and the 4S RNA lost 45% of its specific radioactivity when polyadenylated RNA was removed. These findings suggest that in the rat gastrocnemius muscle polyadenylated RNA may have a heterogenous size distribution, appearing in all three RNA peaks and making up a large portion of the 4S fraction when it may be present as breakdown products of heavier polyadenylated RNA molecules. Polyadenylated RNA in this tissue thus made a significant contribution to specific radioactivities in RNA fractions that are considered to represent mainly ribosomal and transfer RNAs. Therefore, any investigations that were of ribosomal RNA exclusively required the removal of this contaminating RNA fraction as a first step.

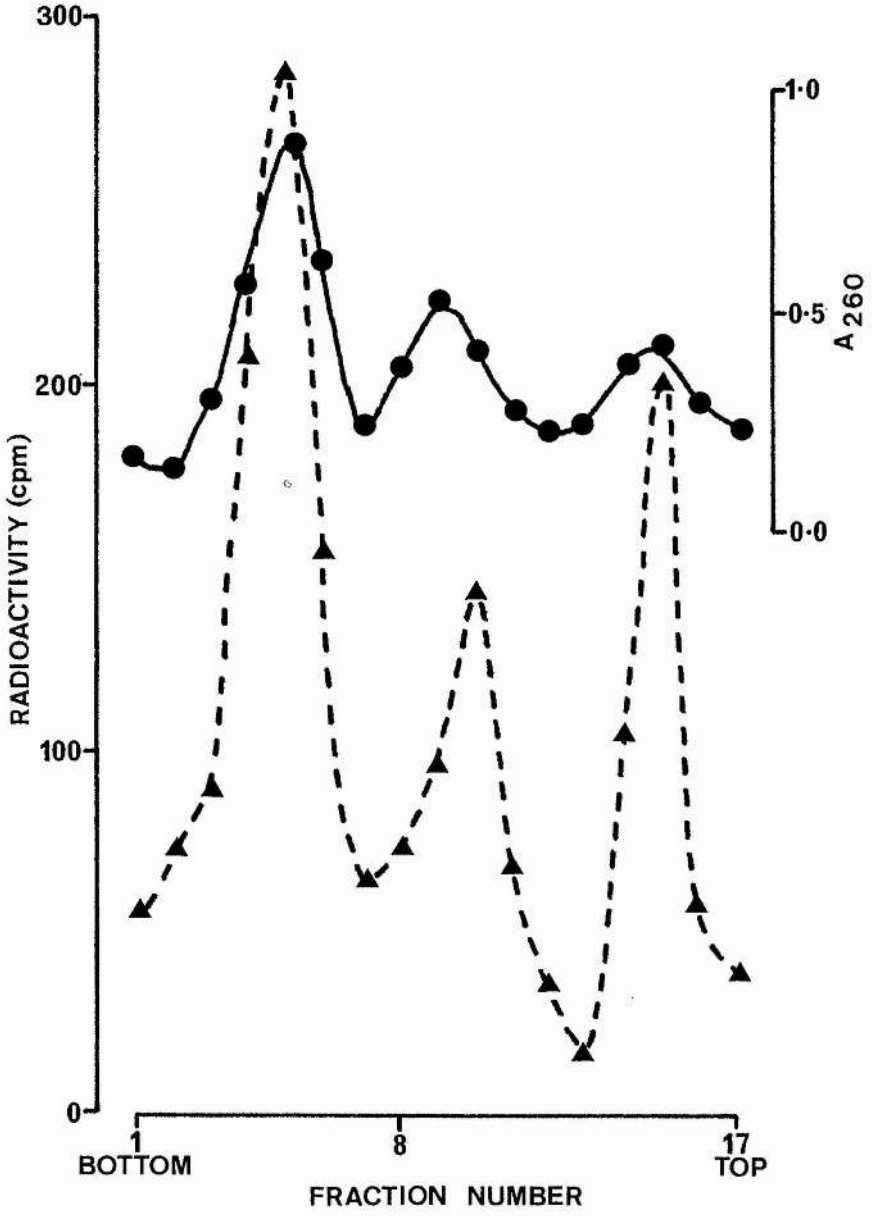


FIG. 26A Level of radioactivity of cytoplasmic RNA from gastrocnemius muscle of normal rats injected with 20μCi of ^{3}H-Orotic Acid 48 h before sacrifice. RNA was isolated from the tissue by Procedure C on page and centrifuged on a 5 - 20% (w/w) sucrose density gradient at 5°C for 7 h at 60,000g ave. Radioactivity (-▲-▲-) was measured as described on page 98 and RNA peaks were identified by U.V measurements at 260nm (-●-●-)

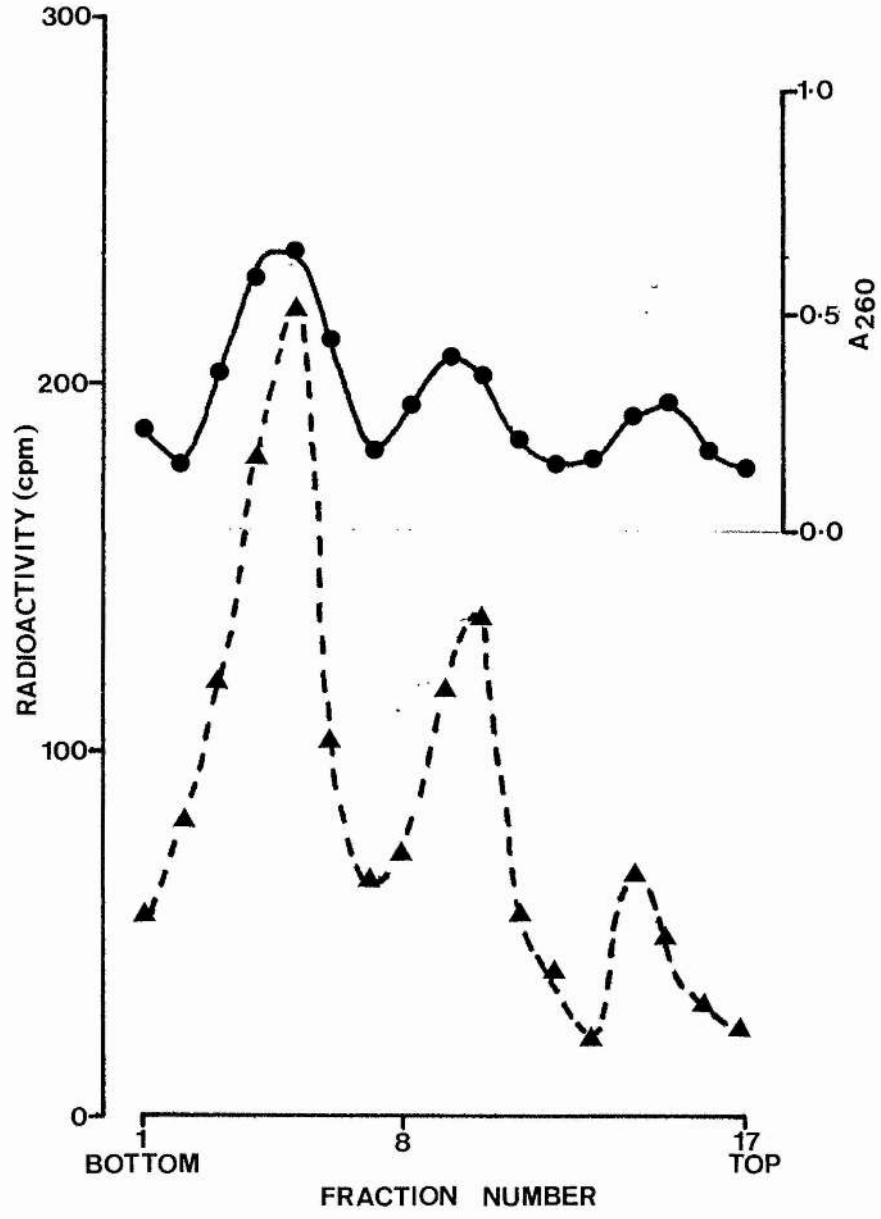


FIG. 26B Level of radioactivity in cytoplasmic RNA preparations from which polyadenylated RNA had been removed.
Experimental details are as for Fig. 26A except that the RNA sample was passed through an oligo(dT)-cellulose column, as described on page 71 before sucrose gradient centrifugation. (-▲-▲-) refers to radioactivity and (-●-●-) refers to A_{260}.

TABLE 19. EFFECT OF THE REMOVAL OF POLYADENYLATED RNA ON THE SPECIFIC ACTIVITY OF GASTROCNEMIUS MUSCLE RNA FRACTIONS

	SPECIFIC ACTIVITY (cpm/μgRNA)		
	28S RNA	18S RNA	4S RNA
PolyA-RNA present	1.04	0.98	1.41
PolyA-RNA removed	0.85	0.92	0.77

RNA was extracted using Procedure C (page 71), from muscle of rats treated with a single injection of 20μCi of ^{3}H-Orotic acid 48 before sacrifice. An aliquot of the RNA in 0.01M Tris-HCl buffer pH 6.0 was fractionated by sucrose gradient centrifugation (page 73). Another aliquot was freed of polyadenylated RNA by passage through a column of Oligo(dt)-cellulose (page 71) before sucrose gradient analysis. RNA was measured by ultraviolet absorption at 260nm and a representative fraction from each RNA peak was meásured for radioactivity as described on page 98.

EFFECT OF PREDNISOLONE ADMINISTRATION ON THE TURNOVER OF RIBOSOMAL RNA IN RAT MUSCLE

Several studies have provided evidence that glucocorticoids have a pronounced effect on liver ribosomal RNA (Ottolenghi and Barnabei, 1970; Mishra and Feltham, 1973; Schmid and Sekeris, 1975). These hormones tended to increase the amount of liver ribosomal RNA either by causing an increase in its rate of synthesis or a decrease in its rate of breakdown. Since, from the results quoted on page 13 and also from the finding of other workers (Manchester, 1967; Young, 1970), ribosomal RNA constitutes the major fraction of RNA found in the gastrocnemius muscle, it seemed pertinent to investigate the turnover of ribosomal RNA in the muscle following prednisolone administration.

Rats were given an injection of ^{3}H-Orotic acid and after 4 days half the animals were injected daily with prednisolone acetate and the remainder, the control animals, were given daily injections of 0.8%(w/v) saline. Intact and undegraded cytoplasmic RNA was extracted from the gastrocnemius muscle of control and treated rats using the phenol/chloroform procedure described on page 71 . The extracted RNA was dissolved in 0.01M sodium acetate buffer pH 6.0 and passed through a column of oligo(dT)-cellulose, as described on page 71 , to remove polyadenylated species of RNA. The RNA recovered in the effluent from this column was subjected to sucrose density gradient centrifugation to fractionate the ribosomal RNA from lower molecular weight RNA. Finally, the fractions containing 28S and 18S RNA were pooled, precipitated with ethanol and their content of radioactivity measured as described on page 98.

The results of these investigations are presented in Table 20 and in Figs. 27 and 28. The specific activity of ribosomal RNA in muscles of control rats was found to decrease progressively from the 4th to the 12th day after ^{3}H-Orotic acid injection. On the 12th day the specific activity was 35% of that observed on the 4th day after the injection of label. There was a marked decrease also in the specific activity of muscle ribosomal RNA in rats which had received a single injection of prednisolone (i.e. rats killed on day 6). At this stage, the specific activity of ribosomal RNA in the control and treated animals were not significantly different. The decrease in specific activity of muscle ribosomal RNA from the 6th to the 12th day however was much less in the treated animals than in the controls.

Over the experimental period gastrocnemius muscles of prednisolone-treated rats exhibited a 51% loss of total RNA content while in control rats a small increase (4.6%) was found which was not statistically significant. Since over 80% of skeletal muscle RNA is ribosomal (Manchester, 1967; Young, 1970) alterations in total RNA in muscle can be taken to give an indication of the behaviour of ribosomal RNA. In the present work therefore, in order to obtain an estimate of the effect of prednisolone treatment on the turnover of ribosomal RNA, the value of the product of total RNA per muscle per 100g initial body weight X the specific activity of ribosomal RNA was taken as total muscle ribosomal RNA radioactivity and graphed against days of experiment. In the case of muscles from control animals the plot of log total muscle ribosomal RNA radioactivity decreased in a linear manner with

TABLE 20. LOSS OF RADIOACTIVITY FROM RIBOSOMAL RNA IN RAT GASTROCNEMIUS MUSCLE FOLLOWING PREDNISOLONE TREATMENT

Days after Orotic acid Injection	Specific Activity (cpm/μg RNA)		Total Activity (cpm/ muscle/100g initial body weight)	
	Control	Prednisolone -treated	Control	Prednisolone -treated
4	1.6 ±0.1	-	1811 ±66	-
6	1.2 ±0.1	1.0 ±0.1	1234 ±72	951 ±118
8	0.9 ±0.1	1.0 ±0.1	1029 ±52	750 ±83
10	0.7 ±0.1	1.0 ±0.1	797 ±105	659 ±133
12	0.6 ±0.1	0.9 ±0.1	633 ±64	499 ±43

Each animal received a single dose (20μCi) of ^{3}H-Orotic acid intraperitoneally. Commencing 4 days afterwards each prednisolone-treated animal received 0.06ml prednisolone acetate (25mg/ml in saline) while controls received the same volume of 0.85%(w/v) saline. RNA was isolated as described on page 71 and measured for radioactivity as described on page 98 . Each result is the mean from 4 experiments ± standard deviation.

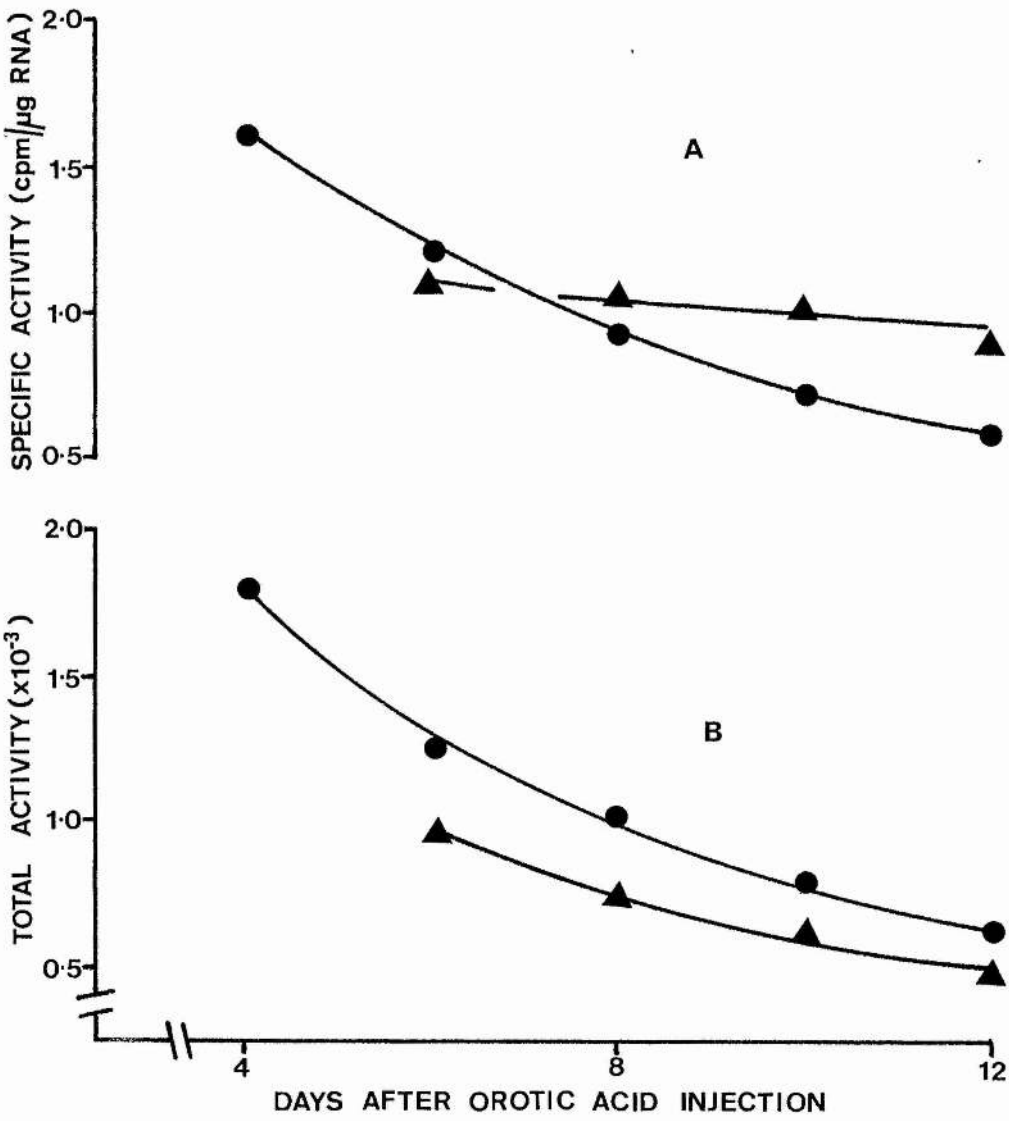


FIG. 27 Loss of radioactivity from rat gastrocnemius muscle ribosomal RNA following prednisolone treatment. Experimental details are as described for Table 20. Triangles indicate results from prednisolone-treated animals and circles indicate controls. Total activity was the cpm/muscle/100g initial body weight.

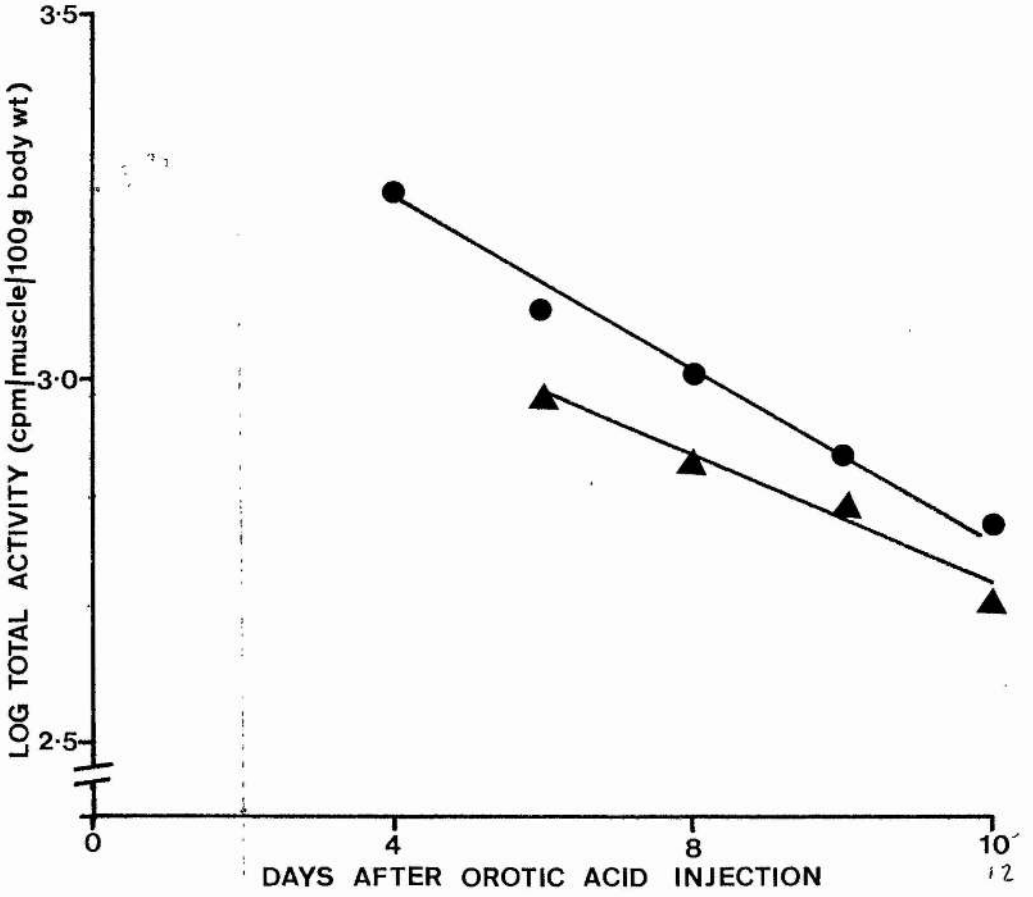


FIG. 28 Loss of radioactivity from ribosomal RNA in rat gastrocnemius muscle following prednisolone-treatment:
Experimental details are as described for Table 20. Triangles indicate results from prednisolone-treated animals and circles denote controls. Each slope was plotted by method of least squares.

time (Fig. 28). Assuming that first order kinetics applied, the rate constant for the biological degradation and the biological half-life for muscle ribosomal RNA in control rats were calculated to be 0.1439 and 4.8 days respectively. Since there was no significant alteration in the muscles of the control rats, it can be assumed that the rate of synthesis of muscle ribosomal RNA in these animals must also be of the order of their degradation rate. This was calculated to be 0.16mg RNA per day per muscle per 100g initial body weight. When the results obtained from rats given prednisolone were treated similarly, a somewhat different picture emerged. Rats killed on day 6, i.e. after they had received one prednisolone injection, retained significantly less radioactivity in muscle ribosomal RNA than did their controls (Table 20; Fig. 27B). When the logarithm of total muscle ribosomal RNA was plotted against time, it became apparent that from day 6, there was a less marked depletion of radioactivity from ribosomal RNA of prednisolone-treated animals than occurred in control animals. During the period from day 6 to day 12 the loss of radioactivity from ribosomal RNA in the prednisolone-treated rats followed first order kinetics. The rate constant for degradation of ribosomal RNA and the biological half-life of ribosomal RNA in these animals were calculated to be 0.0978 and 7.08 days respectively. The loss of RNA from the muscles of the treated animals during the period was equivalent to 0.36mg per muscle per 100g initial body weight. The amount of ribosomal RNA synthesised during this period can be calculated from the relationship NET LOSS OF RNA = AMOUNT DEGRADED - AMOUNT SYNTHESISED. Using this relationship, the

amount of ribosomal RNA synthesised per day during this period was found to be 0.03mg per muscle per 100g initial body weight. The amount of ribosomal RNA synthesised in the control rats per day over the same period was 0.16mg per muscle per 100g initial body weight. There would thus appear to be a marked dimunition in ribosomal RNA synthesis as a result of prednisolone treatment.

In the period from day 4 to day 6, when the treated animals had received two daily injections of prednisolone, there was a net loss of 38% of the specific radioactivity in the muscle (Table 20). During the same period, the control rats showed a specific radioactivity loss of 25%. This picture would suggest an increased synthesis of new ribosomal RNA in the treated animals during this period. However, their level of ribosomal RNA had been shown to drop during this period (Table 21). Thus, it would seem that the initial response of muscle ribosomal RNA metabolism to prednisolone is a stimulation of synthesis accompanied by an even greater increase in the rate of degradation. This is followed by a period when ribosomal RNA synthesis falls to a very low level compared to that in untreated animals.

TABLE 21. EFFECT OF PREDNISOLONE TREATMENT ON RIBOSOMAL RNA LEVELS IN THE RAT GASTROCNEMIUS MUSCLE

Days after Orotic acid Injection	Ribosomal RNA content (mg/muscle/100g initial body weight)	
	Control	Prednisolone-treated
4	0.73 ± 0.04	-
6	0.72 ± 0.06	0.64 ± 0.03
8	0.71 ± 0.06	0.56 ± 0.07
10	0.71 ± 0.07	0.49 ± 0.05
12	0.72 ± 0.05	0.34 ± 0.07

Experimental details are as described for Table 20.

EFFECT OF PREDNISOLONE TREATMENT ON THE ACID-SOLUBLE NUCLEOTIDE POOL OF RAT LIVER AND MUSCLE

In interpreting the results of the experiments on RNA turnover reported on page 118 , consideration must be given to possible reutilisation of RNA breakdown products in the nucleic acid precursor pool. If there is significant reutilisation, the half-lives calculated for the nucleic acid would have been overestimated. Furthermore the effects noted with prednisolone on RNA turnover may be due to alterations in the free nucleotide pool in the tissue rather than a direct effect on RNA synthesis or breakdown. These considerations prompted a study of the radioactivity in the RNA precursor pool in rat liver and gastrocnemius muscle during the experimental period and of the effect of prednisolone treatment on these pools. Total acid-soluble radioactivity and, more significantly, radioactivity present in acid-soluble pyrimidine nucleotides were measured.

Table 22 and Figs. 29A and 29B show the results of assays carried out on the rat gastrocnemius muscle. Significant levels of radioactivity were still present on the second day after ^{3}H-Orotic acid was injected. Thereafter, radioactivity levels of the total acid-soluble pool from the control animals fell by almost 50% over the next 4 days. A further loss of radioactivity occurred but at a much slower rate over the last 4 days of the experiment and, on the tenth day, the radioactivity remaining in the pool was still 40% of that present on the second day. The possibility therefore exists that there was some reutilisation of RNA breakdown products in new RNA synthesis. The pattern of loss of

TABLE 22. EFFECT OF PREDNISOLONE ADMINISTRATION ON THE RADIOACTIVITY OF THE TOTAL ACID SOLUBLE POOL AND THE ACID-SOLUBLE NUCLEOTIDE POOL OF RAT GASTROCNEMIUS MUSCLE

Days after Orotic Acid Injection	Total acid-soluble pool (cpm/g muscle x 10^{-3})		Acid-soluble nucleotide pool (cpm/g muscle x 10^{-3})	
	Prednisolone -treated	Control	Prednisolone -treated	Control
2	-	2.9	-	2.2
4	2.0	1.9	1.6	1.4
6	1.7	1.5	1.3	1.2
8	1.5	1.2	1.2	1.0
10	1.6	1.2	1.1	0.9

Each animal received a single dose of ^{3}H-Orotic acid (20μCi) intraperitoneally. Commencing 2 days after the orotic acid injection each prednisolone-treated animal received daily subcutaneous injections of 0.06ml of prednisolone acetate (25mg/ml in 0.85%(w/v) saline), while the control received the same volume of 0.85%(w/v) saline. Total acid soluble pool and acid-soluble nucleotide pool were prepared as described on page 82 and radioactivity was measured as described on page 98. Each result is the mean value from two experiments.

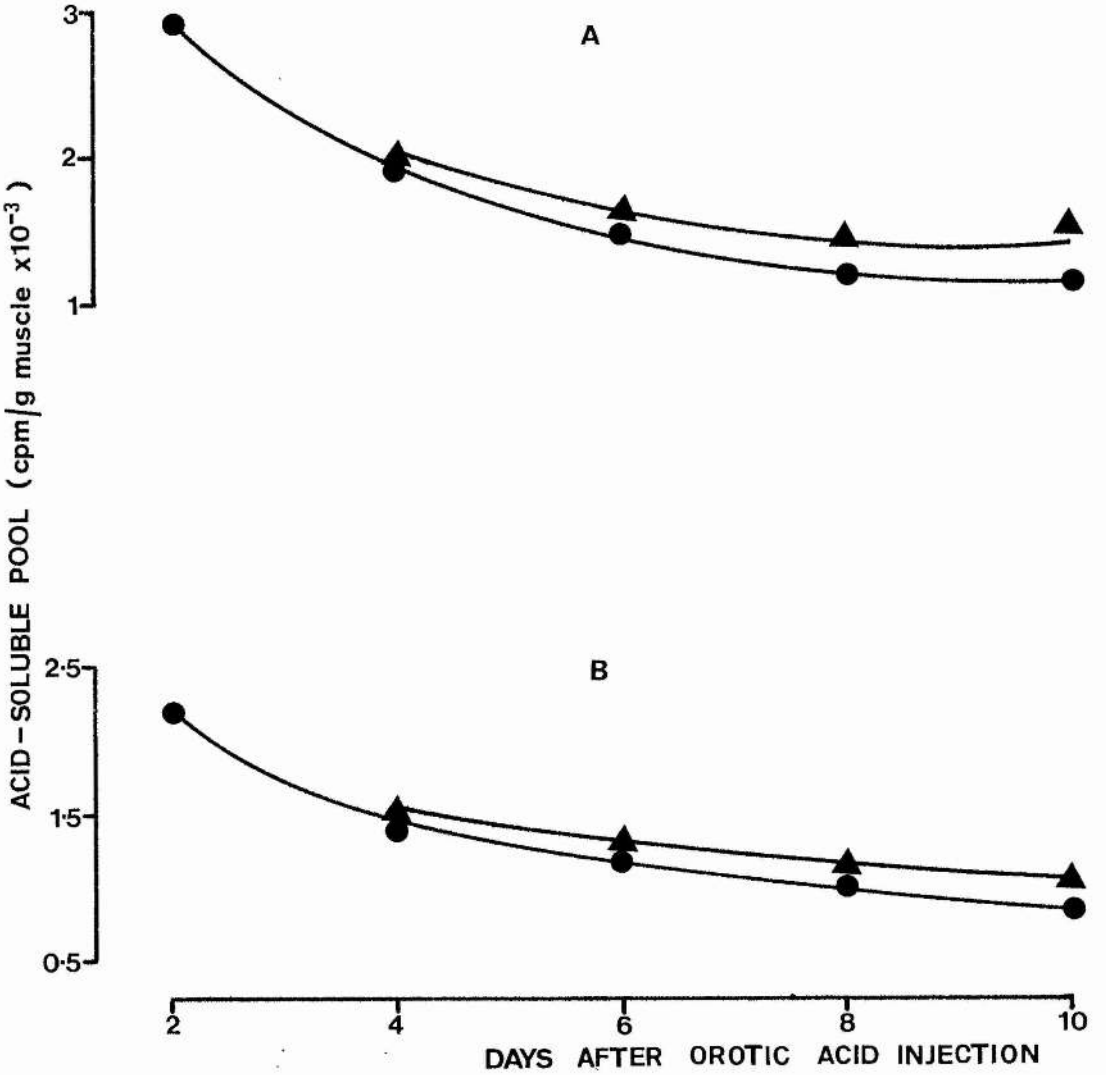


FIG. 29 Effect of prednisolone treatment on the radioactivity of the acid-soluble pool in rat gastrocnemius muscle.
Experimental details were as described on Table 22.
(A) shows total acid-soluble pool results while (B) shows results with the acid-soluble nucleotide pool.
Triangles represent results obtained with prednisolone-treated animals while circles indicate control values.

radioactivity from the total acid-soluble pool from the muscle of the prednisolone-treated animals closely paralleled the results obtained with the control group, a sharp drop occurring between the second and the sixth day after ^{3}H-Orotic acid administration and a slower fall continuing until the end of the experiment.

The levels of radioactivity in the rat muscle nucleotide pool amounted to more than 70% of the total acid-soluble pool (Table 22), an indication that most of the labelled nucleosides were in the phosphorylated form. Radioactivity levels in the nucleotide pool, as in the total acid-soluble pool dropped more sharply between the 2nd and 6th day and showed a less marked decline during the last four days of the experiment. A 46% drop in radioactivity occurred between days 2 and 6 compared with a fall of 25% over the last four days. Again, prednisolone administration caused no significant alteration of this pattern.

The results of similar experiments with rat liver are presented on Table 23 and, graphically, in Figs. 30A and 30B. In the control group of animals levels of radioactivity in the total acid soluble pool fell by over 65% between the 2nd and 6th day and by only a further 35% in the last four days. Over the entire experimental period the total acid soluble pool of the prednisolone-treated animals lost radioactivity at approximately the same rate as was observed the control group. This pattern was essentially the same when the liver nucleotide pools were examined and no significant differences could be found in this pattern of loss of radioactivity between control and prednisolone-treated animals.

TABLE 23 EFFECT OF PREDNISOLONE TREATMENT ON THE TOTAL ACID-SOLUBLE POOL AND THE ACID-SOLUBLE POOL IN RAT LIVER

Days after Orotic Acid Injection	Total Acid-Soluble pool (cpm/g liver x 10^{-3})		Acid-Soluble Nucleotide pool (cpm/liver x 10^{-3})	
	Prednisolone -treated	Control	Prednisolone -treated	Control
2	-	45.1	-	16.9
4	14.9	19.1	8.7	10.1
6	13.4	15.4	9.4	10.5
8	12.4	10.9	8.3	8.8
10	12.4	9.9	8.1	8.0

Experimental details were as described on Table 22.

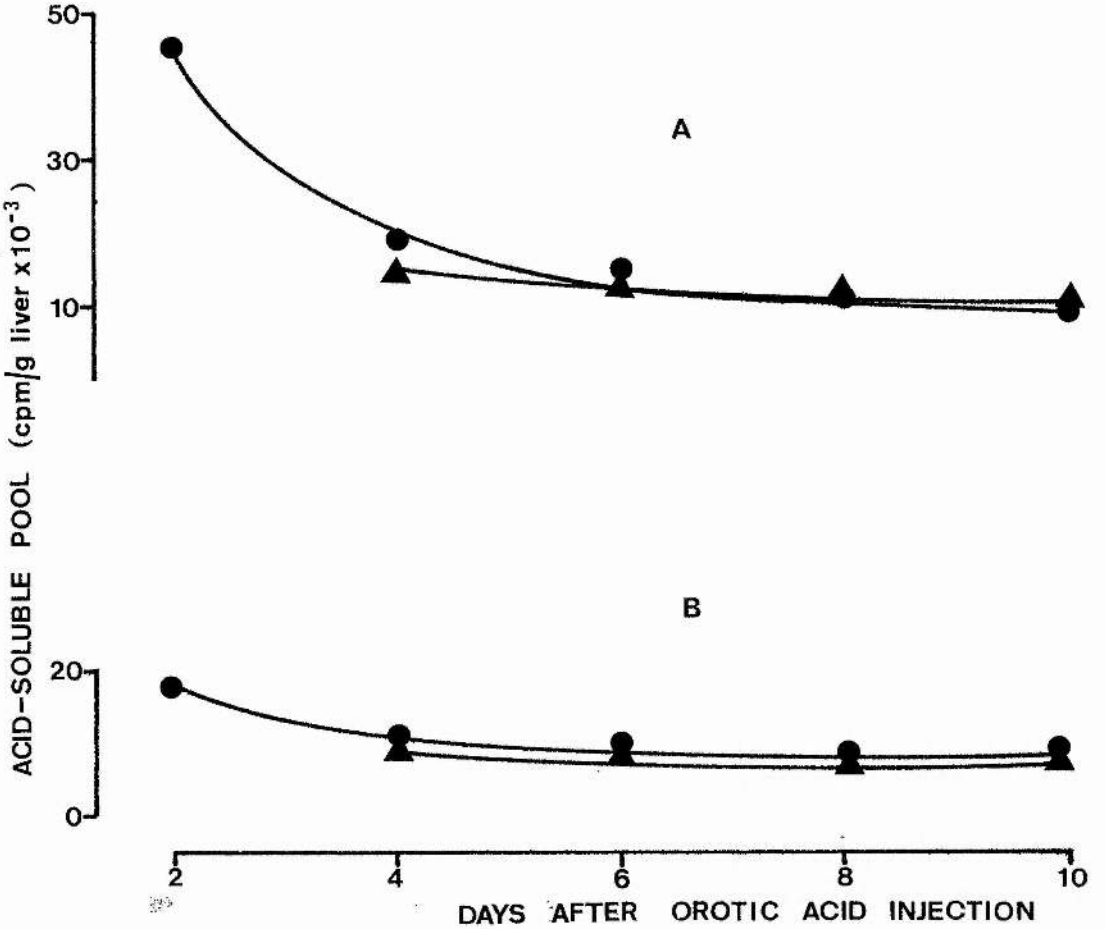


FIG. 30 Effect of prednisolone treatment on the radioactivity of the acid-soluble pool in rat liver.
Experimental details were as described on Table 22.
(A) refers to total acid-soluble pool while (B) shows results with the acid soluble nucleotide pool.
Triangles indicate results obtained with prednisolone-treated animals while circles represent control values.

In these experiments, the peak level of radioactivity in the tissue nucleotide pool due to the injection of ^{3}H-Orotic acid was not estimated. Thus, although the results presented above suggest the possibility of some reutilisation of RNA breakdown products, it is difficult to assess the significance of the absolute levels of radioactivity observed on individual days with respect to the labelling of any RNA synthesised during that period. Hirsch and Hiatt (1966A) studied the turnover of liver ribosomes, after administration radioactivity-labelled orotic acid under conditions similar to those carried out here and considered the possible effect of reutilisation of label. They also studied ribosome turnover in the presence of [6-^{14}C]-arginine where the protein components of the ribosome would be labelled and where reutilisation of any label would be minimal since any ^{14}C-arginine released by protein broken would be eliminated as urea. Since the half-lives of ribosomes obtained with ^{3}H-Orotic acid or ^{14}C-arginine were almost identical these workers concluded that reutilisation of the nuclei acid label was not occurring to any great extent. It would appear that separate synthetic and catabolic pools for RNA exist or alternatively that unlabelled pools may have effectively diluted labelled RNA precursors.

It was evident, from the results in both the muscle and liver that prednisolone treatment did not cause significant changes in RNA precursor pool labelling in either tissue. The alterations observed in RNA turnover following prednisolone administration might therefore be attributable to more direct effects on RNA synthesis and breakdown.

EFFECT OF PREDNISOLONE ADMINISTRATION ON RNA POLYMERASE ACTIVITY IN NUCLEI ISOLATED FROM RAT LIVER AND MUSCLE

DNA-dependant RNA polymerase (RNA nucleotidyl transferase E.C. 2.7.7.6) is the key enzyme implicated in the first step of genetic information transfer from DNA to RNA (Biswas et al, 1975). Although comparatively little is known about the regulatory role of this enzyme in eukaryotic RNA synthesis, there is evidence that RNA synthetic capacity of the rat liver nuclei (Pogo et al, 1966; Yu and Feigelson, 1971) and rat muscle nuclei (Sobel and Kaufman, 1970; Baieve and Florini, 1970) can be related to alterations in the RNA polymerase reaction in these tissues. There is evidence also that administration of glucocorticoids causes a decrease in RNA polymerase activity in the rat thymus (Drews and Wagner, 1970; Bell and Borthwick, 1976) and an increase in this activity in hepatic nuclei (Barnabei et al, 1965; Yu and Feigelson, 1971). In view of the alterations in RNA metabolism observed in the liver and gastrocnemius muscle of rats given prednisolone, the effect of this steroid on RNA polymerase activity in these tissues was studied. Following prednisolone administration, nuclei were isolated from liver and gastrocnemius muscle as described on page 84 and the activities of both the Mn^{++} activated and the Mg^{++} activated RNA polymerase were measured as described on page 87. The Mg^{++} activated enzyme has been shown to catalyse the transcription of ribosomal RNA genes while the Mn^{++} activated polymerase the transcription of messenger RNA (Nair et al, 1967; Drews and Wagner, 1970).

It was shown by Neal and Florini (1975) that differential mechanical and DNAse-catalysed damage to DNA during nuclei isolation

procedures could cause artefactual differences in RNA polymerase activities in normal and dystrophic chick muscle preparations. Preliminary experiments were therefore performed to establish the time course of the system employed to assay RNA polymerase activity in muscle of both control and prednisolone-treated animals and to ascertain that for both groups of animals there was a linear incorporation of ^{3}H -UTP during the polymerase assay. The results of these experiments (Fig. 31) confirmed that over the first 6 min. the amount of ^{3}H -uridine incorporated into RNA was linearly related to the incubation time in nuclei preparations from control and prednisolone-treated animals. Therefore, in the experiments reported here, the incubation time for muscle RNA polymerase assays was always less than 6 min. The incorporation of ^{3}H -uridine into RNA was shown to be linearly related to the number of nuclei up to and including 1.25×10^{6} (Fig. 32).

There was a statistically significant decrease of 13% in the activity of the Mg^{++} activated enzyme in muscle nuclei from prednisolone-treated animals compared to the levels in the control group (Table 24). This alteration in RNA polymerase activity was observed after two daily injections of prednisolone into the treated animals, a time when cytoplasmic ribosomal RNA levels in these animals had also been found to be lower than control levels (Table 21). No significant differences in the levels of the Mn^{++} activated enzyme activity in the two groups of animals were observed. In the liver, there was a two-fold increase in Mg^{++} activated RNA polymerase activity as a result of prednisolone treatment (Table 24). This observation is in keeping with the

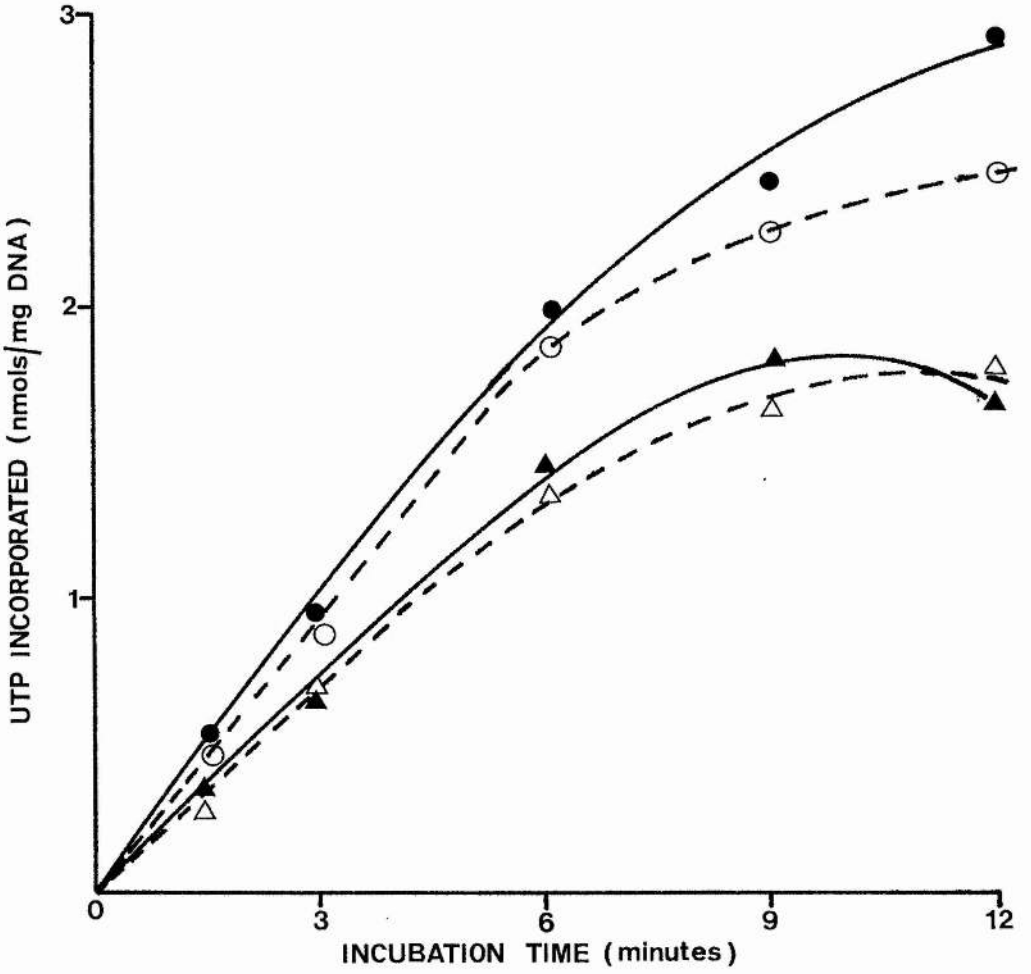


FIG. 31 Time course of the RNA polymerase-catalysed incorporation of UTP into RNA.
RNA polymerase was contained in nuclei isolated from rat gastrocnemius muscle as described on page 84 and was assayed as described on page 87 . Circles refer to activity of the Mn^{++} activated enzyme and triangles indicate the activity of the Mg^{++} activated enzyme. Open symbols refer to prednisolone-treated animals and shaded symbols indicate controls.

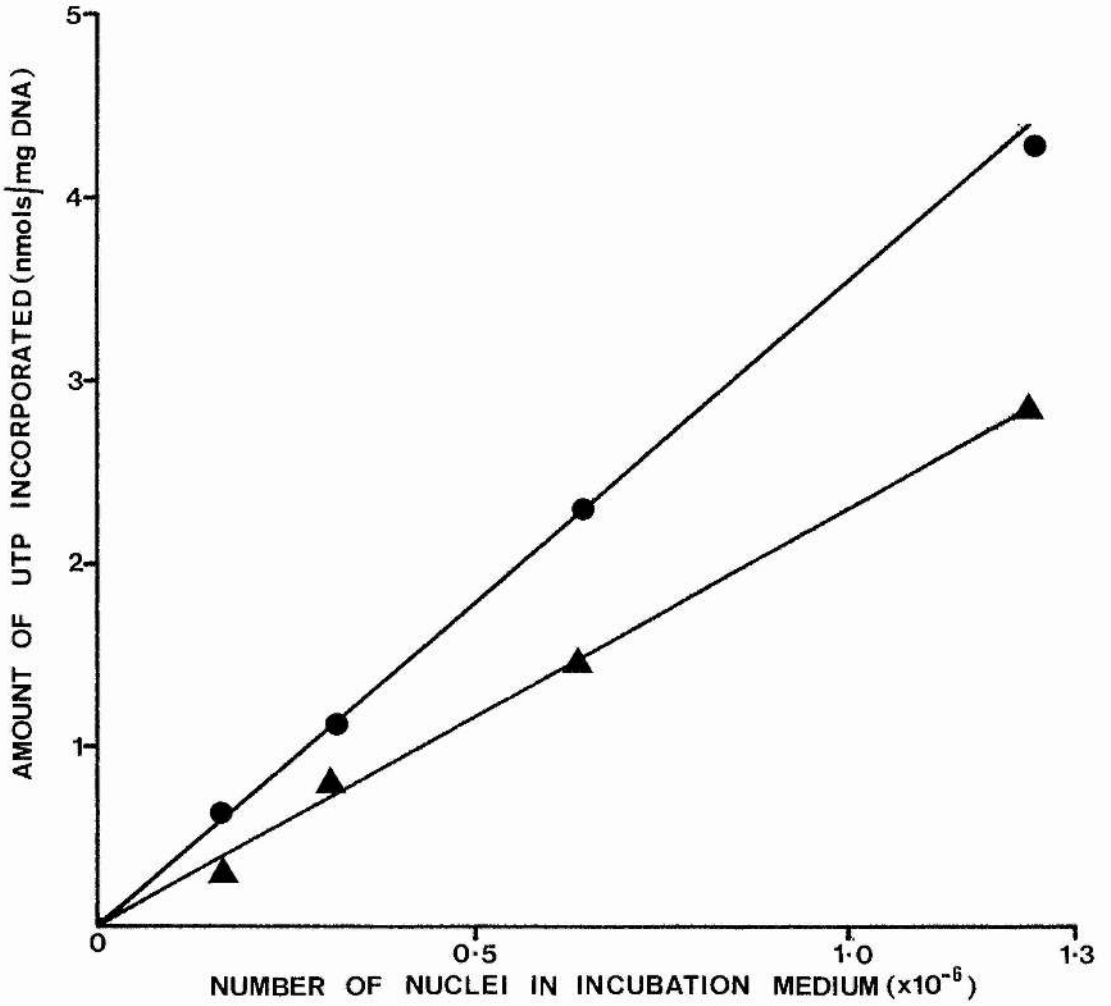


FIG. 32 Linearity of UTP incorporation into RNA with concentration of gastrocnemius muscle nuclei in incubation medium.

Preparation of nuclei and assay of RNA polymerase reaction were carried out as described on pages 84 and 87 respectively.

Circles indicate the activity of the Mn^{++} activated enzyme assay and triangles refer to the Mg^{++}-activated assay.

TABLE 24 CHANGES IN RNA POLYMERASE ACTIVITIES FOLLOWING PREDNISOLONE TREATMENT

	MUSCLE		LIVER	
	Control	Prednisolone -treated	Control	Prednisolone -treated
Mg^{++} activated enzyme	0.83 ±0.03	0.72 ±0.03 (<0.025)	0.17 ±0.01	0.35 ±0.01 (<0.005)
$Mn^{++}/(NH_4)_2SO_4$ -activated enzyme	0.95 ±0.06	0.99 ±0.07 (N.S)	0.33 ±0.01	0.40 ±0.02 (N.S)

Each prednisolone-treated animal received 2 daily subcutaneous injections of 0.06 ml of a solution containing 25mg/ml prednisolone acetate in 0.85%(w/v) saline. Control animals received the same volume of 0.85%(w/v) saline. All animals were sacrificed 24 h after the second injection and nuclei were extracted as described on page 84 . Results are expressed as units of RNA polymerase activity and 1 unit of enzyme activity is the activity, per mg DNA, that incorporated 1 nmole of UTP into RNA under the experimental conditions described on page 87 . Results are the mean values of 4 experiments ± S.E.M. Figures in brackets indicate statistical significance level between control and treated animals, as calculated by the Students paired 't' test.

increase in RNA levels in prednisolone-treated animals and agrees with the findings of other workers studying glucocorticoid-induced increases in Mg^{++} activated RNA polymerase activity in the liver (Barnabei and Ottolenghi, 1968; Yu and Feigelson, 1971). The activity of the Mn^{++} activated enzyme was not significantly altered by prednisolone treatment.

It was evident from these results that prednisolone raised Mg^{++} activated RNA polymerases in the rat liver and depressed this activity in the muscle. The decrease in the Mg^{++} activated RNA polymerase activity in muscle and the increase in this activity in liver observed in rats which had been given two injections of prednisolone acetate and killed 24 h after the last of these is in keeping with the results of the turnover studies of ribosomal RNA in these tissues. Thus when the ribosomal RNA synthesis was observed to be inhibited in muscle and enhanced in liver by prednisolone there were corresponding decreases and increases in the RNA polymerase activity believed to be concerned with the synthesis of ribosomal RNA. However, it is recognised that RNA turnover is regulated by RNA degradative as well as RNA synthetic processes and that prednisolone treatment may also affect RNA degradation.

LEVELS OF RIBONUCLEASE ACTIVITY IN LIVER AND GASTROCNEMIUS MUSCLE OF PREDNISOLONE-TREATED RATS

The decreased protein synthetic capacity of cell-free preparations from the liver of hypophysectomised rats has been attributed to increased levels of alkaline ribonucleases (E.C. 3.1.4-) in these preparations (Brewer et al, 1969) and it has been suggested that the activities of ribonucleases and ribonuclease inhibitors represent a control point in cytoplasmic RNA breakdown (Kraft and Shortman, 1970; Rosso et al, 1973; Lorup, 1977). These inhibitors have been shown to be proteins which inhibit hepatic alkaline ribonucleases (Shortman, 1961) and it has been suggested that corticosteroids may exert their effects by regulating the concentration or effectiveness of RNAse inhibitors in rat thymus cells (Wiernik and Macleod, 1965). The altered levels of RNA in rat liver and muscle following administration of prednisolone could therefore have been the result of alterations in ribonuclease activities in these tissues. This section reports the results of investigations on the activities of the two principal ribonucleases in the mammalian cell the acid- (E.C.3.1.4.23) and the alkaline ribonucleases, following the administration of prednisolone. The influence of the alkaline ribonuclease inhibitor on these enzymic activities was also investigated by measuring the latent activities of the enzymes. Latent activity refers to the enzyme activity liberated by the inactivation of the inhibitor with PCMB which breaks the enzyme-inhibitor bond by blocking -SH groups on the inhibitor protein. The latent activity discussed here must be distinguished from the latent activity released when lysosomal

membranes are destroyed (De Duve, 1969). In the experiments discussed here all tissue homogenates were taken through cycles of freezing and thawing, as described on page 91 , before ribonuclease assays were carried out.

Cytoplasmic 'supernatants' isolated as described on page 91 from control and prednisolone-treated animals were incubated in the presence of yeast RNA and the extent of hydrolysis of this RNA under defined experimental conditions (page 91) was taken as a measure of the activity of the particular ribonuclease. The linear relationship (Fig. 33) between the quantity of 'supernatant' employed for enzyme assay and the observed changes in the extent of RNA hydrolysis confirmed that this assay technique is a reliable measure of acid and alkaline ribonuclease activities. One unit of ribonuclease activity was defined as the enzyme activity that caused an increase of 0.1 in the absorption at 260 nm of the perchloric acid supernatant under the experimental conditions.

In a preliminary investigation the effect of KCl on ribonuclease activities was studied. The addition of KCl to homogenising media to a concentration of 0.3M or higher facilitates solubilisation of muscle proteins. In the presence of 0.3M KCl, there was a complete suppression of both 'free' and latent ('Total' minus 'free') muscle acid ribonuclease activity (Table 25). This finding is consistent with the inhibition of acid ribonuclease in liver homogenates by monovalent cations (Rahman, 1966). Small but measurable 'free' and 'latent' ribonuclease activities were observed when KCl was omitted from the homogenisation medium. The salt also caused some inhibition of alkaline ribonuclease activities,

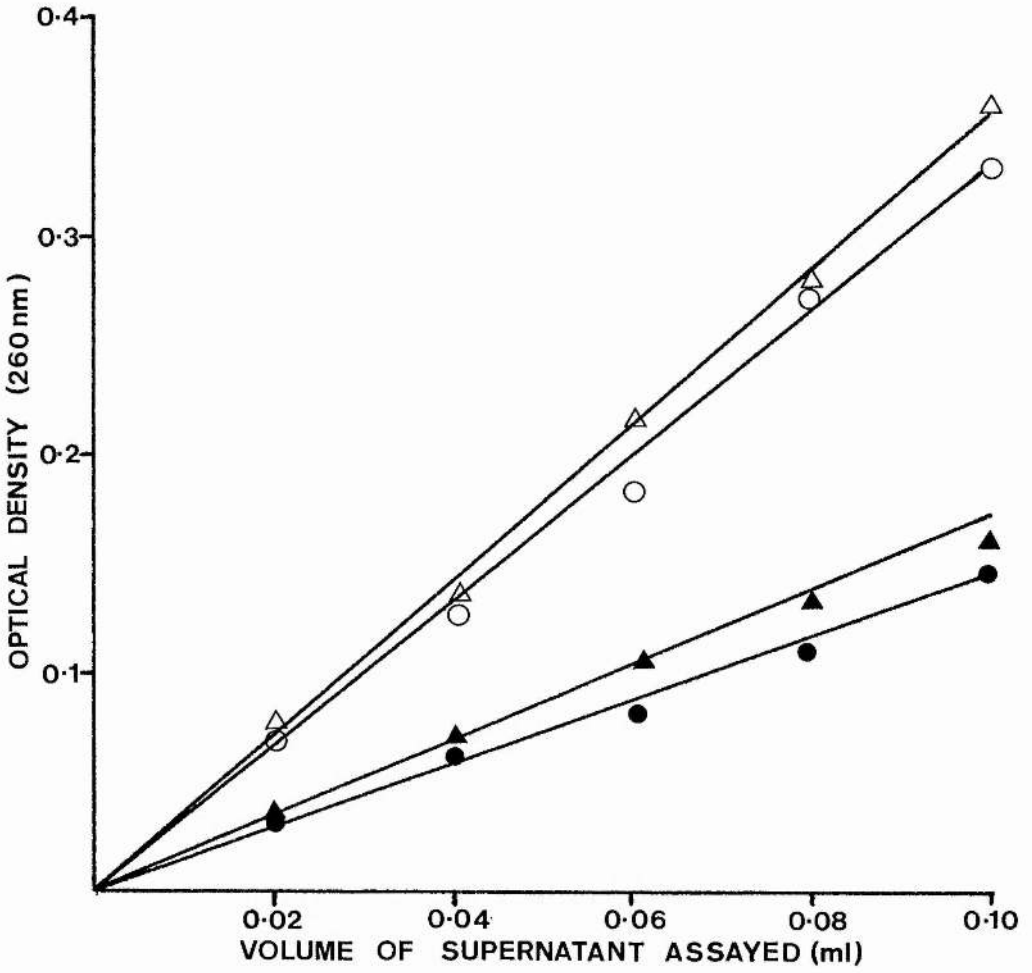


FIG. 33 Relationship between ribonuclease concentration and increase in absorbance at 260nm of the acid ethanol-soluble fraction from ribonuclease assays.

The 'supernatant' was extracted from rat liver as described on page 91 0.1ml of serial dilutions of this supernatant with water was assayed for ribonuclease activity as described on page 91. Triangles represent alkaline ribonuclease activity and Circles represent acid ribonuclease activity. Shaded symbols refer to 'free' ribonuclease and open symbols to 'total' ribonuclease assays.

TABLE 25 EFFECT OF KCl (0.3M) ON RIBONUCLEASE ACTIVITY IN RAT GASTROCNEMIUS MUSCLE

	Acid Ribonuclease (units/g muscle)		Alkaline Ribonuclease (units/g muscle)	
	'free'	'total'	'free'	'total'
KCl present	0.0	0.0	61.3	130
No KCl	7.0	69.6	74.4	282

Enzyme activity measurements were performed as described on page 91 using 'supernatants' extracted from gastrocnemius muscle as described on page 91.

'Free' activity refers to enzyme activity in the absence of PCMB; Total activity was measured in the presence of PCMB, to inactivate the enzyme inhibitor.

One unit of ribonuclease activity was the enzyme activity that caused an increase of 0.1 in the A_{260} of the acid ethanol-soluble fraction under the conditions described on page 91 .

suppressing the 'free' enzyme activity by 18% and the 'latent' activity by 67% (Table 25). The inclusion of KCl in homogenisation media was therefore discontinued in these enzyme studies.

The levels of ribonuclease activity in gastrocnemius muscle following 8 days of prednisolone administration are shown in Table 26. 'Free' acid ribonuclease activity in treated animals was only 22% of the level in controls. 'Latent' acid ribonuclease was also lowered as a result of prednisolone administration and the level in the treated animal was only 12% of the level in the control animal. The observation of 'latent' activity by acid ribonuclease was unexpected as this enzyme was believed to be insensitive to PCMB (Roth, 1957). However, 'latent' acid ribonuclease activity has been reported in rat liver homogenates by Shortman (1961). Free alkaline ribonuclease activity in the prednisolone-treated animals was only 62% of the level in the control group. Latent alkaline ribonuclease activity also dropped due to prednisolone treatment and level in the control animals were about 1.4 times higher than levels in the treated animals (Table 26). Since latent ribonuclease activity is an indirect measure of the RNAse inhibitor activity, the lower latent activity in treated animals, in comparison with controls, suggests a lower inhibitor activity in these animals. It follows also that the decrease in the free ribonucleases in this prednisolone-treated group was not due to an increase in RNAse inhibitor activity.

The results of investigations into liver ribonucleases are presented in Table 27. Free acid ribonuclease activity in the liver, after 8 days of prednisolone treatment, was only 41% of the

TABLE 26 RIBONUCLEASE ACTIVITY IN GASTROCNEMIUS MUSCLE IN RATS GIVEN PREDNISOLONE FOR 8 DAYS

	Acid Ribonuclease (units/g muscle)		Alkaline Ribonuclease (units/g muscle)	
	'Free'	'Total'	'Free'	'Total'
Prednisolone -treated	1.5 ± 0.5	9.0 ± 4.2	43.6 ± 7.3	192 ± 8
	(< 0.005)	(< 0.005)	(< 0.005)	(< 0.005)
Control	6.6 ± 1.2	67.3 ± 9.5	70.2 ± 8.4	278 ± 14

Each prednisolone-treated animal received daily subcutaneous injection of 0.06 ml prednisolone acetate (25mg/ml) over 8 days while the control animal received the same volume of 0.85%(w/v) saline. All animals were sacrificed on Day 9.

One unit of ribonuclease activity is the enzyme activity that caused an increase of 0.1 in A_{260} under the experimental conditions described on page 91.

'Free' ribonuclease activity refers to the activity assayed in the absence of PCMB while 'Total' ribonuclease activity was assayed in the presence of PCMB.

Each result is the mean value from 4 experiments $\pm$ standard deviation. The figures in brackets indicate the level of statistical significance between control and prednisolone-treated animals.

TABLE 27 RIBONUCLEASE ACTIVITIES IN LIVER IN RATS GIVEN PREDNISOLONE FOR 8 DAYS

	Acid Ribonuclease (units/g liver)		Alkaline Ribonuclease (units/g liver)	
	'Free'	'Total'	'Free'	'Total'
Prednisolone -treated	283 ± 40	659 ± 66	268 ± 32	1047 ± 46
	(<0.005)	(<0.005)	(<0.005)	(<0.005)
Control	684 ± 69	1473 ± 55	782 ± 61	1922 ± 58

Experimental details were as described on Table 26

TABLE 28 RIBONUCLEASE ACTIVITY IN PANCREAS IN RATS GIVEN PREDNISOLONE FOR 8 DAYS

	DNA Content (mg/per pancreas)	Acid Ribonuclease (units/mg DNA x 10^{-1})	Alk. Ribonuclease (units/mg DNA x 10^{-1})
Prednisolone -treated	4.1 ± 0.1	419 ± 31	531 ± 29
	(N.S)	(N.S)	(N.S)
Control	4.1 ± 0.1	394 ± 26	563 ± 32

Experimental details were as described on Table 26.
DNA was measured as described on page 46.

activity in the control animals. The latent activity of this enzyme in the treated animals was 48% of the activity level in the control group. Alkaline ribonuclease activities also decreased as a result of prednisolone treatment, the free activity dropping by 65% and the latent activity by 32%. It was evident from these results that in the rat liver, as in the gastrocnemius muscle, there was a loss of both acid and alkaline ribonuclease activities as a result of the prednisolone therapy. Furthermore, this loss was not due to increased ribonuclease inhibitor activity.

It has been suggested (Bartholeyns et al, 1975) that liver ribonuclease may have an extra-hepatic origin and may be derived from the pancreas. The pancreas is a rich source of alkaline ribonuclease and the enzyme in this gland resembles liver alkaline ribonuclease in its stability to heat and sulphuric acid as well as in the effects of salts and buffers (Roth, 1957; Bartholeyns and Baudhuin, 1977). It is conceivable, therefore, that any changes in ribonuclease activities in the rat liver may be a reflection of the changes in the pancreatic enzyme activities. These considerations prompted an investigation of ribonuclease activity in the pancreas following prednisolone administration. The rat pancreas is a diffuse and lobulated tissue extending from the concavity of the duodenum to the spleen and it is interspersed to varying degrees by webs of gelatinous connective tissue. This nature of the gland makes estimations of its actual weight often inaccurate. Therefore, in these studies, units of pancreatic ribonuclease activity have been expressed in terms of the DNA content of the gland rather than in terms of wet weight.

The effect of 8 days of prednisolone treatment on total pancreatic ribonuclease activity is seen from the results presented on Table 28. Small and statistically insignificant differences were observed in both the acid and alkaline ribonuclease activities in the pancreas of control and prednisolone-treated animals. It is unlikely therefore that the alteration of ribonuclease activities in the rat liver and gastrocnemius muscle are related to the pancreatic enzyme levels.

The reduced ribonuclease activity in the liver of prednisolone-treated animals is in keeping with increased RNA levels in the liver of these animals and this result is consistent with those from the study of RNA turnover which indicated that prednisolone treatment caused an increased synthesis and a reduced breakdown of RNA in rat liver (page 118). The finding that ribonuclease activity in the gastrocnemius muscle of prednisolone-treated rats was less than that in control animals may reflect the tissue's response to the diminished synthesis of new ribosomal RNA as indicated by the decreased RNA polymerase activity in the muscles of this group of animals (page 170).

COMPARATIVE STUDIES OF PREDNISOLONE-RECEPTOR COMPLEXES FROM RAT LIVER AND MUSCLE CYTOSOLS

It is generally believed that the events which follow steroid administration to animals are connected to the transfer of cytoplasmic receptor-hormone complexes to the cell nucleus (Beato et al, 1970; O'Malley and Means 1974; O'Malley and Buller, 1977). In particular, it has been shown that cytoplasmic glucocorticoid receptors are necessary for the response the hormone produces in its target cells (Baxter and Tomkins, 1970 and 1971; Rousseau et al, 1972). It has also been suggested that the biological response elicited by a glucocorticoid may depend on the actions of the glucocorticoid-receptor complexes as allosteric effectors (Rousseau et al, 1972) and that organ specificity of steroid action may be related to qualitative or quantitative differences between the steroid binding entities in the target organ (Mayer et al, 1974; Kondo et al, 1975). If indeed glucocorticoid-receptor complexes act as allosteric effectors, possible differences in their physico-chemical characteristics may affect their allosteric properties and, consequently, could explain the different responses glucocorticoids evoke in different target cells. It was therefore of interest to compare some of the properties of prednisolone receptors in the cytoplasm of rat liver and muscle, two tissues in which the hormone produced opposite responses. These considerations prompted investigations the results of which are presented in this section.

Liver and muscle cytosols were prepared as described on page 94 and the prednisolone-receptor complexes, formed after exposing the

cytosol preparation to tritium-labelled prednisolone, were measured using the activated charcoal adsorption technique described on page 94. This technique depends on the fact that the charcoal will adsorb free but not receptor-bound prednisolone. Therefore, determination of the radioactivity in the charcoal-free supernatant, after the prednisolone-binding reaction, is a measure of the bound steroid and a quantitative monitor of prednisolone receptors in the cytosol preparations (Baxter and Tomkins, 1971; Roth 1974; Agawaral, 1975).

To establish the validity of this assay technique, a series of preliminary experiments were performed. The first of these was designed to investigate the efficiency of activated charcoal as an adsorbent for free prednisolone. An appropriate dilution of the cytosol preparations was made to ensure a fixed protein concentration of 2.0mg per assay. This diluted cytosol was exposed to 9.6μM prednisolone (specific activity = 173μCi/mg) and the amount of radioactivity remaining in the charcoal-free supernatant following the removal of unbound steroid with varying concentrations of the activated charcoal was measured. As the results presented in Fig. 34 show, the radioactivity levels remaining in the diluted liver cytosol preparation after the charcoal treatment decreased in a linear manner when the amount of activated charcoal used per assay was less than 20mg per ml of the cytosol preparation. Thereafter, further increases in the charcoal concentration did not significantly alter these radioactivity levels. It would appear, therefore, that under the experimental conditions all unbound prednisolone was adsorbed when charcoal was added to the assay

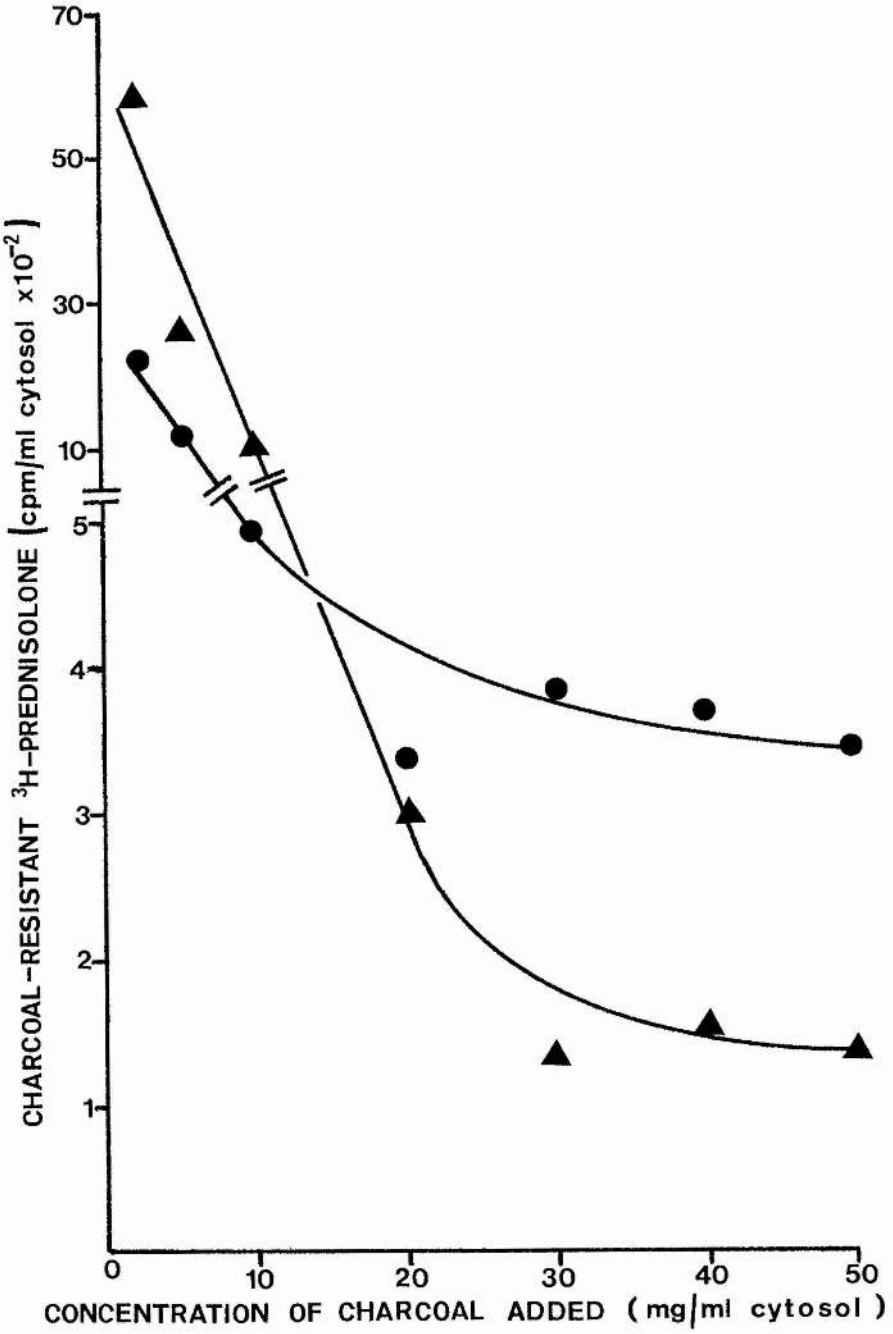


FIG. 34 Effect of charcoal concentration on the measurement of receptor-bound prednisolone in rat liver and muscle cytosols. Prednisolone binding was performed as described on page 94 and unbound steroid was removed using varying concentrations of activated charcoal. Prednisolone was 9.6μM in each assay and cytosol concentration was 2.0mg protein per assay. The circles indicate liver cytosol assays and the triangles the muscle cytosol assays.

mixture in any amount greater than 20mg/ml. Similar experiments with muscle cytosol preparations showed (Fig. 34) that the radioactivity levels remaining in the preparations after charcoal treatment also dropped in a linear manner as the amount of charcoal used as free steroid adsorbent was increased progressively up to 30mg per ml of cytosol. Above this concentration, further increases in charcoal concentrations per assay did not significantly alter these radioactivity levels. Starting with the same concentration of liver and muscle cytosol preparations (2.0mg protein) and working with a charcoal concentration that effectively removed all unbound prednisolone (37.5mg charcoal per ml cytosol preparation), it was observed, by comparing radioactivity levels in the two preparations, that the muscle cytosol preparation bound only 42% of the amount of prednisolone bound by the liver cytosol preparation. This observation may reflect the presence of fewer prednisolone binding sites in the muscle.

In the second series of preliminary experiments cytosol preparations of varying protein concentrations were incubated with a fixed amount (9.6μM) of prednisolone and, in a subsequent step, with a fixed quantity (37.5mg per ml of the cytosol preparation) of activated charcoal. Finally, the radioactivity in the charcoal-free supernatant was measured. Results obtained with liver cytosol preparations are presented in Fig. 35 and show that the amount of receptor-bound prednisolone measured by this assay technique was linearly related to the protein concentration of the cytosol preparation up to a concentration of 4.0mg protein per assay. This linearity was lost when cytosol preparations

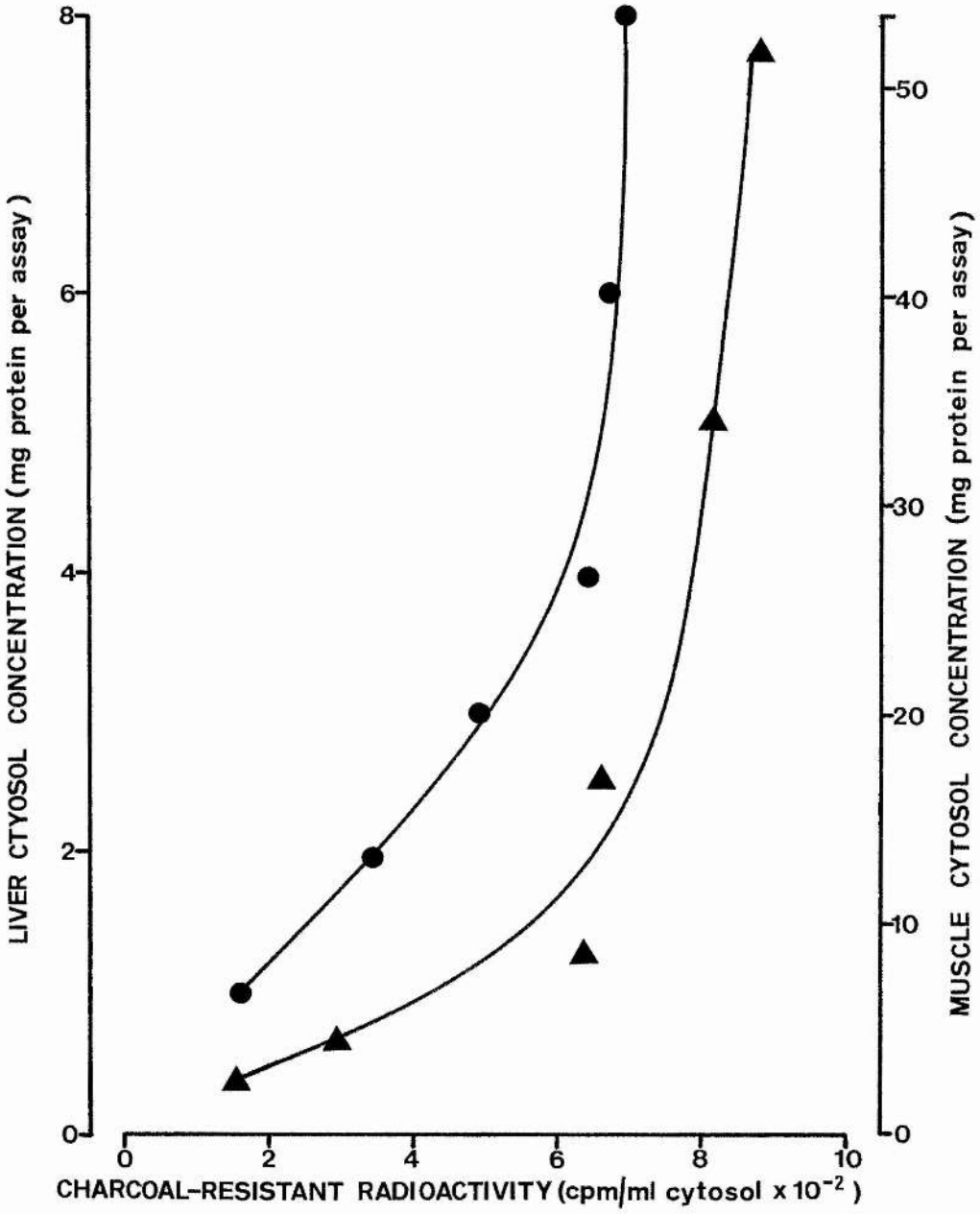


FIG. 35 Relationship between cytosol concentration and prednisolone binding in rat liver and muscle cytosol preparations: Prednisolone binding at various cytosol concentrations was measured, using 9.6μM prednisolone and activated charcoal at a concentration of 37.5mg per ml of cytosol, as described on page 94 . Circles indicate results obtained with liver cytosol and triangles represent results obtained with muscle cytosol.

containing higher protein concentrations were assayed. In experiments with muscle cytosol preparations, linearity of receptor-bound radioactivity with cytosol concentration was maintained up to a cytosol concentration of 8.6mg protein per assay. A plateau began to develop when protein in the cytosol preparation exceeded this concentration (Fig. 35).

The results of these preliminary experiments confirmed that quantitative measurements of prednisolone receptors in the rat liver and muscle cytosol preparations could be obtained using the activated charcoal adsorption technique under the experimental conditions described on page 94 . Under those conditions, activated charcoal should be used at concentrations above 20mg per ml of liver cytosol and above 30mg per ml of muscle cytosol preparations. As the results in Fig. 35 indicate, the protein concentration of the liver cytosol preparation should not exceed 4.0mg per assay and muscle cytosol should be diluted to give a concentration below 8.6mg protein per assay. In subsequent work, both liver and muscle cytosols were diluted to the same protein concentration (4.0mg per assay) for use. The results obtained in these conditions were reproducible and the charcoal adsorption technique proved to be a simple and speedy method of measuring prednisolone receptors in rat liver and muscle cytosol preparations.

Characterization of liver and muscle prednisolone receptors by ion-exchange chromatography

The series of preliminary experiments which were reported in the preceding section demonstrated the ability of prednisolone to

bind to receptors located in the cytosol of rat gastrocnemius muscle. Those results support earlier suggestions (Simpson and White, 1973; Mayer et al, 1974 and 1975) that this tissue, since it possesses glucocorticoid binding proteins like the liver, is a target tissue for these hormones.

To characterise these prednisolone receptor moities with respect to heterogeneity and ionic charge the liver and muscle prednisolone-receptor complexes were subjected to ion-exchange chromatography as recommended by Agawaral (1976). This technique fractionates the receptor subpopulations on the basis that the order in which the receptors are eluted from the column by a linear ionic gradient depends on each receptor's overall ionic density and the tenacity with which it is held on the column. Following exposure to prednisolone, and charcoal treatment to remove unbound steroid, the cytosol preparation was loaded on a DE-52 cellulose anion exchange column which had been packed and pre-equibrated with 0.001M sodium phosphate buffer, pH 7.5. After an initial prewash with 60ml of the 0.001M phosphate buffer, elution was effected by a linear gradient formed by mixing 60ml of this buffer with 60ml of 0.2M phosphate buffer, pH 7.5 in a gradient former. Radioactivity measurements of the sample before it was loaded on column and of the fractions collected after the gradient elution confirmed that no significant radioactivity remained on the column after the elution.

With the liver sample (Fig. 36) this chromatographic analysis resulted in the resolution of two distinct peaks of radioactivity. The first and larger zone was eluted with the initial wash (0.001M

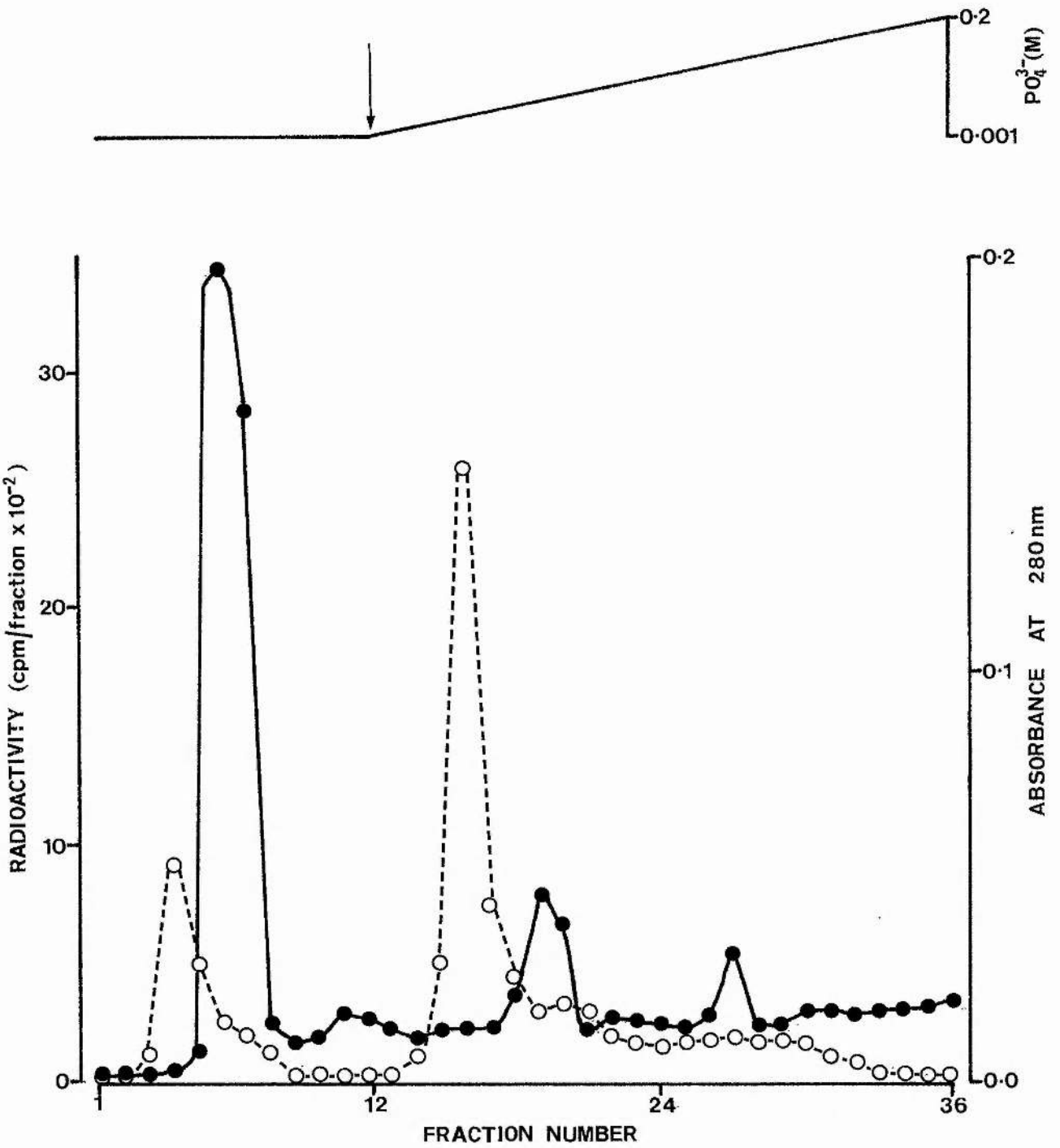


FIG. 36 DE-52 column chromatography of rat liver prednisolone receptors.
The liver was processed for chromatography as described on page 94. 3.5ml of the sample, containing 1.0mg protein per ml was applied to the column and, after the initial prewash, gradient elution was begun at the arrow. (●—●) represents radioactivity and (-O-O-) represents absorbance at 280nm.

phosphate) and the second eluted at a phosphate concentration of 0.069M. A third zone, which was eluted with 0.019M phosphate was occasionally observed. This third zone showed extreme variability in amount. The results of the experiments with muscle receptors are presented in Fig. 37. Two zones of radioactivity were observed and their elution profile followed the pattern observed with liver samples. There was, however, no indication of a third zone in any of the experiments with muscle receptors. The first zone of muscle receptors was only 43% of the same zone of liver receptors, in terms of their radioactivity levels. This quantitative difference was not due to a difference in the cytosol protein concentrations which were the same in both preparations. Since it had also been confirmed that no radioactivity was retained on the column after the elution, this difference may reflect the difference in ability or capacity of each tissue to bind prednisolone.

The data in Fig. 36 and Fig. 37 also indicate that the prednisolone-binding proteins in both liver and muscle cytosols constitute only a very small proportion of the cytoplasmic proteins in these tissues. In the liver, two major protein fractions could be separated by this chromatographic technique but neither of these peaks coincided with the peaks of radioactivity. In the muscle cytosol, three major protein fractions were detected at positions which did not coincide with the radioactivity peaks.

Characterization of liver and muscle prednisolone receptors by ultracentrifugation on a sucrose gradient

To obtain an estimate of the molecular sizes of the prednisolone-receptor complexes in the muscle and liver cytosol

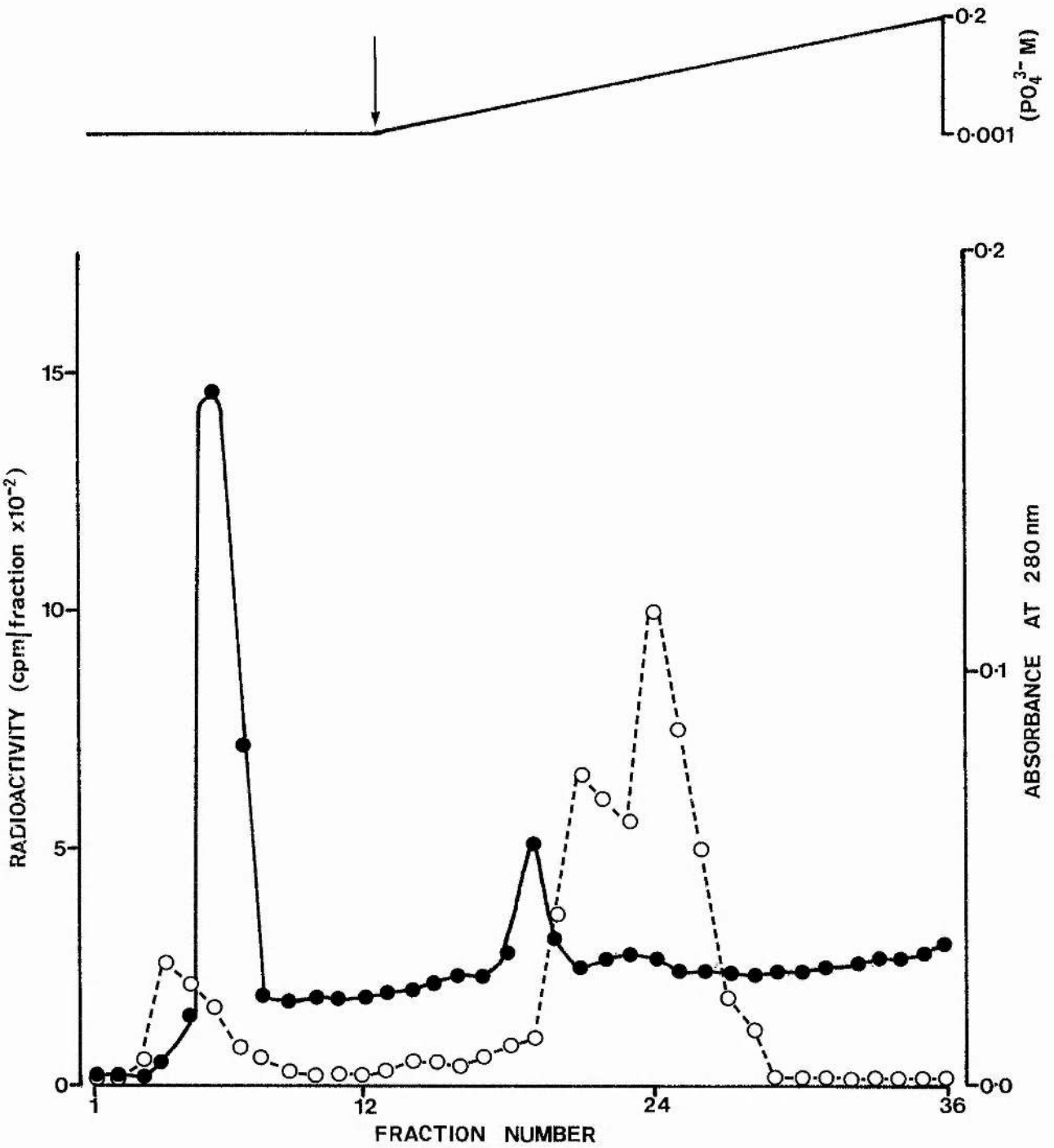


FIG. 37 DE-52 column chromatography of rat gastrocnemius muscle prednisolone receptors.
The muscle was processed for chromatography as described on page 94. 3.5ml of the sample, containing 1.0mg protein per ml was applied to the column and, after the initial prewash, gradient elution was begun at the arrow. (-●-●-) represents radioactivity and (-O-O-) represents absorbance at 280nm.

preparations, the sedimentation pattern of these complexes on a linear 5 - 20% sucrose gradient was studied using the ultracentrifugation technique described on page 73. The sucrose gradient served as a supporting medium through which the complexes were moved by centrifugal force and fractionated on the basis that the rate at which they travelled down the gradient was a function of their molecular sizes (Martin and Ames, 1961). Pre-treatment of the samples consisted of exposing the cytosol preparation to ^{3}H-prednisolone and, thereafter, to activated charcoal to remove unbound steroid. The charcoal-free supernatant was loaded on the sucrose gradient and centrifugation was performed at 5°C for 24 h at 60,000g ave. fractions collected after centrifugation were analysed for radioactivity, as described in 'Methods' section, to locate the prednisolone-receptor complexes. The sedimentation coefficients of these complexes were calculated by comparing the distance they moved from the meniscus with that travelled by the standard (bovine albumin) under identical conditions. The molecular weights of these receptor complexes were calculated from the sedimentation coefficients as recommended by Martin and Ames (1961).

Analysis of the fractions of the gradient obtained with the liver sample revealed a single peak of radioactivity (Fig. 38). The position of this peak in comparison with the bovine albumin peak indicated that it had a sedimentation coefficient of 5.9S. This value is larger than the value of 4S reported by Petrovic and Markovic (1975) for cortisol receptors in rat liver but is within the range (5S to 8S) reported by Baxter and Tomkins (1971); Beato

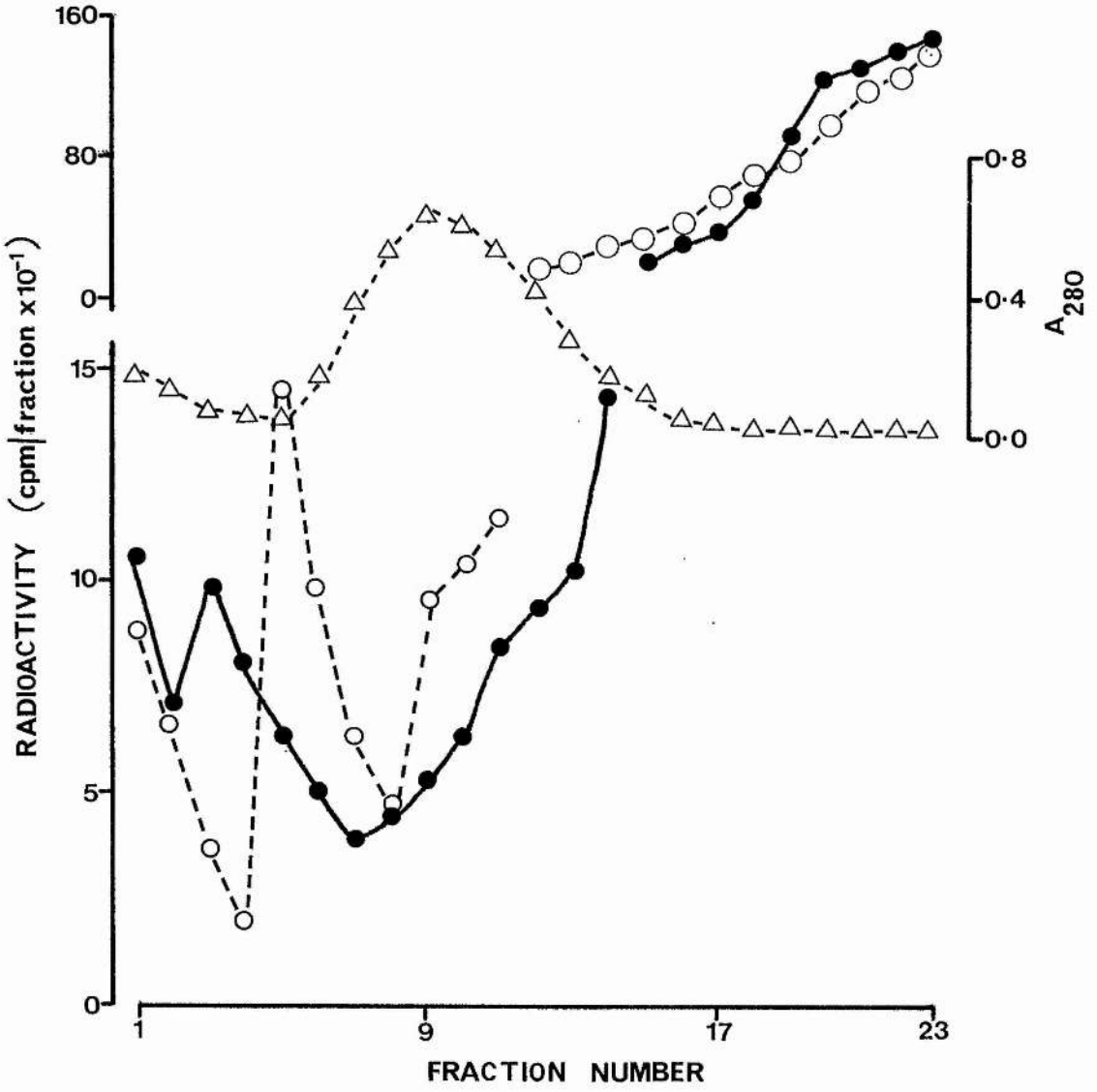


FIG. 38 Sedimentation Profile of liver cytosol prednisolone binding receptors in 5% - 20% (w/v) sucrose gradients.

1.0ml of the sample, prepared as described on page 94 was layered on the gradient and centrifuged at 60,000g ave. for 24 h at 2°C. 25-drop fractions were collected and assayed for radioactivity. - (-○-○-) indicates gradients containing no KCl and (-●-●-) indicates gradients containing 0.3M KCl. The sedimentation peak of bovine albumin (-△-△-) was identified by ultraviolet absorption measurements at 280nm.

and Feigelson (1972) and Agawaral et al, (1970). A protein with an S value of 5.9 would have a molecular weight of about 97,200. This peak of radioactivity constitutes only a very small fraction of the radioactivity present in the gradient and the rest of this radioactivity remained at the top of the gradient (Fig. 38). This picture suggests that considerable dissociation of the hormone-receptor complexes occurred during the lengthy (24 h) centrifugation as has been observed by Smart et al, (1970), who studied the sedimentation behaviour of corticosterone-binding proteins in rat liver. With the muscle prednisolone-receptor complexes no reproducible peak could be detected under the existing experimental conditions and almost all the radioactivity remained at the top of the gradient. Mayer and Rosen (1975) encountered the same problem under similar experimental conditions and detected a peak only when they included KCl (0.3M) in the sucrose gradient. It was, therefore, decided to examine the effect of the presence of KCl on the gradient analysis of muscle prednisolone-receptor complexes. This salt was included, at a concentration of 0.3M, in both the homogenisation buffer used in the preparation of the cytosol and in the sucrose gradient on which the sample was loaded. In the presence of 0.3M KCl, a definite peak was observed in the 3.9S region after centrifugation (Fig. 39). This result is in good agreement with that obtained by Mayer and Rosen (1975) in experiments with dexamethasone receptors in rat gastrocnemius muscle cytosol. A sedimentation coefficient of 3.9 indicates a molecular weight of about 52,300 for these muscle prednisolone-receptor complexes. When the liver cytosol prednisolone-receptor complexes

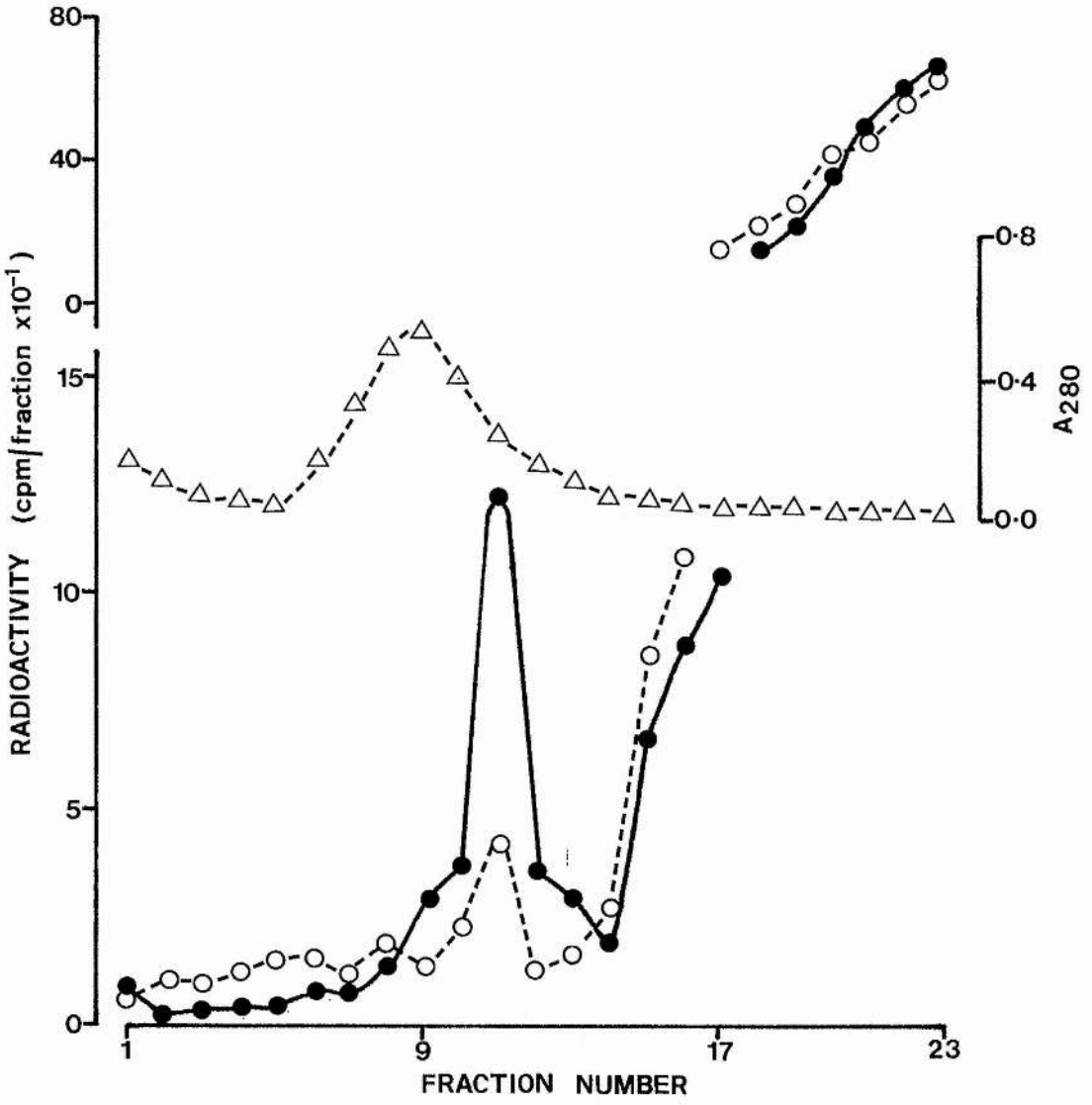


FIG. 39 Sedimentation Profile of Muscle Cytosol prednisolone binding receptors in 5% - 20% (w/w) sucrose gradients.

1.0ml of the sample, prepared as described on page 94 was layered on the gradient and centrifuged at 60,000g ave. for 24 h at 2°C. 25-drop fractions were collected and assayed for radioactivity. (-O-O-O-) indicates gradient containing no KCl and (-●-●-●-) indicates gradient containing 0.3M KCl. The sedimentation peak of bovine albumin (-△-△-) was identified by ultraviolet absorption measurements at 280nm.

were prepared and centrifuged in the presence of 0.3M KCl a slightly faster-sedimenting peak (6.5S) was obtained. This observation contrasts with the findings of Beato and Feigelson (1972). These workers found that, in the presence of 0.3M KCl, dexamathasone-binding receptors in rat hepatoma cells tended to dissociate or were otherwise converted to 4S forms. The ionic environment and the centrifugation process itself may, therefore, be critical factors in the results obtained in these experiments.

Possible differences between rat liver and gastrocnemius muscle prednisolone receptors

The results of investigations reported in this section indicated that more prednisolone was bound by the liver than was bound by the muscle. Since there is a dose-related response to glucocorticoids (Locker and de Wever, 1970), the response of rat liver and muscle to prednisolone may be related to the different amounts of the steroid that is retained by the tissue. The sucrose gradient centrifugation experiments indicated that the prednisolone receptor in the liver was a bigger molecule than the muscle receptor. This liver prednisolone receptor also appeared to be less readily dependent on the prevailing ionic environment than the muscle receptor. This was evident from the effect of the presence of KCl on the sucrose gradient profile of prednisolone receptors in the two tissues. The liver prednisolone receptor may, therefore, be more stable than the muscle receptors. Such possible difference in the relative stabilities of prednisolone receptors in rat liver and muscle may also influence the response the steroid elicits in the respective tissues.

4. DISCUSSION

The data presented on page 111 clearly indicate that daily subcutaneous injections of prednisolone acetate, at a dosage of 10mg per kg initial body weight and over an 8-day period, caused a loss of protein and RNA in the gastrocnemius muscle of rats as well as a net loss in their body weight. Although glucocorticoids are known to cause fluid retention (Sayers and Travis, 1970), in these experiments no attempt was made to estimate any increase in water content in the body as a whole or in individual tissues. The depletion of body weight observed may thus be an underestimation of the amount of tissue wasting which had occurred. These effects of prednisolone administration are in agreement with the findings of other workers (Mayer et al, 1976; Shoji and Pennington, 1977).

Both prednisolone-treated animals and their controls received and consumed the same quantity of food over the experimental period. The observed response to prednisolone could not therefore have been influenced by the nutritional status of the treated animals. Goodlad and Munro (1959) in fact found that cortisol-induced changes in body and liver weights in rats were independent of dietary protein and energy.

Different muscles vary in their response to the action of glucocorticoids. Smith (1964), studying the histological and histochemical changes in the muscles of rabbits treated with trianicinolone, reported more wasting in pale muscle fibres than in the dark or red fibres; D'Agostino and Chiga (1966) reported that corticosteroids predominantly affected red muscle fibres in rabbits and Afifi and Bergman (1969) reported that there was no predilection to wasting in any one type of muscle fibre. In more recent studies

with tumour-bearing rats, which also show a depletion of muscle mass and muscle protein, it was demonstrated that the ability to resist the cachectic effect of the tumour varied with the type of muscle involved and that soleus muscle with predominantly dark fibres showed a greater resistance to this cachectic effect than the gastrocnemius muscle which consists mainly of white fibres (Clark and Goodlad, 1971; Goodlad and Clark, 1972). In the present studies it was shown that gastrocnemius was very responsive to the administration of prednisolone. This muscle type therefore appeared suitable for a study which was concerned primarily with the nature of the response to prednisolone rather than with the relative susceptibilities of different muscle types to the steroid.

The progressive decrease in protein content in the gastrocnemius muscle demonstrated in this study indicates that changes may have occurred in protein metabolism in this tissue in response to the administration of prednisolone. A net loss of muscle protein could be the result of (a) an increased rate of protein breakdown with no change in the rate of protein synthesis, (b) a decrease in the rate of protein synthesis with no change in the rate of its breakdown or (c) a combination of increased rate of protein breakdown and decreased rate of protein synthesis. In the absence of data on the effect of prednisolone on the amino-acid pool in muscle or on protein metabolism in this tissue, it is not possible in the present work to be definitive about the mechanism of protein loss in muscle following the administration of prednisolone. However, other workers have shown that in response to the administration of cortisol or cortisone there was a reduced incorporation of amino-acids into

muscle protein (Hanoune et al, 1972; Shoji and Pennington, 1977) as well as increased degradation and decreased synthesis of protein in the tissue (Goldberg, 1969). Previously, Kaplan and Schimizu (1963) had reported that the loss of muscle protein occurred in the face of increased concentrations of free amino-acids in the tissue and it is therefore not due to unavailability of these protein precursors.

The fact that RNA levels also fell during the experimental period is a strong hint that prednisolone-mediated changes in protein levels may be related to changes in the metabolism of nucleic acids. That the effect of steroids on protein metabolism are preceded by effects on RNA metabolism was first proposed by Karlson (1963) and this view has received considerable support from subsequent related studies (Kidson, 1967; Amaral and Moriber, 1967; Young, 1970; Nakanishi et al, 1977). Only total cellular RNA levels were measured in these investigations and it was not possible to be more specific about the RNA species affected by the administration of glucocorticoids. However it is probable that the loss of RNA in muscle of prednisolone-treated animals involved ribosomal RNA principally since this RNA species constituted more than 80% of the total RNA in skeletal muscle (Manchester, 1967; Young, 1970). This probability does not rule out possible accompanying effects of prednisolone on other RNA species.

In rat liver there was an increase in mass, protein and RNA as prednisolone treatment progressed. These effects in the liver were demonstrable two days after the initial prednisolone injection and before statistically significant changes were observed in the gastrocnemius muscle. It would seem therefore that the protein and

RNA precursors in the liver were not derived from gastrocnemius muscle. The observation that cortisol and triamcinolone bind to distinct cytoplasmic receptors in rat skeletal muscle (Mayer et al, 1976) and the presence in liver cytosol of cortisol-specific receptors (Beato et al, 1971; Rao et al, 1976) support the belief that rat liver and gastrocnemius muscle are direct target organs for the action of prednisolone and that the steroid-mediated responses in both tissues occur independently.

It is significant that the alterations observed in both the liver and gastrocnemius muscle occurred without any change in the amount of DNA present in these tissues. A change in DNA concentration is an indication of cell turnover and, conversely, a constant DNA level reflects a constant cell population (Enwonwu and Sreebny, 1970). It is evident therefore that the prednisolone-mediated changes observed in the rat liver and gastrocnemius muscle were due to changes in cellular components rather than to changes in cell number.

In these studies prednisolone acetate was administered at a dosage of 10mg per kg initial body weight of the animals. This dosage was within the recommended pharmaceutical range for humans (Wilson, 1977) and was the basis for any comparison of the effects of this steroid in rats and in humans. It should however be pointed out that different amounts of a glucocorticoid may elicit quite different responses in experimental animals (Loeker and De Wever, 1970).

The alterations in liver and gastrocnemius muscle RNA following prednisolone treatment prompted an investigation of the effect of the steroid on RNA turnover in the two tissues. It was found that

the administration of prednisolone to these animals had an effect on the rates at which RNA turned over but in a somewhat different fashion in the two tissues. The result of the investigation of total RNA turnover in rat liver (page 118) suggests that in the livers of prednisolone-treated rats there was a reduced rate of RNA breakdown and an increased rate of RNA synthesis, compared with that in the control rats. It would appear therefore that a factor in the prednisolone-induced increase in RNA levels in rat liver could be a slowing down of the rate at which cellular RNA was being degraded together with an increase in the synthetic rate of this RNA. When RNA turnover in the ribosomes of rat liver was examined in a similar series of experiments a resemblance between the turnovers of ribosomal and total RNA was observed. This finding is what would be expected since most of liver RNA is ribosomal (Blobel and Potter, 1967). Whether or not these changes in RNA metabolism involve changes in the activities of ribonucleases and RNA polymerases in the liver will be discussed in subsequent sections.

Administration of prednisolone appeared to have a more complex effect on the turnover of RNA in the gastrocnemius muscle. The total radioactivity in the muscle ribosomal RNA of prednisolone-treated rats was always lower than control levels (page 157) and over the first two days of prednisolone treatment, when the estimated amounts of ribosomal RNA showed only a slight decrease, the specific activity was also lower in the treated animals. RNA turnover in rat gastrocnemius muscle would seem to follow a biphasic pattern during prednisolone treatment, the initial event being an increase in RNA synthesis and renewal. If during this first phase RNA was also

degraded at a rate exceeding the synthesis rate the net result would be a lowering of total radioactivity and amount of RNA in the tissue. In the absence of precise data on the kinetics of loss of radioactivity *in vivo* from the muscle in the period immediately following the first prednisolone injection, this reasoning is speculative at the present time. However such a picture would be similar to that shown to occur in rabbit lymph node cells under the influence of cortisol (Kidson, 1967). This hormone was found to cause a net decrease in the rate of RNA synthesis but the immediate response of the cells was to increase RNA synthesis within seconds of exposure to the steroid. In the period from the second day of prednisolone administration to the end of the experiment a reduced rate of RNA synthesis appeared to be occurring. The observed reduction in RNA polymerase activity, particularly that believed to be responsible for the synthesis of ribosomal RNA (Table 24) supports this view. Thus a possible regulatory site for the action of prednisolone in rat gastrocnemius muscle is at the level of transcription of ribosomal RNA and the alterations observed in cytoplasmic RNA may be related to nuclear interactions and/or alterations in RNA polymerase activities.

These conclusions about the effect of prednisolone on RNA turnover in the liver and gastrocnemius of rats could be subjected to one general criticism - the steroid may have altered radioisotope uptake into the precursor pool for RNA leading to alterations in RNA labelling independent of the rate of RNA turnover. In this connection it is of interest that the administration of cortisone was shown to cause a marked increase in the uptake of glycine for

purine biosynthesis in rat liver (Feigelson and Feigelson, 1963). Stimulation of endogeneous synthesis of RNA precursors is thus a possibility that could result in dilution of labelled RNA precursors in the pool and cause alteration in radioisotope uptake into RNA. Moreover, any alteration in the re-utilisation of labelled nucleotides arising from RNA degradation would lead to apparent changes in the half-life of the RNA (Blobel and Potter, 1968). However, the investigation of the specific activity of RNA precursor pools in both rat liver and gastrocnemius muscle carried out in the present work shows that prednisolone does not alter these precursor pools significantly during the experimental period. The pools of labelled precursors in both control and prednisolone-treated rats were found to be essentially the same and it would thus appear unlikely that any changes in RNA turnover could be related to alterations in the RNA precursor pool in the treated animals.

Greenman et al (1965) showed from studies of the action of hydrocortisone on rat liver that this steroid altered the synthesis of all major RNA species in the tissue. It cannot however be concluded that the action of prednisolone on RNA is also indiscriminate. Selective alteration of particular RNA species is a possibility that would not be detected in the initial experiments which investigated total RNA turnover. Subsequent study of polyadenylated and ribosomal type RNAs showed that, at least in the rat gastrocnemius muscle the administration of prednisolone affected these two RNA species differently. It was shown that although polyadenylated RNA constituted only a very small proportion by weight of cytoplasmic RNA ($< 3\%$), it was highly labelled and consequently made significant

contributions to the specific radioactivity of total RNA in gastrocnemius muscle. Two days after the injection of the radioisotope, the specific radioactivity of polyadenylated RNA is more than three times that of other RNA species (Table 17) and over a ten-day period this level of radioactivity did not fall but rose slightly (Fig. 25). It is a well-documented fact that polyadenylate sequences exist covalently bound at the 3′-OH end of most eukaryotic messenger RNAs (Sheldon et al, 1972; Darnell et al, 1973; Brawerman, 1974). RNA containing these sequences are therefore often regarded as messenger RNA (Singer and Penman, 1973; Robbins and Heywood, 1978). The presence of these sequences in rat muscle messenger RNAs has been demonstrated by Kaufmann and Gross (1974) and they have been utilised to provide specific methods for isolating these RNA species from muscle tissue (Neal and Florini, 1975; Heywood et al, 1975). There are two points of view concerning the regulation of polyadenylated RNA degradation in eukaryotic cells. The first is that the degradation of this RNA species is not subject to regulation and occurs randomly once ribosome initiation sites are empty (Haywood, 1974). The second viewpoint is that the stability of polyadenylated RNA is regulated by translation. After translation the polyadenylated RNA is degraded but if translation is inhibited this RNA remains intact (Arbuzov 1976 & 1977). A study of the cytoplasmic distribution of polyadenylated RNA revealed that transcription products which eventually form myosin messenger RNA can be temporarily sequestered from activation as messengers and accumulate in the cytoplasm of calf muscle cells (Buckingham et al, 1974 & 1976). This RNA

presumably retains large portions of polyadenylate segments and thus prolongs its biological lifetime (Marbaix et al, 1977). Myosin is a major protein component of skeletal muscle myofibrils (Potter, 1974) and it follows that there may exist in the cytoplasm of rat gastrocnemius muscle polyadenylated RNAs which have a much slower turnover than other RNA species in the cell. This RNA species may have contributed to the specific radioactivity of total RNA in gastrocnemius muscle, to the extent observed in Fig. 22.

Another possible explanation for the difference in the observed specific radioactivities of polyadenylated RNA and other RNA species in the gastrocnemius muscle could be that separate RNA precursor pools exist in the muscle cell and that these pools are characterised by the species of RNA for which they provide the precursors. Weigers et al (1976) showed from a study of RNA synthesis in Hela cells, in culture, that messenger RNA was formed from precursors located in a large pool of pyrimidine nucleotides while a smaller pyrimidine pool served to supply ribosomal RNA synthesis. Since the bulk of cellular RNA is believed to be ribosomal (Blobel and Potter, 1967; Manchester, 1967; Young, 1970) the small pyrimidine pool would have a higher turnover than the pool from which messenger RNA is formed. This difference in precursor pool size could thus influence the availability of labelled precursors and the overall rate of incorporation of these labelled precursors into the various RNA species. A compartmentalisation model for RNA precursors has also been described by Plagemann (1971) who examined nucleotide pools in rat hepatoma cells and by Goody and Ellem (1975) who studied the entry of RNA precursors into nucleotide pools in 6C3HED lymphoma

cells. However, the difficulty in measuring the absolute size and the specific radioactivity of those RNA precursors directly involved in new RNA synthesis precludes an accurate assessment of the contribution of exogeneous RNA precursors to this pool (Goody and Ellem, 1975). The views on the influence of RNA precursor pools on the uptake of labelled nucleotide by cellular RNA species must therefore remain speculative.

In summary, the results of the present work suggest that RNA levels in the liver of prednisolone-treated animals rose as a result of a reduced degradation and an increased synthesis of ribosomal RNA. In the gastrocnemius muscle the picture appears to be more complex but the net result seems to be a reduced synthesis of ribosomal RNA. It is not possible to be completely unequivocal about the relationship between prednisolone-mediated alterations of RNA turnover and the observed alterations in protein levels in the muscle and liver of experimental animals. The two events may be unrelated, the prednisolone effect on RNA turnover representing a response emanating from a selective steroid effect at the genetic level and the effect on cellular protein concentrations reflecting an independent action of the steroid on, for example, amino-acid pools or the energy requirements for protein synthesis. However, available evidence would appear to argue against such a view. Protein levels can be related to changes in RNA turnover and significant correlation has been observed between the fractional rate of protein synthesis and RNA concentration (Garlick et al, 1976; Kaplan and de Nicola, 1976; Carinci et al, 1976; Varga and Sinitsin, 1978). It has also been demonstrated that the activity

of the entire protein synthetic machinery is influenced by transcriptional events and messenger RNA synthesis (Grieninger and Granick, 1975). Control mechanisms for protein synthesis may also involve small RNA molecules acting as promoters of translation (Fraser, 1975; Heywood et al, 1975) and the capacity of any tissue for protein synthesis depends on the integrity of the RNA in the ribosomes (Hirsch and Hiatt, 1966; Layman et al, 1976). These considerations as well as results which showed alterations in RNA levels wherever protein levels were altered lead to the conclusion that the means by which prednisolone affects these two processes may be closely related.

Considerable difficulty was encountered in isolating cytoplasmic RNA species from rat gastrocnemius muscle in the course of this work. Firstly, RNA prepared by most of the conventional methods was significantly contaminated by other cellular components. From inspection of the A_{260}/A_{280} ratios of the isolated materials these contaminants were, at least partially, protein in nature. Polysaccharides were other possible contaminants since extraction with methoxyethanol improved the specific activity of the material. Traces of the sodium dodecycl sulphate used in the tissue homogenisation medium may also be present as contaminants in the isolated material and may account for the high ultraviolet absorption at the top of sucrose gradients following the centrifugation of the material on these gradients (Fig. 24C). This detergent absorbs light at 260nm and it is a compound which, once introduced, is not easily completely removed from solutions. The second difficulty in this series of experiments was in obtaining undegraded RNA consistently by

some of the methods employed. Enzymic degradation by ribonucleases may account for this RNA degradation. Contrary to an early report (Kirby, 1957) ribonucleases are neither completely removed nor permanently inactivated by phenol and several workers (Littauer and Eisenberg, 1959; Brownhill et al, 1959; Ralph and Bellamy, 1964) have reported enzymic degradation of RNA extracted from plant and animal sources by phenol methods. Kirby himself noted later (1965) that ribonucleases are relatively stable, can survive phenol extraction and, on removal of the phenol, can degrade RNA. There is evidence also that RNA itself may protect ribonucleases from reagents employed to remove the enzymes (Robins and McNutt, 1974). Krechetova et al (1972) held the view that some of the tissue ribonucleases are structural proteins of the small ribosomal subunit and that the proximity of the larger ribosomal subunit renders the enzyme inactive. This inactive state is maintained only as long as the ribosome structure is preserved. RNA degradation by ribonucleases was therefore always a likely possibility during the isolation procedures employed in the course of this work.

Ribonucleases may be inhibited by several reagents. Sodium dodecycl sulphate (Crestfield et al, 1955), Polyvinyl sulphate (Neal and Florini, 1975), Zn^{++} (Brownhill et al, 1959), Bentonite (Huppert and Pelmont, 1962) and copolymers of glutamate and tyrosine (Sela, 1962) have all been employed to inhibit ribonucleases. Diverse mechanisms have also been suggested to explain the inhibitory effects of these various agents. Thus the acidic copolymers of glutamate and tyrosine may inhibit ribonucleases through electrostatic competition with the substrate for the enzyme. Such an inhibition will be

pH-dependent since the ability of the inhibitor to bind enzyme will depend on the ionisation of dissociable groups on the enzyme. Bentonite is a form of aluminium silicate and its ribonuclease inhibitory ability has been attributed to its protein adsorbing properties (Darrow and Collowick, 1962). Other proteins in the environment may therefore affect the efficiency of bentonite as a ribonuclease adsorbent by competing for adsorption sites on the bentonite. From these considerations, it would seem that the effectiveness of a ribonuclease inhibitor depends on the prevailing experimental conditions. A medium which favours good yields of RNA from a tissue may not be ideal for the action of a particular ribonuclease-inhibiting agent. For example, in the presence of a high concentration of KCl, the adsorptive capacity of bentonite is much reduced (Darrow and Collowick, 1962) and the degradation of RNA is increased (Ito, 1977). This is the same experimental condition required to cause maximal release of RNA from muscle proteins (Manchester, 1967). Furthermore, the action of a ribonuclease inhibitor depends on the concentration of inhibitor employed as well as on the particular ribonuclease and its source (Littauer and Sela, 1962; Neal and Florini, 1975). In the course of the present work, the choice of a ribonuclease-inactivating agent was, therefore, largely a matter of trial and error. The problems of RNA contamination and degradation were however solved by adopting the RNA isolation method reported by Perry et al, (1972) and modified as described on page 68. This method involves the use of a phenol/chloroform mixture which prevents the interaction of specific proteins with certain regions of the polynucleotide (Perry et al,

1972) and ensures a good recovery of undegraded RNA. The RNA extraction was performed at pH 6.0 where the activity of the so-called alkaline ribonucleases were low, and polyvinyl sulphate was included in the tissue homogenisation medium as additional protection against ribonuclease degradation. The purity of the RNA isolated by this technique was high as indicated by the A_{260}/A_{280} ratio of 1.98 obtained with the isolated material. The sucrose gradient profile of the material did not show any significant RNA contamination by sodium dodecyl sulphate.

After it was established that prednisolone altered RNA metabolism in both rat liver and gastrocnemius muscle attention was next focussed on two enzyme systems which may be involved in the regulation of RNA turnover in these tissues. The effects of administration of prednisolone on the activities of RNA polymerases and ribonucleases were therefore investigated.

Alterations of RNA polymerase activity in rat liver by glucocorticoids have been widely reported (Barnabei et al, 1965; Jacob et al, 1969; Yu and Feigelson, 1971). It has also been demonstrated that, following administration of prednisolone, there was a specific and preferential reduction in the activity of the RNA polymerase responsible for ribosomal RNA synthesis in rat thymus cells (Drews, 1969; Drews and Wagner, 1970). Data obtained in the present work indicate that prednisolone treatment causes a decrease in the activity of Mg^{++} activated (but not the Mn^{++} activated) RNA polymerase in rat gastrocnemius muscle. In rat liver this enzymic activity was increased (Table 24). The preferential localisation of Mg^{++} activated RNA polymerase in the nucleolus (Maul and Hamilton,

1967) and reports that this enzyme is responsible for ribosomal-type RNA production (Widnell and Tata, 1966; Jacob et al, 1969) would suggest that the changes observed in the present study reflect some fundamental alterations in the ribosomal RNA synthetic machinery as a result of prednisolone administration. It is not clear whether prednisolone altered RNA polymerase activity by altering the DNA template or whether the steroid acted directly on the enzyme protein. Several possibilities exist as to how prednisolone may alter DNA template activity. These include displacement of chromatin-associated proteins, unwinding of DNA, facilitation (or impediment) of movement of RNA polymerase along the transcribable regions of DNA or a combination of these changes. Unfortunately, these in vivo processes could not be investigated in the in vitro studies undertaken in the present work. Factors which control RNA synthesis in vivo may have been removed from the system by the isolation procedures. Moreover, the osmotic or mechanical disruption of nuclei during such isolation procedures may have further complicated the picture (Guzak and Anderson, 1976). RNA polymerase activity in the nucleus can be modulated by changing the ionic environment (Widnell and Tata, 1964 and 1966) and it has been shown in rat liver that nucleolar RNA polymerase activity is very dependent on ions (Grummt and Lindigkeit, 1973; Laval et al, 1976). It is thus possible that prednisolone treatment altered nucleolar RNA polymerase activity by causing alterations in the nuclear ionic environment. It is not certain how this could be achieved but Laval et al (1976) have suggested that in a high ionic environment there is a marked alteration of the ultrastructure of

rat liver nucleolus which results in a stimulation of nucleolar RNA polymerase activity.

Prednisolone may have produced the observed changes in RNA polymerase activities by altering the concentration of the enzyme in the nucleus. It has been demonstrated that the increased activity of RNA polymerase observed in the liver of carcinogen-treated rats is due to an increase in the amount of enzyme in the nucleus (Leonard and Jacob, 1977). A study by Nakagawa and White (1971) indicated that the decreased RNA polymerase activity in rat thymic nuclei exposed to cortisol was the result of an alteration in the enzyme itself. These data would appear to rule out the DNA template as the prime site of action of the steroid. However, the demonstration by the same workers (Nakagawa and White, 1970) that the degree of the cortisol effect was different in isolated intact nuclei and in aggregate enzyme preparations suggested that the action of the steroid may not be explicable solely on the basis of alteration of the enzyme concentration.

In view of the different effects which prednisolone treatment had on RNA polymerase activities in rat liver and muscle, it is difficult to visualise an identical mode of action of this steroid in the two tissues. Some effect of prednisolone which precedes the change causing alterations in RNA polymerase activity may explain the tissue-specificity of the steroid. One such event may be at the level of the steroid receptor system. It is conceivable that prednisolone-receptor complexes in the rat liver and gastrocnemius muscle are different in some way and therefore affect different portions of the DNA template. More light will be shed on this

subject with a better understanding of the regulation of RNA synthesis in eukaryotes. All that can be said at the present time is that prednisolone would appear to affect rat liver and muscle ribosomal RNA at the transcriptional level through alterations of RNA polymerase activities.

An interest in ribonucleases in the present work arose from suggestions that these enzymes may play a role in the intracellular degradation of ribonucleic acids (Shortman, 1962; Kraft and Shortman, 1970). The present study showed that following administration of prednisolone the activities of acid and alkaline ribonucleases were depressed in rat gastrocnemius muscle and liver (Tables 26 and 27 respectively). The decrease in ribonuclease activity observed in the liver of prednisolone-treated animals is in keeping with the increased level of RNA observed in this tissue. Barnabei and Ottolenghi (1968), in a study of the response of rat liver to cortisone also observed an inverse relationship between RNA levels and ribonuclease activity. The changes observed in the present work in the activities of acid ribonuclease were unexpected since this enzyme is usually associated with RNA digestion in cell death or lysis (De Duve et al, 1955) and not with regulation of cellular RNA levels. However the activities of ribonucleases are poorly understood at the present time and it is possible that acid ribonucleases are involved in the regulation of RNA levels in the cell. In this regard it may be significant that the activity of acid ribonuclease in rat liver, thymus, heart and brain was observed to increase whenever RNA levels were high (Shortman, 1962; Kraft and Shortman, 1970). The results of investigations of 'latent'

ribonuclease activities in the liver may explain how prednisolone reduces ribonuclease activities in this tissue. If prednisolone reduced ribonuclease activity by increasing ribonuclease inhibitor activity, this reduction would be accounted for by increased 'latent' ribonuclease activity. In this work however, 'latent' ribonuclease activity decreased as a result of prednisolone administration. It would appear therefore that prednisolone regulated ribonuclease activity directly rather than at the level of the ribonuclease inhibitor. The steroid may exert this control by reducing the synthesis or increasing the destruction of these enzymes. It has been suggested that liver ribonucleases may have a pancreatic origin (Bartholeyns et al, 1975; Bartholeyns and Baudhuin, 1977). Thus the observed prednisolone effects on hepatic ribonucleases might reflect the effect of the steroid on these enzymes in the pancreas. It was found, however, that pancreatic ribonuclease activities were not altered by prednisolone treatment (Table 28). It would appear therefore that the steroid had a direct effect on liver ribonucleases and that the pancreatic enzyme may be quite different as the immunological studies of the enzymes from the two tissues had suggested (Zan-Kowalczewska et al, 1974).

In the gastrocnemius muscle, the administration of prednisolone caused a depression of ribonuclease activity. The lower 'latent' ribonuclease activity observed in this tissue suggests that a lowering of the amount of ribonuclease inhibitor accompanied the reduction in ribonuclease activity. This reduction in ribonuclease activity may be an attempt to maintain normal levels of RNA in gastrocnemius muscle following prednisolone treatment.

In summary it appears that RNA polymerase and ribonucleases play a role in bringing about changes in RNA metabolism in rat liver and gastrocnemius muscle following administration of prednisolone. In the liver of prednisolone-treated animals, the observed changes in the activities of the two enzyme systems could explain both the increased synthesis and the reduced degradation of RNA indicated by studies of turnover of RNA in this tissue carried out earlier in the present work. In the gastrocnemius muscle, the lowered activity of RNA polymerases would explain the fall in RNA levels following prednisolone treatment. The decreased activity of ribonucleases in this tissue may reflect an attempt to restore to normal the depleting levels of RNA resulting from decreased RNA synthesis in the muscle of prednisolone-treated animals.

An investigation of prednisolone-receptor complexes in the cytosol of rat liver and gastrocnemius muscle was carried out in an attempt to explain the different responses which prednisolone elicited in the two tissues. The formation of a steroid-cytosol receptor complex is essential for transporting the steroid to the nucleus and for its subsequent effect on nuclear transcription (Raspe, 1971). Responses to steroids may be regulated by changes in the intracellular concentration of the steroid receptor (Kirkpatrick et al, 1972; Rosenau et al, 1972; Cake et al, 1973; Lippman et al, 1973; Chomczynski and Zwierzchowski, 1971) and differences in the amount of steroid-receptor complexes which are formed in target tissues may therefore be sufficient to determine the response of each target tissue to the steroid. In the present

work, it was observed that the amount of prednisolone bound to receptors in the rat liver cytosol was more than twice the quantity bound in gastrocnemius muscle cytosol of the same protein concentration. This finding would suggest that there are fewer prednisolone-binding sites in the muscle than exist in the liver. A similar conclusion was made by Roth (1974) with regard to dexamethasone. It is recognised that differences in quantities between steroid-receptor complexes in cytosols from different tissues do not necessarily imply the same variation in the amount of receptors for the steroid in the cell as a whole. The differences observed in the present study could reflect, for example, differences in distribution of prednisolone receptors between the cytoplasm and the nucleus of the tissues investigated. However, it is generally accepted that it is the cytoplasmic receptor that is transformed and translocated to the nucleus (Munck et al, 1972; Litwack et al, 1973; Kondo et al, 1975). The binding to the cytoplasmic receptor enhances the nuclear binding of the hormone (Abraham and Sekeris, 1973) and changes in the capacity of cytoplasmic receptors to bind a hormone closely resemble changes in the nuclear binding capacity (Lipp and Van der Muelen, 1977). The quantity of cytoplasmic prednisolone-receptor complexes formed in the rat liver and gastrocnemius muscle is therefore a relevant consideration in the present study and may be significant in the different responses observed in the two tissues. Tissue-specific differences in the biological response elicited by a steroid may also result from qualitative differences in the steroid-receptor complexes formed in each tissue. Such differences

may be reflected in the sedimentation properties of the complexes in a sucrose gradient, their elution profiles on an ion-exchange column or in their stability in particular ionic environments and may affect the capacity of these complexes for transfer to the nucleus (Bird et al, 1976) or their ability to survive cellular degradative processes (Mayer et al, 1974). In the present study no significant differences in the ionic nature of prednisolone-receptor complexes in rat liver and gastrocnemius muscle were observed from their behaviour on ion-exchange chromatography. The complexes from each tissue behaved like single proteins when analysed by sucrose gradient centrifugation. The detection of two peaks of radioactivity by ion-exchange chromatography would seem to contradict the view that there was one class of prednisolone receptors in the cytosol of rat liver and muscle. However the appearance of more than one peak in an ionic gradient elution profile has been explained by the gradual disaggregation of steroid-receptor complexes as the ionic strength of the eluent is increased (Lea, 1973). This might occur in the present study if some aggregation of prednisolone-receptor complexes took place in the low ionic environment in which the complexes were formed and if differently aggregated complexes were differently retained on the DE 52-cellulose column. Nicholas and Morishige (1978) have also reported the presence of only one class of dexamethasone receptors in the cytoplasm of bovine trachealis muscle. The sedimentation coefficients calculated for muscle and liver prednisolone receptors indicated that these receptors may be different proteins. The intergrity of prednisolone-receptor

complexes prepared from the cytosol of rat gastrocnemius muscle during sucrose gradient centrifugation appeared to depend on the presence of 0.3M KCl during both the preparation of the complexes and their centrifugation on the gradient. Liver receptor complexes on the other hand were readily detectable on the gradient in the absence of KCl. This observation suggests that the muscle prednisolone-receptor complexes were less stable than the liver receptor complexes in a low ionic environment. Glucocorticoid receptors depend for their stability on the intergrity of their sulphydryl groups (Mayer and Rosen, 1975; Rao et al, 1976; Granberg and Ballard, 1977) and there is evidence that rat liver contains high concentrations of non-protein sulphydryl groups (Granberg and Ballard, 1977). The liver may therefore maintain the sulphydryl groups of its prednisolone receptors at the expense of these non-protein sulphydryl groups and may thus acquire a greater stability than the receptors in the muscle.

In summary, the differences observed in the prednisolone-binding capacity of rat muscle and liver cytosols and in the stability of their prednisolone-receptor complexes in a low ionic environment may be important in determining the responses of each tissue to prednisolone. However, it is difficult without further work to relate these responses unequivocally to differences in the receptors in the two tissues.

It should be emphasized that consequent upon the administration of a glucocorticoid to an animal several responses occur in the endocrine system which make it difficult to establish precisely those effects attributable to the administered steroid alone. These

responses include a suppression of segments of the hypothalmic-pitiutary-adrenal axis; a stunting of growth consistent with antagonistic action to growth hormone; a partial suppression of the release mechanisms of both the thyroid and the melanocyte stimulation hormone; and a potentiating effect on the pressor activity of norepinephrine (David et al, 1970; Baxter and Forsham, 1972; Thompson and Lippman, 1974). The effects of the administration of prednisolone to rats may therefore have reflected an interaction of hormones as well as the individual action of prednisolone. This concept of a system of hormonal programming of cellular response is demonstrated in the severe hyperinsulinemia induced by the administration of glucocorticoids (Owen and Cahill, 1973; Curry and Bennett, 1973; Altszuler et al, 1974; Gunn et al, 1975; Millward et al, 1976). In the skeletal muscle insulin enhances protein synthesis, suppressing protein breakdown and amino-acid release (Felig and Wharen, 1974). There is also evidence that following the administration of glucocorticoids to rats the release of amino-acids from skeletal muscle into circulation was increased while the incorporation of amino-acids into the tissue was decreased (Manchester et al, 1959; Smith and Long, 1967). It is likely therefore that following the administration of prednisolone to rats there may exist in vivo a situation in which a potentially catabolic steroid (prednisolone) and a protein synthesis-enhancing hormone (insulin) are present simultaneously. It was demonstrated in the present work that in the gastrocnemius muscle the net result was decreased protein and RNA levels with the catabolic effects of prednisolone prevailing

over the effects of any secondary hyperinsulinemia. Tissue specificity appears to be an important factor in these responses and it has been shown that in cardiac muscle the net result was anabolic with insulin completely neutralising the effect of hydrocortisone on cardiac protein balance (Griffin and Widenthal, 1978). In the rat liver where prednisolone treatment resulted in higher RNA and protein levels, the induced insulin presumably exerts a co-operative effect by promoting conditions which enhance protein synthesis (Felig and Wharen, 1974; Reed and Grisham, 1975). Any potentially catabolic action of prednisolone in rat liver is thus offset by insulin and a new state of homeostasis, in favour of anabolism, is established in the tissue.

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