ABSTRACT

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The Effect of Cortisone on Hexosamine and Mucopolysaccharide Metabolism

Radioautographic studies indicated that the synthesis of sulfated mucopolysaccharides (MPS) in cartilage, skin, aorta and liver was decreased in rats treated with relatively large doses of cortisone. Chemical analyses showed that the hexosamine and hexuronic acid content of aorta and skin was decreased as was also the glucosamine content of liver in the treated animals.

The activity of fructose-6-p-D-glutamine amidotransferase was decreased in supernatants from the liver homogenates of rats treated with cortisone for three days or longer. Similarly, the amidotransferase activity was decreased on addition of the soluble hydrocortisone succinate to normal liver supernatants, although the two effects may have different mechanisms of action.

Studies with glucosamine-^{14}C indicated that cortisone treatment caused no additional block in the hexosamine pathway.

Thus, one way by which cortisone exerts its influence on MPS metabolism is by inhibiting the first step of the hexosamine biosynthetic pathway.

THE EFFECT OF CORTISONE ON

HEXOSAMINE AND MUCOPOLYSACCHARIDE METABOLISM

by

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PREFACE

The study described in this thesis is a continuation of a connective tissue study described in a thesis submitted in partial fulfilment of the requirements for the M.Sc. degree in the Department of Biochemistry of McGill University in the spring of 1966. The latter arose out of an investigation that had been in progress in the Department for several years under the direction of Professor O.F. Denstedt in the biochemical etiology of haemorrhagic vascular disease. A survey made by workers in the Department, with an eye open for biochemical alterations, revealed the prominent involvement of connective tissue in the blood vessels during haemorrhagic states.

The present writer undertook a study on the changes of connective tissue in vascular disease, particularly atherosclerosis, which demonstrated the importance of alterations in the metabolism of hexosamines. On completion of these investigations it was considered that a further study on the influence of anti-inflammatory steroids on the metabolism of hexosamines and mucopolysaccharides may lead towards a better understanding of the role of these substances in diseases of connective tissue. The author was privileged to continue this phase of the work as an 'out of residence graduate student' in Rockefeller University, New York, in association with Professor D. Dziewiatkowski. During the past year the study has been continued in McGill University, with special emphasis on how

cortisone influences the biosynthesis of compounds with hexosamine in their structure.

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INTRODUCTION

1. NOMENCLATURE.

The nomenclature of the carbohydrates containing amino-sugars is still in a state of flux. Names such as mucoprotein, mucolipid, mucosubstance, mucoid and others have been used in the past rather indiscriminately and the precise meaning has varied depending on the author. However, the term 'acid mucopolysaccharide' (MPS) has been used consistently to refer to long chain polymers, sulfated or not, containing alternating units of hexosamine and hexuronic acid. In one compound (see Table I) the hexuronic acid is replaced by a neutral sugar. More recently, Jeanloz (1) has proposed that the term 'acid mucopolysaccharide' be discarded in favour of the term 'glycosaminoglycan'. The following Table summarizes the composition of the main MPS (glycosaminoglycans).

TABLE I

Components of Some Intercellular Acid Mucopolysaccharides Produced by Connective Tissue Cells (2)

Polysaccharide	Hexosamine	Hexuronate	Hexose	Sulfate/Period* Ester	Amide
Hyaluronate	Glucosamine	Glucuronate	-	-	-
Chondroitin-4-Sulfate	Galactosamine	Glucuronate	-	1.0	-
Chondroitin-6-Sulfate	Galactosamine	Glucuronate	-	1.0	-
Dermatan-4-Sulfate	Galactosamine	Iduronate	-	1.0	-
Heparin	Glucosamine	Glucuronate	-	1.5	1.0
Heparitin Sulfate	Glucosamine	Glucuronate	-	0.5	0.5
Keratan Sulfate	Glucosamine	-	Galactose	1.0	-

* Sulfate groups per disaccharide unit

Two other terms are in common use. 'Glycoprotein' refers to proteins which contain neutral sugar and/or hexosamine and/or sialic acid either as single units or in relatively short chains. The term 'glycosaminolipids' refers to lipids which contain aminosugars. These terms are used in the present thesis.

In recent years workers have shown that in tissues the MPS (glycosaminoglycans) sometimes occur attached to protein through short carbohydrate chains. The linkages joining these short chains to protein and to the MPS are of a covalent nature. These complex molecules are referred to as 'protein-polysaccharide' (2, 3).

While the term glycosaminoglycans is more precise for referring to macromolecules which contain aminosugars and are unassociated with proteins, it is not entirely suitable when referring to 'protein-polysaccharide' complexes. In view of the fact that the nomenclature of these substances is not entirely settled, the author prefers to use the term mucopolysaccharide instead of glycosaminoglycans. The terminology for the individual MPS is as given in the foregoing Table.

2. BIOSYNTHESIS OF HEXOSAMINES AND THEIR DERIVATIVES.

(1) The Origin of the Hexosamine Carbon Atoms.

The evidence that the carbon skeleton of glucose is not broken during the formation of glucosamine was reported first by Becker and Day (4). These workers fed either ^{14}C-1-glucose or ^{14}C-1-glucosone to rats, isolated the glucosamine from the blood serum, and measured its radioactivity. They found that nearly all the radioactivity from both of the precursors was on the C-1 carbon of glucosamine and they concluded that the glucose carbon skeleton had been incorporated intact. They observed further that the specific activity of the isolated glucosamine derived from glucosone was higher than that derived from glucose, thus suggesting a more efficient utilization of glucosone and the possibility that it might be an intermediary in glucosamine biosynthesis. However, the validity of the latter suggestion is questionable. Glucosone is known to be a toxic substance (5) probably because of its competitive inhibition of the phosphorylation of glucose (6). Moreover, glucosone may interfere also with glucose transport, and it is known that the pool sizes of the two are widely different (7). For these reasons glucosone could not be considered with certainty to be involved in hexosamine biosynthesis.

Roseman, Dorfman and associates have published a series of comprehensive papers pertaining to the origin of the carbon skeleton of glucosamine and the incorporation of hexosamines into MPS (8-11). These studies with group-A haemolytic streptococcus concerned the incorporation of labelled precursors in the biosynthesis of hyaluronic acid. The following observations were among their findings and conclusions:

The specific activity of the carbon-1 of the aminosugars was found to be essentially the same as that of the glucose in the medium, thus indicating the unlikelihood of the involvement of another intermediary. Moreover, as the medium contained a large proportion of unlabelled amino acids they inferred that compounds, such as serine, do not play an important role in hexosamine biogenesis. Both glucosamine and N-acetylglucosamine could function as precursors of the glucosamine moiety in hyaluronic acid. In experiments with ^{14}C- and ^{15}N-labelled glucosamine they demonstrated that the aminosugar is incorporated into hyaluronic acid without first undergoing deamination. They established further that glucosone is not a precursor in hexosamine synthesis. As to the origin of the hexuronic acid and the acetate moieties, they found that hexuronic acid also is derived from glucose without randomization of the carbon chain, and that the acetyl radical in N-acetyl-glucosamine was derived from the acetate in the medium.

These studies have been confirmed for a number of mammalian tissues including rat liver (12), where the incorporation of glucose into the glucosamine moiety in liver glycoproteins was investigated.

(2) Role of Glutamine.

In 1953 Boström and Mansson (13) found that the addition of a liver homogenate, to a system containing slices of costal cartilage and $^{35}SO_4$, greatly stimulated the rate of incorporation of labelled sulfate into the chondroitin sulfate of the cartilage. The active principle was purified by fractional precipitation, and resolved by paper chromatography into several ninhydrin-positive spots, one of which contained the activity (14). The results of preliminary tests suggested that the compound appeared to be glutamine (15). In 1957 the factor was isolated in crystalline form and proven to be glutamine, on the basis of the melting point, x-ray diffraction pattern, elemental analysis and its activity in stimulating the ^{35}S-uptake by cartilage slices (16). Lowther and Rogers (17) investigated the problem from a different approach. They studied the source of the hexosamine nitrogen in the hyaluronic acid synthesized by Streptococci from ^{15}N-labelled substrates. The young cells were found to utilize the ammonium radical and L-glutamate, or glutamine alone, for the synthesis of hyaluronic acid. Aged cells, however, were found to have an absolute requirement for L-glutamine. With the younger cells, glutamine and ammonium-glutamate gave the same rate of glucosamine formation and were found to be utilized in equimolar proportions. The addition of the medium of methionine sulfoxide, which is known to inhibit glutamine synthesis from ammonium glutamate (18), was found to inhibit also the synthesis of hexosamine when ammonium glutamate is used as the substrate. It was apparent therefore that L-glutamine, rather than ammonium glutamate, is the direct precursor

source of the nitrogen in the hexosamine moiety of hyaluronic acid.

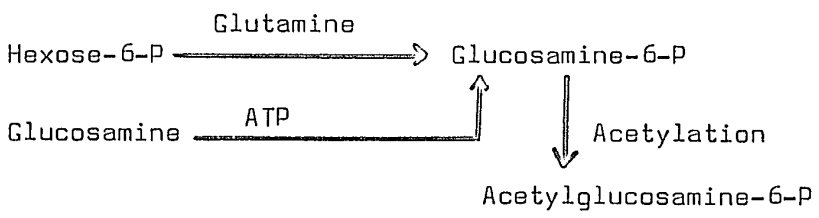
Lowther and Rogers (18) confirmed the findings by incubating young and old cells with $^{15}NH_4$ glutamate. They observed that in neither case was the ^{15}N-label in the glucosamine diluted out on addition of cold glutamate. Thus they established that, not the alpha-amino group of glutamic acid, but the amide nitrogen of glutamine is the source of the nitrogen in the glucosamine moiety.

(3) Biosynthesis of Glucosamine-6-phosphate.

(a) Early investigations.

An important early contribution in this field was the work of Leloir and Cardini (19) in 1953. With a cell-free and partially purified extract from the mycelium, Neurospora crassa, they demonstrated the biosynthesis of a hexosamine product from added glucose-6-P and glutamine, or with fructose-6-P in place of glucose-6-P. However, as the preparation contained phosphoglucoisomerase also, there was uncertainty as to the specificity of the enzyme involved in the synthesis. Furthermore, the enzyme proved to be very labile even when kept at 5°C for a few hours. The hexosamine product was tentatively identified as glucosamine-6-P.

Leloir and Cardini (19) studied also the phosphorylation of glucosamine and acetylation of glucosamine-6-P. They found that the extracts from Neurospora catalyzed the phosphorylation of glucosamine, thus confirming the results of the work of Harpur and Quastel with brain (20). However, while the Neurospora extract catalyzed the acetylation of both glucosamine and glucosamine-6-P in the presence of added CoA, ATP and Mg^{++}, it could not phosphorylate acetylglucosamine in the presence of added ATP and Mg^{++}. Leloir and Cardini concluded therefore that glucosamine-6-P is formed prior to the acetylation of the glucosamine. They propose the following scheme of reactions to account for these findings:



(b) Purification and specificity.

The earliest description of the biosynthesis of glucosamine-6-P in mammalian cells was reported by Pogell and Gryder in 1957 (21). These workers observed that only a small amount of Elson-Morgan positive chromogen was obtained on incubation of whole rat-liver homogenates with glucose-6-P and glutamine. However, if the homogenate, prepared with a medium containing 0.154 M KCl and 0.001M sodium ethylenediamine tetra-acetate, pH 7, was centrifuged at 18,000 x g for 90 minutes to remove particulate matter, the supernatant fraction proved to be much more active than the whole homogenate in its capacity to synthesize hexosamines. The particulate fractions were devoid of this capacity. The enzyme proved to be very unstable, and completely lost activity during dialysis over-night at 4°C, or even on storage at -20°C. Purification leading to a three-fold increase in activity was achieved by removing the material precipitable with 1.7 - 2.3 M ammonium sulfate from the 18,000 x g fraction. This material was relatively stable at 0°C and could be dialyzed prior to use. The pH optimum of the enzyme system was between 7.4 and 8.0 with the 18,000 x g supernatant fraction, and 7.5 with the ammonium sulfate precipitable fraction.

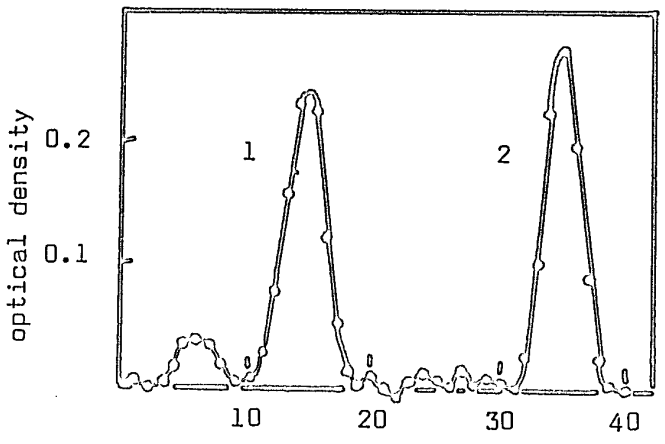
Experiments to determine the specificity of the hexose-6-P substrate showed that glucose-6-P was more efficiently utilized than fructose-6-P with the more highly purified fractions. For example, the ratio of the concentration of hexosamine formed by the 18,000 x g supernatant from fructose-6-P to that from glucose-6-P was 0.88. The ratio with the dialyzed ammonium sulfate precipitated fraction was 0.70 and with a dialyzed and lyophilized ammonium sulfate fraction, 0,57.

Study of the protective influence of the presence of substrate on the stability of the enzyme appeared to support the view that glucose-6-P was the superior substrate. When prepared in the presence of this substance the enzyme proved to be more stable to heat treatment than when prepared in the presence of fructose-6-P.

Identification of the hexosamine product was carried out with the aid of ion exchange chromatography. The product was treated with wheat-germ acid phosphotase, and the resulting preparation chromatographed on a Dowex-50 column (H^+). Gradient elution was carried out with an increasing gradient of H^+. The results obtained by Pogell and Gryder are illustrated in the following figure for purpose of comparison.

Figure 1

Ion Exchange Chromatography of Hexosamine Product (21)
(After hydrolysis with wheat germ acid phosphatase)



Peak No.1 in the figure corresponded to glucosamine-6-P, and peak No.2, to glucosamine. The small peak to the left probably represents hexose-6-phosphate. The identity of the compound represented by peak No.2 as glucosamine, was further substantiated with the aid of paper chromatography with a 3-solvent system. Thus it was established that the hexosamine product formed by rat-liver fractions from glucose-6-P and glutamine is glucosamine-6-P. The authors referred to the enzyme which catalyzes this reaction as aminotransferase.

Gryder and Pogell (22) achieved a 25-fold purification of the aminotransferase with a procedure involving alcohol fractionation and ammonium sulfate precipitation. Some of their fractions gave a higher activity with glucose-6-P, while other fractions were more active with fructose-6-phosphate. The latter fractions yielded the highest overall activity. The authors suggest that two enzymes may be involved, one requiring glucose-6-P and the other, fructose-6-P as the substrate.

Further study of the protective action of aminotransferase by glucose-6-P revealed that when the liver was frozen before homogenization only small traces of aminotransferase activity persisted in the 18,000 x g supernatant fraction. They surmised that an 'inactivator' substance may be released on freezing of the preparation. On testing the effect of addition of various subcellular liver fractions to a purified aminotransferase preparation (i.e. decreased formation of hexosamine product from glucose-6-P and glutamine) they found that addition of the 2,000 x g residue (nuclear plus mitochondrial elements), or the 18,000 x g residue (microsomal elements) caused inactivation. However, only with the former fraction was inactivation prevented by the addition

of glucose-6-P. No inactivation occurred if the aminotransferase was preincubated with the 18,000 x g supernatant. The authors do not provide an explanation for this inactivation. This phenomenon will be referred to later in the 'Discussion' section of this thesis.

The problem of specificity of the enzyme responsible for glucosamine-6-P synthesis was further investigated by Roseman and his associates (23). These workers purified the enzyme from Neurospora crassa, Escherichia coli and from rat liver. The rat liver enzyme was prepared from the 18,000 x g supernatant fraction (Fraction I) from a homogenate in 0.125 M KCl, 0.004 M EDTA and mercaptoethanol. On further fractionation, active fractions were obtained as follows:

Fraction II: The supernatant after precipitation with polymyxin sulfate;

Fraction IV: after absorption and elution from DEAE cellulose;

Fraction V: after dialysis against 0.005 M potassium phosphate, EDTA and mercaptoethanol;

Fraction VII: after absorption and elution from a calcium phosphate gel;

Fraction VIII: on dialysis as with Fraction V.

Each of the fractions was assayed with fructose-6-P, glucose-6-P and mannose-6-P as the substrate, with the results indicated in Table II.

TABLE II

Purification of Aminotransferase from Rat Liver (23)

Fraction	Total activity	Specific activity with*		
		Fructose-6-P	Glucose-6-P	Mannose-6-P
	units	units/mg	units/mg	units/mg
I	20.2	0.023	0.023	0.018
II	20.2	0.032	0.032	0.019
IV	11.4	0.44	0.00	0.040
VII	5.4	4.7	0.00	0.00
VIII	1.5	2.1		

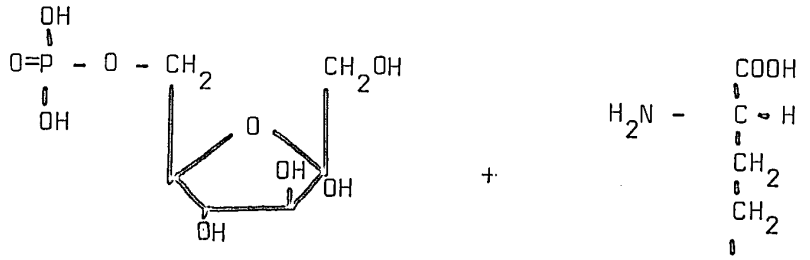
* Fractions which were inactive with fructose-6-P showed no activity either with glucose-6-P or mannose-6-P.

It is of interest that with increasing purification, activity was obtained only with fructose-6-P as the substrate. Comparable results were obtained with various fractions on purification of the enzyme from N.crassa and E. coli. The authors call the enzyme L-glutamine-D-fructose-6-phosphate transamidase. The K_m values for the rat-liver enzyme were respectively 1.6×10^{-3} M for glutamine and 3.8×10^{-4} M for fructose-6-P. The activity of the enzyme was inhibited by 6-diazo-5-oxo-L-norleucin (DON). It is noteworthy that even the purest fractions of the rat-liver enzyme

were unstable. The activity was reduced to half on storing for 24 hours at -18° or 0°C, and was completely lost at 48 hours. The addition of glucose-6-P, fructose-6-P, glutamine or a variety of other substances had no stabilizing influence on the enzyme.

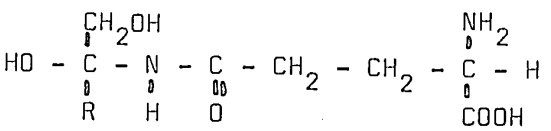
(c) Mechanism of action.

The precise mechanism of action of the transamidation of a hexose-6-phosphate to yield glucosamine-6-P has not as yet been elucidated. The possibility still exists that the reaction *in vivo*, is catalyzed by more than one enzyme. The instability of the enzyme even in the purest fractions complicates the problem of specificity studies. The following two-stage mechanism of the transamidase-catalyzed reaction with fructose-6-P as the substrate was proposed by E.A. Davidson (24): First, addition of the glutaminyl residue to C2 of fructose-6-P.



Fructose-6-P

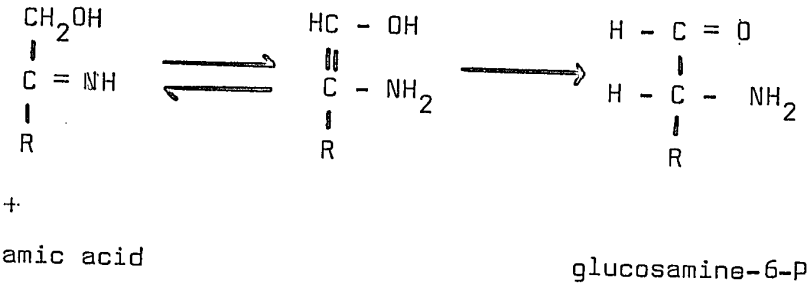
glutamine



Where 'R' represents the rest of the fructose-6-P molecule (from C3 on).

Cleavage of the amide bond of glutamine would be irreversible as there is insufficient energy in the bond between the C2 of fructose-6-P and the nitrogen to permit resynthesis of glutamine.

Secondly, formation of an intermediary imine followed by a tautomerization:



glutamic acid

glucosamine-6-P

The above reaction is presumed to involve an isomerase type of mechanism. One of the hydrogens in C-1 of the fructose-6-phosphate would ultimately appear at the C-2 of the glucosamine-6-P. This could be determined by the use of an appropriately labelled tritium substrate. Davidson (24) suggests that the energy for the overall conversion might be derived from the formation of a carbonyl group at C-1, as well as the amide bond of glutamine.

Note on nomenclature:

The name adopted recently for the enzyme which catalyzes the synthesis of glucosamine-6-P from fructose-6-P and glutamine, is L-glutamine D-fructose-6-phosphate amidotransferase (E.C.2.6.1.16). However, the problem of the hexose-6-P specificity in crude or partially purified preparations has not been entirely resolved. For the sake of convenience,

this enzyme will be referred to as 'amidotransferase' from hereon, except where otherwise noted.

(d) Factors which control the activity of the amidotransferase.

(i) Induction and repression.

Pasternak and Clark (25) obtained a preparation with amidotransferase activity from Bacillus subtilis. They observed that when the micro-organism was grown in the presence of exogenous aminosugar the activity of amidotransferase decreased presumably due to decreased synthesis of the enzyme. In subsequent studies (26) they separated the amidotransferase (activity) from glucosamine-6-P deaminase (activity) by fractionation with protamine sulfate. When the micro-organism was grown in the presence of an aminosugar the induction of the glucosamine-6-P deaminase was accompanied by a repression of the amidotransferase. Furthermore, the induction of glucosamine-6-P deaminase is repressed when glucose is added to the medium.

(ii) Feedback control.

The main pathway for the biosynthesis of the active precursors for mucopolysaccharide and glycoprotein synthesis in mammals is believed to be as follows:

Fructose-6-P (glucose-6-P) + glutamine →

Glucosamine-6-P → N-acetylglucosamine-6-P → N-acetylglucosamine-1-P

→ UDP-N-acetylglucosamine. (See page 22).

Kornfeld et al. (27) showed that when ^{14}C-labelled glucosamine was administered to normal rats as a marker, both the total radioactivity and the specific activity of the labelled UDP-N-acetylhexosamine derivatives increased for about 15 minutes and then declined rapidly. If puromycin was administered to the animals the total counts in the sugar-nucleotide pool remained high, thus suggesting diminished utilization of the UDP-N-acetylhexosamines. The specific activity remained high also throughout the two-and-a-half hour experimental period. As the turnover of the sugar-nucleotide is of the order of two hours, this observation would indicate that the rate of synthesis of the UDP-N-acetylhexosamines was diminished. Presumably, the decreased utilization of the sugar-nucleotides was due to insufficiency of glycoprotein acceptor molecules attributable to the inhibition of protein synthesis by the puromycin. However, when both ^{14}C-glycerol and ^{3}H-glucosamine were administered simultaneously to normal and to puromycin-treated animals the incorporation of the ^{14}C label into UDP-N-acetylhexosamines of the puromycin-treated animals was decreased, as was also the $^{14}C/^{3}H$ ratio of the incorporated labels. The authors suggest therefore that a step between the utilization of the ^{3}H-glycerol and the formation of glucosamine-6-P must have been inhibited. However, the possibility of an effect of puromycin on the glycerol incorporation into hexose-6-P, or the effect of dilution of that pool cannot be excluded. The ^{14}C-glucosamine would be

incorporated into sugar-nucleotides by bypassing this step. It was found further that when UDP-N-acetylglucosamine (2×10^{-5} M) was added to a preparation of the amidotransferase, the activity of this enzyme was suppressed. They postulated, therefore, that UDP-N-glucosamine may act as a feedback inhibitor in its own synthesis by suppressing the activity of the amidotransferase.

Recently, these studies (28) have been extended to the amidotransferase system in a number of species. It was found that while the enzyme from mammalian sources (rat liver and *He La* cells) is subject to feedback inhibition by UDP-N-acetylglucosamine, the enzyme from bacteria is not. The molecular weight of the amidotransferase from mammalian sources is about 340,000, while that of the enzyme from bacteria is approximately 100,000. The authors suggest that the mammalian enzyme molecule may represent an aggregate of several units. Kinetic studies on the rat liver enzyme have shown that the presence of UDP-N-acetylglucosamine increases the K_m for fructose-6-P without affecting the maximum velocity, and that it does not affect the K_m for L-glutamine.

Bates *et al.*(29) have studied also the control of the formation of UDP-N-acetylhexosamine in rat liver. They found that rats, fed a purified diet containing orotic acid, accumulated relatively large amounts of UDP-N-acetylhexosamine in the liver, as a result of increased synthesis, *de novo*. Apparently, under these conditions the feedback control of UDP-N-acetylglucosamine did not function. Administration of glucosamine by injection decreased the incorporation of labelled fructose into the glucosamine of UDP-N-acetylglucosamine. 'Duazomycin-A', (N-acetyl-6-diazo-5-oxo-L-norleucine), an analogue of glutamine, was found to inhibit the amido-

transferase-catalyzed reaction and to reduce the level of UDP-N-acetylhexosamine in the liver, and thus depress glycoprotein synthesis. Duazomycin-A,however, does not interfere with the incorporation of glucosamine into UDP-N-acetylglucosamine.

Other examples also of feedback inhibition involving sugar nucleotides have been reported. For example, CMP-sialic acid is inhibitory to UDP-N-acetylepimerase (27), d-TDP-L-rhamnose is inhibitory to d-TDP-D-glucose pyrophosphatase (30), and CDP-paratose to CDP-D-glucose pyrophosphorylase (31).

(iii) Influence of drugs.

Several of the anti-inflammatory drugs are known to influence the activity of amidotransferase. Gold sodium chloride, added to whole homogenates of liver and connective tissue, inhibits the activity of the enzyme (326). Similarly, injected gold thiomalate (in vivo) was found to produce inhibition of the enzyme in whole homogenates prepared from connective tissue but not that in the liver preparation. It was observed (32) further that high concentrations of salicylates (4.2×10^{-2} M) caused 50% and 60% inhibition of the transamidase of liver and connective tissue, respectively. Phenylbutazone, in a concentration of 2.6 mg/ml, similarly inhibited the enzyme. The degree of inhibition was greater with the enzyme in connective tissue preparations than with that in the liver preparations.

Jacobson and Borström (33) reported that the amidotransferase in partially purified extracts from calf aortic and pulmonary valves was in-

hibited by a relatively high concentration of sodium salicylate (e.g. 5 µmoles salicylate in 2.93 ml incubation mixture caused 46% inhibition). Similar results were obtained with fetal calf cartilage. p-Hydroxybenzoic acid, an isomer of sodium salicylate, is relatively less inhibitory. Chloroquine diphosphate likewise is inhibitory only in high concentration (e.g. 50 µmoles in 3.2 ml incubation mixture caused 58% inhibition).

The amidotransferase activity has been found to be decreased in extracts from epiphyseal plates of animals treated with lathyrogenic factors, such as β-aminoproprionitrite and aminoacetonitrite (34). However, when these substances are added to cell free systems they are without inhibitory effect on amidotransferase.

The influence of corticosteroids on the activity of amidotransferase is discussed on page 70.

(4) Other Intermediaries in the Biosynthesis of Phosphorylated Hexosamines.

The biosynthesis of the hexosamines in glycoprotein and MPS of mammalian tissues proceeds largely from glucosamine-6-P through a number of steps, involving phosphorylated intermediates, to the UDP-N-acetylhexosamine derivatives which, in turn, are considered to be active precursors of polymer synthesis. As this topic has been the subject of several reviews, only a general review of the literature relevant to the author's work will be given here.

(a) Pathways of synthesis.

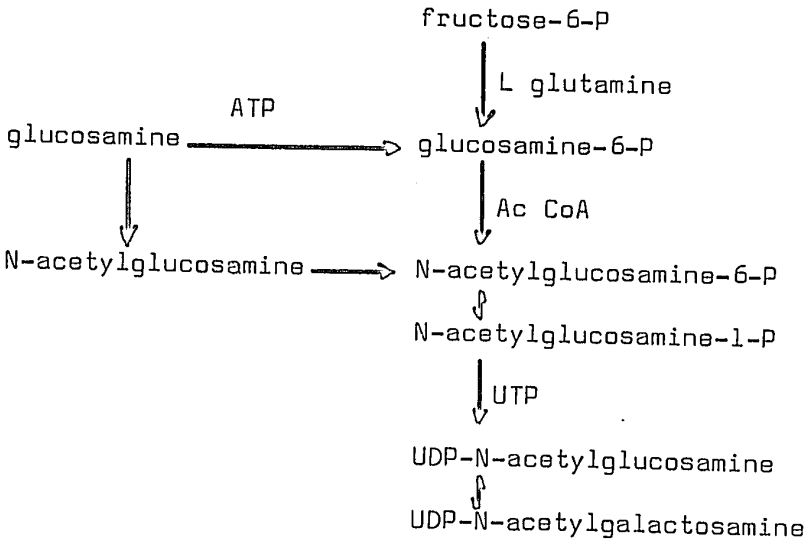
The initial finding of Leloir and Cardini (19) that glucosamine-6-P can be acetylated to N-acetylglucosamine-6-P has now been confirmed for a number of tissues (35, 36). The acetyl donor is acetyl coenzyme A (37). Davidson, Blumenthal and Roseman (38), in 1957, obtained two fractions of liver with the capacity to acetylate hexosamines, but with different specificities. One fraction could acetylate free glucosamine and aromatic amines, while the other fraction was specific for the acetylation of glucosamine-6-P. Several authors have reported also the observation of phosphorylation of N-acetylhexosamines in the presence of ATP (39). There is evidence that glucosamine can be phosphorylated directly (20, 40) prior to acetylation. A specific kinase for this reaction has been found to be present in Schistosoma mansoni (41). The kinase in the mammalian system appears to be less specific as to substrate since it has

been shown that both glucosamine and glucose can compete for the glucokinase. The particular function of these kinases is not well understood. Free acetylhexosamines or hexosamines may be derived from the catabolism of MPS or glycoproteins, or, they may be absorbed in small amounts from the diet. However, there is little doubt that the major pathway of hexosamine synthesis arises through the intermediary of the hexose-6-phosphates (42).

The carbon skeleton of N-acetylglucosamine-6-P can therefore be derived from at least two sources. Phosphorylated hexose intermediates can contribute to it by way of the amidotransferase catalyzed reaction, or it may arise from endogenous or exogenous glucosamine, or in two ways as indicated in Figure 2.

Figure 2

Some Pathways of Synthesis of Phosphorylated Hexosamine Intermediaries in Mammalian Systems



A number of mutases are known (43) which can catalyze the interconversion of N-acetylglucosamine-6-P and N-acetylglucosamine-1-P. Studies with partially purified preparations of some of these enzymes (44) have indicated a requirement of the mutases for either glucose-1, 6-diphosphate or glucosamine-1,6-diphosphate, thus suggesting that an intermediate phosphoenzyme complex is formed in the course of the reaction. In bacteria glucosamine-6-P can undergo epimerization to form other hexosamine-6-phosphates.

The conjugate, UDP-N-acetylglucosamine, was discovered and characterized by Cabib, Leloir and Cardini (45) in 1952. It has been shown that N-acetylglucosamine-1-P can be converted to UDP-N-acetylglucosamine in the presence of UTP by a number of tissues (46). UDP-N-acetylglucosamine can be converted to UDPN-acetylgalactosamine (47). These two amino-sugar nucleotides are considered to be the precursors of hexosamines in MPS and probably glycoproteins (see page 26).

Glucosamine-1-P can be converted also toUDP-glucosamine by extracts of yeast (46). The biological significance of this compound has not been elucidated.

(b) Inter-relationships of the phosphorylated hexosamine intermediates.

McGarahan and Malley (48) made use of glucosamine-1-^{14}C to study the inter-relationships of the phosphorylated hexosamine intermediates in rat liver. The radioactive glucosamine is used as a tracer, either in

in vivo or *in vitro* experiments, and acid soluble extracts are prepared and fractionated on Dowex-1-formate columns with an increasing concentration of the formate ion. In this manner, the various phosphorylated hexosamine intermediates have been separated. These workers found that the major products isolated after the intraportal injection of glucosamine-1-^{14}C were N-acetylglucosamine, N-acetylglucosamine-6-P and UDP-N-acetylhexosamines. Little or no radioactivity could be detected in the hexoses, hexosephosphates or UDP-glucose. However, the degree of phosphorylation of glucosamine to glucosamine-6-P was found to be very small. The authors offer the explanation that free glucose competitively inhibits this step. The K_i for glucose was found to be 1.1×10^{-4} M, while the K_m for glucosamine phosphorylation was 5.5×10^{-4} M. Thus one molecule of glucose would inhibit the phosphorylation of five molecules of glucosamine to the extent of about 50%. They suggest further that the rate-limiting steps in the metabolism of glucosamine in rat liver are the initial ones: the acetylation of glucosamine to N-acetylglucosamine and phosphorylation of the latter to glucosamine-6-phosphate. Since the glucose concentration in the liver is sufficient to inhibit the phosphorylation of glucosamine, the major pathway would appear to proceed by way of acetylation (see Figure 2, p.22).

Subsequent studies with perfused livers (49) confirmed these findings, and indicated that during a four-hour perfusion with glucosamine-1-^{14}C the ^{14}C-label was incorporated also into sialic acid and UDP-N-acetylglucosamine, and, to a lesser extent, into N-acetylglucosamine-6-P. With the non-mammalian system of *Aspergillus parasiticus* (50) nearly all

of the radioactivity incorporated into the phosphorylated hexosamine intermediates from labelled glucosamine, was found in the UDP-N-acetyl-glucosamine.

3. INCORPORATION OF HEXOSAMINE INTERMEDIATES INTO GLYCOPROTEINS AND MPS.

Some aspects of the incorporation of hexosamines into MPS are discussed in the section on Sulfation (p. 29).

Relatively little is known about the mechanism by which sugars are incorporated into glycoproteins. Three hypotheses have been proposed (51): First, oligosaccharide units may be incorporated in a stepwise manner at the end of a growing oligosaccharide chain. Second, an entire oligosaccharide chain is synthesized from sugar nucleotide precursors, prior to incorporation. Third, a combination of these events may occur, so that the 'inner' monosaccharides are first attached to the peptide and,subsequently, short oligosaccharide fragments are added which have, in turn, been synthesized from the nucleotide precursors.

The liver is the major site of glycoprotein synthesis (52-54). It is now clear that the primary subcellular fraction of liver cells responsible for hexosamine incorporation into glycoproteins is the one containing the microsomes (55, 56). Evidence, based on the kinetics of the incorporation of glucosamine-^{14}C into protein bound hexosamine of rat liver subcellular fractions, indicates that the hexosamine is incorporated into a growing polypeptide chain attached to the ribosomes (55, 57). More extensive studies (57) have suggested that the hexosamine is incorporated in the channels of both the rough and smooth surfaced endoplasmic reticulum, whereas sialic acid is incorporated primarily within the smooth surfaced endoplasmic reticulum.

Studies with electron microscope radioautography on exocrine cells would appear to be in agreement with the view that carbohydrate moieties are being added to a formed polypeptide(58, 59). The Golgi apparatus plays an important role in the incorporation of carbohydrate into glycoprotein, at least in the intestinal goblet cell (60-62). However, what proportion of hexosamine is incorporated into the rough and smooth endoplasmic reticulum as compared to the Golgi is not as yet resolved.

Recent structural studies on liver glycoprotein moieties, obtained from microsomal fractions of liver cells, have provided some interesting information. There is evidence (63, 64) that certain microsomal bound glycoproteins, which are considered to be precursors of the 'mature' glycoproteins, lack sialic acid at the carbohydrate chain terminals. On the other hand, glycoproteins isolated from plasma, in general have a sialic acid-galactose-N-acetylglucosamine sequence at the nonreducing ends, while hexosamine and mannose residues are in the inner core of the molecule (65). Very recently the isolation of small glycopeptides from rat liver microsomal fractions has been reported (66). These glycopeptides contained mannose and acetylglucosamine residues but lacked sialic acid. A sequence of these residues has been proposed (66).

The tentative concept that emerges from these studies is as follows: The polypeptide part of the glycoproteins is synthesized in the rough endoplasmic reticulum in association with the ribosomes. As the polypeptide moves along the rough and the smooth endoplasmic reticulum towards the Golgi apparatus hexosamine and mannose residues are attached first. Sialic acid is attached during the latter part of the process.

The glycoprotein finally traverses the Golgi apparatus and is secreted out of the cell. The precise role that the Golgi plays in glycoprotein synthesis may vary from one cell to another.

4. THE METABOLISM OF SULFATED MUCOPOLYSACCHARIDES.

The recent advances in knowledge of this field are covered in several reviews (67-70). The survey that follows pertains more particularly to the author's area of investigation which concerns the incorporation of $^{35}SO_4$ into the cartilage, skin, aorta and liver of the rat.

Historical.

Baurmann (71), as far back as 1876, surmised that inorganic sulfate is the precursor of the various ester sulfates in mammalian tissues. However, there was no way of resolving such questions until the 'isotope tracer technique' was introduced and $^{35}SO_4$ became available. In 1949, Dziewiatkowski (72) demonstrated that ^{35}S can be taken up by animal tissues. He showed that the isotopic 'label' was rapidly excreted in the urine and feces in the rat, and that, concomitantly, there was a rapid fall in the radioactivity in the liver and the blood. In the bone, on the contrary, the radioactivity continued to increase for about 8 hours, and in the bone marrow for about 24 hours after the administration. Later, he used $^{35}SO_4$ to study the conjugation and excretion of phenols in the rat (73). Part of the label was found to be excreted in the urine as sulfate, conjugated with the phenols. Furthermore, the specific activity of the various sulfate fractions in the urine - inorganic sulfate, total sulfate and ester sulfate - were closely similar, thus suggesting that the origin of the ester sulfate was indeed derived from exogenous sulfate. A number of mesenchymal and nonmesenchymal tissues were investigated by Layton (74), and Singher and Mari-

nelli (75), with respect to their ^{35}S uptake. They found that the uptake by cartilage, blood vessels, skin and bone marrow was very active, while that of the liver and muscle was relatively slower.

Proof that the administered ^{35}S-sulfate label was incorporated into sulfated MPS was obtained by Dziewiatkowski (76) in 1951, by the isolation of ^{35}S labelled chondroitin sulfate from epiphysial cartilage, and by Bostrom (77), in 1952, from rib cartilage. Four years later, Schiller et al. (78) demonstrated that ^{35}S, administered to rats, appears in the chondroitin sulfate of the skin. These workers demonstrated also that on administration of ^{14}C-glucose as a tracer the rate at which the ^{14}C appeared in the glucosamine and the uronic acid moieties of chondroitin sulfate was comparable to that of the appearance of ^{35}S into the chondroitin sulfate. Moreover, the concentration of these labels in the tissue was observed to decrease at about the same rate.

From the mid nineteen-fifties onwards, investigators in this field became interested in certain more or less well defined areas of research. Some groups studied the chemical and physical characteristics of the sulfated MPS from connective tissues with a view to gaining a better understanding of their structure, and of the nature of the associated proteins. Important advances were made also in studies on the sulfated MPS from cartilage. These advances are referred to in a later section (p. 35). Still other workers investigated the intermediary metabolism of the sulfated MPS in a variety of connective tissues, with the aid of $^{35}SO_4$. The labelled MPS were either isolated or studied radioautographically. The discovery of 'active sulfate' by Lipmann's group in 1957, stimulated interest in the mechanism of sulfation and in the identity of the 'acceptor' molecules.

(1) Activation of Sulfate.

In 1953, De Meio (79) demonstrated that ATP can serve as a source of energy for sulfate activation in cell-free systems. Three years later, Lipmann's group (80-83) found that the reaction between ATP and sulfate yielded pyrophosphate and a compound containing adenylic acid. The compound, first, was thought to be the 5'-adenosine phosphosulfate (APS) derivative. However, it was shown to contain two phosphate groups per adenine moiety, and that treatment with 3'-nucleotidase released one equivalent of phosphate. These results suggested that the active sulfate compound was adenosine-3'-phosphate-5'-phosphosulfate (PAPS). The structure, later, was proven by synthesis (84). The sequence of reactions leading to formation of PAPS would then be (85):

(1) $ATP + SO_4 \xrightarrow{\text{Sulfurylase}} APS + PP_i$

(2) $APS + ATP \xrightarrow{\text{APS-kinase}} PAPS + ADP + H^+$

(2) Sulfate Acceptors.

Although PAPS is unquestionably the donor of the sulfate, there is still some uncertainty as to the exact point where 'active sulfate' enters the biosynthesis of sulfated MPS. There are two views that can be considered. Either sulfation precedes MPS chain synthesis, or the sulfate is incorporated after the polymer formation occurs. Davidson and Meyer (86) initially suggested that polymerization precedes sulfation. They based their opinion on an examination of the isolated sulfated MPS from cornea. The analyses indicated either the absence of sulfate or the presence of one residue per disaccharide unit. They suggested that the partially sulfated molecules may represent acceptors of sulfate. However, Strominger (87) isolated a sulfate-containing uridine nucleotide, UDP-N-acetylgalactosamine sulfate, and suggested that this or similar nucleotides might be the precursors of sulfated macromolecules. Recently Härada et al.(88) isolated and purified an enzyme from the oviduct of the hen that was found to catalyze the transfer of sulfate from PAPS to position -6 of the N-acetylgalactosamine moiety of UDP-N-acetylgalactosamine-4-sulfate. However, it has not been possible to prove a direct role for sulfated nucleotide in the synthesis of sulfated MPS (89).

It is now known that a wide variety of long-chain MPS can serve as sulfate acceptors. Delbruck and Lipmann (90) found that a purified enzyme preparation from embryonic cartilage could transfer sulfate from PAPS to chondroitin-4-sulfate, dermatan sulfate and chondroitin-6-sulfate, or to the corresponding desulfated compounds, but not to hyaluronic acid, heparin or keratan sulfate. Similar results were obtained by Adams (91, 92),

except that he did not obtain sulfate incorporation with chondroitin. Suzuki and Strominger (93, 94) studied chondroitin sulfate and a variety of other long-chain sulfated MPS which were found to incorporate $^{35}SO_4$ from labelled PAPS. However, certain MPS such as hyaluronic acid, keratan sulfate and heparin were found to be inert. With active acceptors a relationship between chain length and velocity of sulfate transfer was established. In general, the larger molecules were sulfated more rapidly than the smaller ones. However, nonsulfated trisaccharides and pentasaccharides (i.e., the ones that contained acetylgalactosamine at the nonreducing end) were sulfated as rapidly as chondroitin sulfate itself. These results were interpreted as indicating that sulfation occurred on the terminal N-acetylgalactosamine moiety of a growing MPS chain (95). Perlman, Tessler and Dorfman (96) were able to prepare a particulate fraction from chick embryo epiphyseal cartilage which could incorporate radioactivity from labelled UPD-N-acetylgalactosamine into high molecular weight polysaccharide. Such particulate fractions, however, could not incorporate radioactive sulfate. The supernatant solution obtained in the preparation of the particles, on the other hand, readily incorporated sulfate in the presence of ATP. The results were considered to indicate that polymerization precedes sulfation. Studies by Silbert (97) on heparin biosynthesis in the mouse mastocytoma have shown that radioactivity from UDP-N-glucosamine-^{14}C could be incorporated into a polysaccharide that behaved chromatographically as a nonsulfated precursor of heparin. Recent investigations have shown that the PAPS-synthesizing system of mastocytoma is located in soluble fractions (98). This appears to be true for other systems as well (99-102). However, the sulfate-transfer system apparently

is distributed in a postmicrosomal fraction (103). Silbert (104) has found that in the mastocytoma both the sulfate incorporation system and the sugar-nucleotide incorporation system are present in the same microsomal fraction. This investigator suggests therefore, that polysaccharide polymerization and sulfation in the cell take place in close proximity at least in the biosynthesis of heparin in the mastocytoma.

Recently, Meezan and Davidson (105) have demonstrated, with the chick embryo cartilage, the presence of a sulfating system, in the supernatant fractions, which lacked the capacity to incorporate sugar-nucleotides. The nonspecificity of the system was indicated by the fact that a variety of types of polysaccharides can act as acceptors. In an extension of the study (106) they fractionated the polysaccharide acceptors with Ecteola cellulose or Sephadex G-50, and found that while the most active acceptors were present in the least sulfated fractions there was no relationship between molecular size and ability to accept sulfate. These workers considered that their findings were consistent with the view that sulfation occurs on the preformed polysaccharide chain.

(3) The Structure and Metabolism of Sulfated MPS of Cartilage, Skin, Aorta and Liver.

(a) Cartilage.

(i) Chemical studies.

Krukenberg (107), in 1884, was the first to show that cartilage contains polysaccharide. In 1891 (108) it was found that the polysaccharide material, now known to be chondroitin sulfate, contained a hexosamine, glucuronate, sulfate and acetate. Levene and La Forge (109) showed that the hexosamine was not glucosamine and in 1946 (110) it was identified as galactosamine. Many of the older workers used relatively strong alkali for extracting the MPS from cartilage (111). In general, efforts were made to remove all traces of protein, which were regarded as contaminating material. Yields of chondroitin as high as 32% of the dry weight of the tissue were obtained (112). However, Partridge (113) and Schubert (114), with milder methods of extraction, obtained a product that consisted of chondroitin sulfate associated with protein. With the improved methods of extraction as used by Schubert's group (115) the yield of the product from cartilage was increased to as high as 40% of the dry weight. The purity of the product could not be improved by fractionation, and it appeared to be a homogenous substance on electrophoresis (116). It was called 'chondromucoprotein', and later, 'protein-polysaccharide' (P-P) (117). The protein moiety was found to contain no hydroxyproline and apparently it was not collagen (118). Similar compounds have been isolated from the nasal septum of the cow (119),

the trachea of the pig (120) and from the nasal septum of the horse (121). P-P can be separated into two fractions, by centrifugation at 100,000 x g, a 'light' component ('PP-L') and a 'heavy' component ('PP-H') (122). This separation was achieved first with bovine nasal cartilage, and later (119)with human costal cartilage. Partridge (124) showed that glucosamine and galactosamine were present in the PP-H, and Gregory and Roden (125) demonstrated that bovine nasal PP-L contained keratan sulfate in addition to chondroitin sulfate. Keratan sulfate has been shown to be present also in human costal cartilage (126). Both the PP-L and the PP-H from human costal cartilage (127) contain chondroitin sulfate and keratan sulfate in the approximate ratio of 5 : 1 . Recently, it has been shown that the chondroitin sulfate of human cartilage is a mixture of chondroitin sulfates-A and -C (128).

Investigations during the past two years have indicated that the structure of the protein-polysaccharide material from cartilage is complex. Schubert and his group (129) have divided bovine nasal septum PP-L into five distinct fractions (PP-L1 to PP-L5) according to sedimentation behaviour with high salt concentrations. The fractions differ in protein content but are very similar in their amino acid profile. Furthermore, it appears that the composition of PP-L varies according to the anatomical site from which it is taken, even within the individual tissue such as bone. For example, calf articular PP-L contains less protein than calf epiphysial plate PP-L. This holds also for the corresponding tissues from fetal calf tissues (130).

As yet little is known about the structure of the protein-sulfated mucopolysaccharides in cartilage. The question is further complicated by

the polydispersity of polysaccharide moieties (131. 132). One must be cautious, therefore, in evaluating fractionation procedures of these complex substances. Although the protein in native PP presumably has at least a basic unit structure, polydispersity of the polysaccharide moiety could influence the sedimentation and the solubility of the complex.

Still another factor that can complicate studies on structure, is the method of extraction. Even with the use of mild extraction procedures, acid proteases may be released from cartilage presumably from lysozomes (133) which can act specifically on PP-L and degrade it, as evidenced by the change in composition, viscosity and migrational behaviour in an electrical field (134). The relationship of lysozomal enzymes to MPS catabolism is discussed more fully in a later section (p.81).

Notwithstanding the difficulties in the interpretation of results of work on the structure of the protein-polysaccharides of cartilage, important advances have been made in the knowledge of the nature of the chemical binding between the protein and polysaccharide moieties. Evidence has accumulated in the past ten years (135-137) that protein and chondroitin sulfate are bound by other than salt linkages. From evidence obtained on digestion of the complex with papain, Muir (138) suggested that the polysaccharide is linked to serine in the protein. Partridge's group studied the degradation products after treatment of cartilage with papain or hyaluronidase and with alkali (139). They obtained evidence of the presence of keratan sulfate in the protein-rich portion of the degradation products. Rodin, Gregory and Laurent (140) isolated and characterized the

glycopeptides of the region involved in the linkage, after degradation of the material with testicular hyaluronidase and certain proteolytic enzymes. They obtained evidence that the chondroitin sulfate side chains of PP-L are joined to galactose by a glucuronidic linkage. The galactose, in turn, is joined to xylose, and the bond between the latter and the protein is a glycosidic linkage with the hydroxyl group of serine (141).

The covalent linkage between sulfated MPS and protein is not peculiar to cartilage. There is now evidence that such linkage may be of common occurrence. For example, it has been shown recently that heparin also occurs in the native state as a covalently bound complex with protein (142). Carbohydrate-serine complexes from heparin have been isolated (143) and characterized (144). Among the types of fragments known to be involved in the linkages are o-beta-xylopyranosyl-L-serine (144). These findings establish that heparin also is linked covalently to protein through a glycosidic bond between xylose and the hydroxyl of serine (145).

The main point that emerges from the above review is that the structure, and presumably the function, of many MPS are closely related to the covalently bound protein. In the subsequent discussion this fact is important to bear in mind when considering the manner by which various substances affect the metabolism of the sulfated mucopolysaccharides.

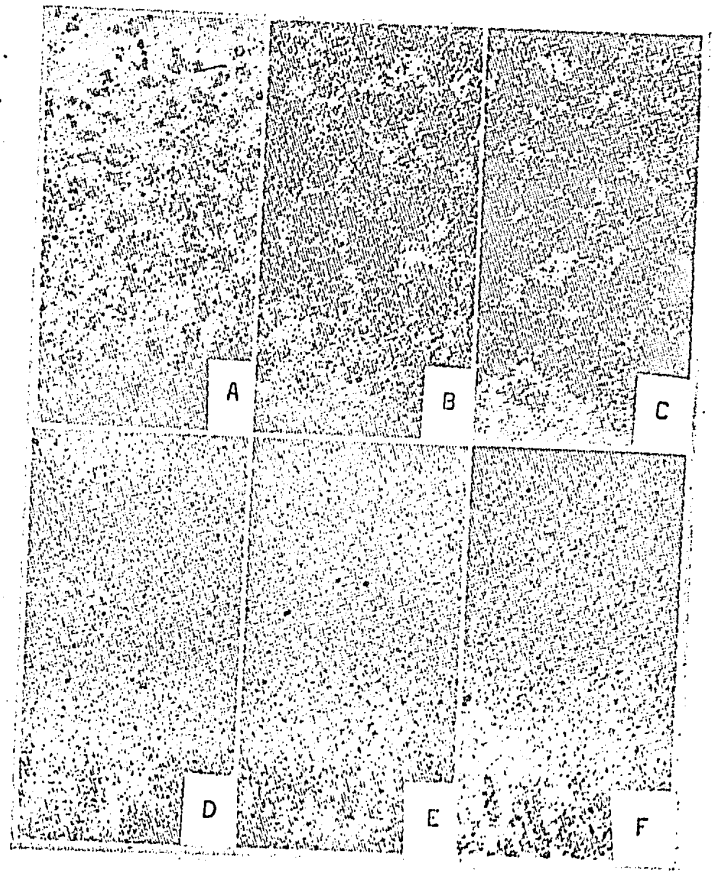
(ii) Metabolic studies.

Labelled $^{35}SO_4$ is taken up readily by cartilage cells and incorporated into chondroitin sulfate (76, 77). The half-life of ^{35}S in chondroitin sulfate is about 17 days (76). The uptake of $^{35}SO_4$ by fresh cartilage slices in vitro decreases with time (146) and is inhibited by oxygen lack, exposure to heat and to respiratory inhibitors such as dinitrophenol (147). The stimulatory effect of glutamine has already been discussed (p. 6). Labelled selenate is not accumulated by cartilage (148).

Information about the metabolism of sulfated MPS of cartilage has been obtained from radioautographic studies. Within a few minutes after injection of ^{35}S, the label is detectable first in the chondrocytes, next in both the chondrocytes and the matrix, and later in the matrix only (149, 150). Campo and Dziewiatkowski (151) demonstrated that the distribution of ^{35}S, as revealed by radioautography, does in fact represent ^{35}S incorporation into chondroitin sulfate. They studied the ^{35}S incorporation into epiphysial cartilage at various intervals up to 120 minutes and found that when the tissues were fixed with formalin saturated with barium hydroxide, instead of formalin alone, the ^{35}S label was practically completely removed, as is indicated in Figure 2 . Treatment with barium hydroxide dissolves the MPS but precipitates the inorganic sulfate. The validity of this experiment was further substantiated by staining corresponding cartilage sections metachromatically with toluidene blue and fixing them again either with 10% formalin or 10% formalin saturated with barium hydroxide. With the latter fixative all the stain in the cartilage

FIGURE 2

INCORPORATION OF ^{35}S-SULFATE INTO EPIPHYSEAL CARTILAGE (151)



A, B, C: Autoradiographs of sections from knee joints of young rats removed after the injection of 25 μC of ^{35}S-sulfate at 30, 60 and 120 minutes, respectively.

Fixation 10 per cent formalin.

D, E, F: Corresponding sections fixed with 10 per cent formalin saturated with barium hydroxide.

was removed with the exception of that from the nuclei of the cartilage cells.

In subsequent studies (152) the authors administered ^{14}C-L-phenylalamine, ^{14}C-L-leucine, ^{35}S-sulfate or $^{45}CaCl_2$ to young rats. Autoradiograms of sections of bones were prepared, and the ^{45}Ca removed from the sections by treatment with dilute acetic acid. Neither the concentration of ^{35}S nor that of ^{14}C was found to be greatly decreased. The ^{35}S could be removed from the demineralized sections by incubation in a medium containing testicular hyaluronidase. The ^{14}C was not disturbed. The authors interpreted these results to indicate that most of the ^{35}S was present in the chondroitin sulfate of the bone and that most of the ^{14}C was in the protein. These findings indicate that the ^{35}S label is indeed incorporated into the sulfated MPS and not into the protein or the inorganic sulfate, since the isotope is removed by the hyaluronidase and by barium hydroxide.

Gross, Mathews and Dorfman (153), in 1960, employed ^{35}S-sulfate and lysine-1-^{14}C and demonstrated that the protein and the chondroitin sulfate of the costal cartilage were metabolized together. Campo and Dziewiatkowski (154) confirmed and extended these findings by incubating cartilage slices with ^{14}C-leucine, ^{14}C-phenylalamine and ^{35}S-sulfate. Isolation and examination of the protein-polysaccharide complex indicated that both the protein and the sulfated polysaccharide were synthesized simultaneously in the cartilage cells. Robinson, Tessler and Dorfman (155), recently have shown that a cell-free preparation from embryonic chick cartilage catalyzes the incorporation of xylose-^{14}C and galactose-^{14}C, from the cor-

responding UDP-derivatives into the TCA-precipitable material. The authors isolated xylosyl-^{14}C-serine and galactose-^{14}C from the precipitate. They regard their results as indicative of the involvement of xylose and serine in the linkage region of chondroitin sulfate to the protein. The protein is distinct from collagen (117). Present evidence suggests that collagen and sulfated MPS are synthesized independently in cartilage (156). Thus, inhibition of collagen synthesis with α_1-α-dipyridyl does not prevent ^{14}C-glucosamine and $^{35}SO_4$ incorporation into the sulfated MPS. Likewise, inhibition of MPS synthesis with 6-diazo-5-oxonorleucine does not affect the biosynthesis of collagen.

The metabolism of ^{35}S in cartilage is reported to be influenced by a wide variety of conditions. Vitamin-C deficiency causes the epiphyseal cartilage matrix to disappear (157). Scorbutic guinea pigs were found to fix only one third as much radioactive sulfur in the cartilage as did the normal controls (158, 159). Vitamin-A deficiency causes decreased uptake of ^{35}S by rat femur or tibia (160). The deficient rats had also a higher serum level of sulfate than the controls. Both the abnormalities were reversed on the administration of vitamin-A (161). The effect of vitamin-A excess will be discussed in the section on lysozomes (p. 81). Vitamin-D deficiency does not seem to affect the rate of synthesis of chondroitin sulfate although rachitic rats tend to have a higher sulfate level than do normal animals (161). However, recent studies indicate (162) that administration of vitamin-D_3 to rachitic chicks increased the level of galactosamine and decreased the level of hydroxyproline and glycine in the epiphyseal cartilage.

A variety of hormones are known to influence sulfate metabolism.

The effect of growth hormone is well recognized even though the nature of 'sulfation factor' is not fully understood. The uptake of sulfate *in vitro* by costal cartilage from hypophysectomized rats is stimulated on the addition of serum from normal rats (163). However, serum from hypophysectomized rats was without effect, unless growth hormone was administered at least 6 hours before the serum was taken. Direct addition of growth hormone to the *in vitro* system was without effect on sulfation. The 'sulfation factor' is dependent upon amino acids, especially serine, for activity (164). The role of growth hormone is not clear, as administration of 100 μg daily for eight days is without effect on normal rats (165). It may be that growth hormone is either a precursor or a stimulator of sulfation factor formation.

Thyroid hormone also affects ^{35}S metabolism of cartilage. Triiodo-L-thyronine strongly accelerates the uptake of intraperitoneally injected sulfate into xyphoid cartilage (166). Thyroidectomy of rats causes a retarded development of the epiphyseal plate and thiouracil causes a reduction in sulfate deposition. The administration of thyroxin counteracts both of these changes (167, 168).

Insulin by itself has no effect on the *in vitro* uptake of sulfate by cartilage from hypophysectomized rats (169). However, insulin can act synergistically with a balanced mixture of amino acids to enhance $^{35}SO_4$ uptake.

A number of steroids have been reported to affect the metabolism of cartilage. Administration of estradiol benzoate to rats, in high doses, reduced the $^{35}SO_4$ uptake by cartilage and aorta, both *in vivo* and *in vitro* (170). As the same result was obtained in normal as well as hypophysectomized

rats, it was assumed that this effect of estradiol was not due to suppression of testosterone. Estradiol benzoate also reduces the specific activity of isolated MPS from bone, following administration of $^{35}SO_4$ or ^{14}C glucose (171). The effects of corticosteroids and ACTH are discussed in detail in a later section (p. 54).

(b) Skin.

(i) Chemical studies .

The chemistry of the MPS of the skin has been reviewed recently by Pearce (172). This section pertains more particularly to some aspects of sulfated MPS.

A number of sulfated MPS have been isolated from mammalian skin. Meyer and Chafer (173) in their earlier investigations isolated two MPS from pig skin. One of them closely corresponded to hyaluronic acid while the other, which resembled chondroitin sulfate from cartilage in chemical composition, differed in optical rotation and was also resistant to the action of hyaluronidase. These findings were confirmed by Pearce and Watson (174) and by Gardell et al. (175). Similar polysaccharides were isolated from calf, rat and rabbit skin (176-178). Even though different methods were used in the various isolations, it became clear that the sulfated polysaccharide of skin differed from that in cartilage. The former therefore was termed 'chondroitin sulfate-B' or 'dermatan sulfate'.

Meyer et al. (179) later modified their isolation procedure and separated the mucopolysaccharides from pig skin by precipitating them with ethanol as calcium salts. The presence of hyaluronic acid and dermatan sulfate was confirmed, but, in addition, a MPS with properties identical with those of chondroitin-6-sulfate was isolated. Further work (180) showed that, in addition to these three MPS, a fourth fraction was present which also was hyaluronidase resistant, but its specific rotation and hexuronic acid composition were different from that of dermatan sulfate.

More recently, Schiller and Dorfman (181) and Schiller, Slover and

Dorfman (182) have demonstrated the presence of heparin also in rat skin. It appears then that mammalian skin contains hyaluronic acid, and sulfated MPS, which include chondroitin-6-sulfate, dermatan sulfate and heparin. In addition, a fifth, as yet not fully characterized MPS may be present. Labelled sulfate, administered to animals, would presumably be incorporated into these sulfated MPS and could be useful for studying their metabolism.

(ii) Metabolic studies.

That $^{35}SO_4$ can be incorporated into the elements of the skin was first shown by Boström (183), then by Boström and Gardell (177), and confirmed by Belanger (184). The localization of ^{35}S in the skin was described more precisely by Montagna and Hill (185). They found that the maximal concentration of ^{35}S occurred at four hours in the ground substance of the papillary layer and to a lesser extent in the reticular layer. Active hair follicles accumulate large amounts of ^{35}S in the dermal papillae, coincident with the appearance of metachromatic substances. These authors noted also that most of the ^{35}S appears from the keratinized parts of the hair follicle about four hours after the administration.

The vitamins, known to affect the ^{35}S metabolism of skin MPS, are vitamins-C and -A. Friberg (186), in 1958, found that the ^{35}S uptake by the skin in scorbutic guinea pigs varies with the duration of the feeding of the scorbutogenic diet. While the skin in the animals that had received the diet for 10 days showed an increased ^{35}S uptake, compared to that of the controls, there was no difference in the uptake between the

two groups up to the seventeenth day. By the 23rd day the uptake in the skin of the scorbutic animals was depressed to less than half that of the controls. However, other workers have found (187) that there is a pronounced decrease in $^{35}SO_4$ uptake by scorbutic animals even after one week on the vitamin-C deficient diet. Depression of $^{35}SO_4$ uptake by granulomas of scorbutic animals has been demonstrated both radio-autographically and by estimation of the MPS (188).

A number of hormones are known to affect the metabolism of MPS of the skin. Rats made diabetic by administration of alloxan demonstrate diminished uptake of acetate-^{14}C into MPS of the skin (189). In these diabetic animals the pool size and turnover of hyaluronic acid are decreased. However, these changes are not as pronounced in the case of chondroitin sulfate. Insulin treatment restores the uptake of the ^{14}C and ^{35}S label towards normal values (190).

Hypophysectomy reduces the half-life of both hyaluronic acid and chondroitin sulfate in rat skin (191). Growth hormone administration to the hypophysectomized animals increases the turnover of chondroitin sulfate towards normal values, but has little effect on hyaluronic acid. Hypophysectomized rats demonstrate increased levels of hyaluronic acid and decreased levels of chondroitin sulfate in the skin. Growth hormone administration causes the levels of chondroitin sulfate to return towards normal, but further increases the abnormally high levels of hyaluronic acid. Schiller (191) suggests that growth hormone stimulates the synthesis of sulfated MPS of the skin, but decreases the breakdown of hyaluronic acid.

The effects of thyroid hormone and thyrotropin on the skin also have been studied. Thyroidectomy performed on one-day-old rats by ^{131}I resulted in a decreased ^{35}S uptake by the MPS of the skin (168). Administration of 5 µg thyroxin resulted in an increase of ^{35}S incorporation in the skin of the thyroidectomized animals. However, surgical thyroidectomy on the 28th day of life had little effect on the ^{35}S uptake of skin (168). Adult rats, made hypothyroid by thiouracil administration, demonstrate an increased ^{35}S incorporation into skin MPS (192). No apparent explanation is available for these discrepancies in findings. Schiller et al. (193) found that administration of propylthiouracil to 48-day-old rats for periods of approximately two, four or five months resulted in an increased concentration of hyaluronic acid, but a decreased concentration of chondroitin sulfate. The turnover of both hyaluronic acid and chondroitin sulfate was decreased in the skins of rats treated with propylthiouracil and these effects were reversed by the administration of thyroxin. These authors (193) felt that the above effects are independent of thyrotropic hormone as administration of two different preparations of thyrotropic hormone to intact rats were without any effect on MPS metabolism. However, other investigators (194) have found that the administration of thyrotropic preparations to dwarf mice increased the hexosamine content of their skins.

Sex steroid hormones also affect skin MPS. Testosterone proprionate causes an increase in the concentration of MPS in the skin of male rats (195) and testosterone administration causes an increase in the hyaluronic acid concentration in cockrels' combs (196). The acid MPS of the sex skin of monkeys accumulate during oestrus (197), and intravenous admin-

istration of Premarin increases the MPS content of the oval mucosa of hamsters, monkeys and humans (198). The effects of glucocorticoids are discussed in a subsequent section (p. 54).

(c) Blood vessel walls.

The composition and metabolism of the MPS of the arterial wall is the subject of several reviews (199-201). The author also has reviewed this topic (202), and therefore only aspects relevant to the present study will be considered here.

That the $^{35}SO_4$ uptake by aorta decreases with age in man (203, 204) and in animals (205) has been shown by incubation of aorta slices with the $^{35}SO_4$ label. The observation has been verified also, in vivo, by by administration of $^{35}SO_4$ to rats. In these studies (206) the half-life of the ^{35}S was found to be 4-5 days in young animals, while in adult animals it is of the order of two weeks (207).

In contradistinction to the diminished rate of sulfate metabolism with aging, Curan and Crane (208) demonstrated an increase in the $^{35}SO_4$ uptake in atherosclerotic areas of the human aorta, as compared to that of the control ('normal') areas. Their results were confirmed by Hauss et al. (204) who showed that the rate of incorporation of ^{35}S into atherosclerotic lesions of the aorta was increased in all the age groups studied, compared to that in the 'normal'areas. The same authors demonstrated that the observations hold also in rats with experimentally induced atherosclerosis. Buck (209) has obtained comparable results with rabbits that were fed added cholesterol in the diet. Increased ^{35}S uptake and increased intensity of metachromatic staining were observed in the aortas of rabbits that were fed a high cholesterol diet, compared to that in the control animals. Further studies (210) revealed that the specific activity of MPS isolated from the aorta of the cholesterol-fed

animals was twice that of the controls. However, the authors found no change in the electrophoretic pattern of the isolated MPS from the two groups. Stimulation of sulfate metabolism in the aorta by cholesterol has been confirmed also by Kowaleski (211) and by Forman et al. (212).

Sulfate incorporation into MPS can be stimulated also by a variety of conditions. Hauss and his coworkers (207) have shown that infection, intravenously administered pyrogens, exposure to whole body irradiation, and in allergic reactions, such as the Arthus and the Shwartzman phenomena (204), all tend to increase the sulfate incorporation into MPS isolated from the aorta, viscera, skin and cartilage of the rabbit. Because the mechanism of action differs with the various agents, and considering the variety of organs affected, the authors suggest that there exists a general, nonspecific mesenchymal reaction to a challenge.

The ^{35}S uptake by arterial tissue can be stimulated also by somewhat more specific means. For example, the small arteries and arterioles of the rat show greatly enhanced $^{35}SO_4$ uptake during experimental steroid hypertension (213). L-thyroxine, administered to normal rabbits, increases the sulfate uptake of the aorta (214). D-thyroxine has a similar effect on sulfate uptake but does not increase the hexosamine content to the degree L-thyroxine does (214). The action of administered adrenaline is similar to that of L-thyroxine and it enhances the action of thyroxine (214, 215). Vitamin-A deficiency increases the incorporation of labelled sulfate into the sulfated MPS of the rat aorta in vitro, but vitamin-A added to the aortic tissue in vitro does not reverse the effect (216). Curiously, large doses of vitamin-A (10-24 mg per day) do not reduce the

MPS content of the aorta of birds on a normal diet, nor that of cholesterol-fed rabbits (217). This observation is unexpected in view of the 'lysozome labilizing action' of vitamin-A (see p. 81) and the demonstrated presence of proteases and cathepsins in aorta (218). When animals are fed Lathyrus odoratus seeds, which contain β-aminopropionitrile and also the γ-glutamyl derivative (219, 220) an increase is noted in hexosamine, uronic acid (221) and metachromatic staining properties of the aorta (222). The epiphyseal cartilage of lathyritic animals, on the other hand, shows both a reduction in the $^{35}SO_4$ uptake and in the galactosamine concentration (224, 225). It appears, therefore, that the response of the MPS in the aorta may be different from that in other tissues during the administration of certain substances. The effects of steroid hormones and corticotropin are discussed in a later section (p. 54).

Thus it is apparent that a variety of substances and conditions can affect sulfate and MPS metabolism in arterial tissue. This, however, is perhaps not surprising when one considers that the turnover of the ester sulfate in connective tissue reflects the metabolism of macromolecular end products, whose rate of synthesis and breakdown, in turn, are influenced by a variety of homeostatic mechanisms.

(d) Liver.

Only two MPS have been isolated from liver and purified: heparin and heparàn sulfate. Heparin was discovered by McLean in 1916 (226). It was obtained from dog's liver, and since has been isolated from liver in a variety of species (227-229). The capsule of the liver contains a high concentration of mast cells (230) and thus has been used for isolation of heparin (231).

A compound similar to heparin but with low anticoagulant activity was isolated by Jorpes and Gardell (232) from beef liver. Meyer et al. (233) introduced the name 'heparan sulfate' for this substance when they discovered that the glucosamine residues in the molecule are acetylated. Cifonelli and Dorfman 234) demonstrated that heparàn sulfate exists in the various liver fractions with varying degrees of sulfation. They suggest that heparin sulfate may be an intermediary in the biosynthesis of heparin.

Relatively little is known about the biosynthesis of sulfated MPS in liver, as most of the work has been carried out with mastocytoma (p. 33.) The work of Lipmann and coworkers, who used rat liver fractions to study the formation of active sulfate, has already been discussed (p. 30). Incorporation of $^{35}SO_4$ into heparin has been demonstrated both in vivo (235) and in vitro (236).

Jaques (237) has shown that heparin is released from the liver of the dog in peptone shock, but that free heparin does not appear normally in the circulation (238). Anastassiades and Denstedt (239) found no free heparin in rat plasma from normal atherosclerotic animals, either in the absence or in the presence of arterial thrombosis.

5. EFFECT OF CORTISONE ON MPS AND HEXOSAMINE METABOLISM.

(1) General Effects of Cortisone on Connective Tissue.

The administration of cortisone to animals and man is known to produce a variety of effects on connective tissue. Clinicians in the late nineteen-forties, observed that the administration of corticosteroids or ACTH caused a dramatic improvement of symptoms in patients with rheumatoid arthritis. The first clinical report was published by Hench, Kendall and coworkers in 1949 (240). In the same year, Ragan et al. (241) published the first report on the effect of cortisone on the connective tissue of experimental animals. These investigators found that the administration of 12.5 mg of cortisone, twice daily, for 5 - 8 days to rabbits, caused a striking depression of new growth of all elements of the connective tissue in surgical wounds of the animals. Histochemically, there appeared to be less ground substance in the cortisone treated rabbits than in the controls, as determined with toluidene blue and Hale's stain. These workers noted also that 1 mg of cortisone, administered daily to 100 g rats, did not affect wound healing, and they suggested that this dosage was too low. A year later, Jones and Meyer (242) reported that local application of cortisone inhibited vasculization of the rabbit cornea, and Howes et al. (243) found that the tensile strength of healing wounds was reduced by cortisone. Subsequently, Layton (244), in 1951, studied the effect of sulfate uptake by a number of tissues,both in vivo and in vitro, after cortisone administration. He found that cortisone in vitro inhibited the uptake of inorganic sulf-

ate by heart and skeletal muscle, but had no effect on the migration of fibroblasts. This author, therefore, suggested that the main action of cortisone may be the inhibition of the synthesis of chondroitin sulfate in connective tissue.

Since 1950, a very large number of reports have appeared regarding the effects of cortisone on connective tissue elements. The observation of inhibition of granulation tissue has been amply confirmed (245-247). The fibroblasts decrease in number (246, 248) and undergo pronounced pathological changes (246, 248, 249). In living tissue pinocytosis and movement of the mitochondria ceases after cortisone treatment (250), and with large doses there is destruction of the fibroblasts (248, 251).

The mast cell also is influenced by cortisone administration. The number of mast cells decreases after cortisone administration (252), and they undergo morphological changes in the granules (252) and nuclei (253). Similar results are observed on local application of cortisone (254),with mastocytoma (255), mast cells in gastric mucosa (256), and in the peritoneal fluid (255).

Corticosteroids are known also to alter the blood elements. ACTH, adrenocortical extract, hydrocortisone, corticosterone, and 11-dehydrocorticosterone induce an erythropoetic effect in hypophysectomized and normal rats (257-258). Blood lymphocytes have been reported to be decreased in number in the rat and other animals, following adrenocorticoid or ACTH administration (259, 260). The lymphopenic response is abolished by adrenalectomy but not by hypophysectomy (259). Neutrophils, on the other hand, increase in number following administration of cortisone or cortisol (261) or ACTH (262). As with lymphocytes, the eosinophils and

basophils are decreased in number following corticosteroid administration (263, 264).

The effect of cortisone on the morphology of the tissue concerned largely with MPS elaboration has been studied to greatest advantage in cartilage and granulation tissue. Cortisone has been reported to narrow the epiphyseal plate and reduce the number of epiphyseal cartilage cells (265). If cortisone is administered to very young rats, the secondary ossification centre in the epiphyses fails to mature and remains cartilagenous (269).

In the chick, cortisone was observed to inhibit chondrogenesis of long bones (266, 267). Addition of cortisone to explants of embryonic chick cartilage caused the cells to become small and underdeveloped (268). Alcian Blue staining for MPS has been reported to be decreased in intensity in hypertrophic zones in epiphysis of cortisone treated rats and cockerels (269).

Cortisone is known to inhibit the development of fibroblasts and blood vessels in the granulation tissue (270) in many species with concomitant reduction in the oxygen consumption (271). The inhibitory effect on the development of granulation tissue has been widely observed also after local application of cortisone or cortisol (272). Simultaneous administration of cortisone and vitamin-C suppress wound healing (273), whereas simultaneous administration of ACTH and vitamin-C stimulate the healing of wounds (274). These effects suggest that cortisone and ACTH have opposing actions on the healing of wounds. It has been demonstrated also that starvation accentuates the interference of cortisone with healing in the rat. Furthermore, retardation of healing due

to protein depletion is accentuated by treatment with adrenocortical hormones (275).

The general antianabolic action of corticosteroids on protein metabolism (276) is well known. Large doses of glucocorticoids (10 mg cortisol/100 g/day) administered to the rat causes reduction in the growth rate, even though the daily food consumption is not decreased (277). Some aspects of the effect of cortisone on protein metabolism, and the effects on carbohydrate and lipid metabolism, are reviewed in a subsequent part of this section.

It is realized that when corticosteroids are administered to an animal, a number of indirect effects also may come into play which can influence connective tissue metabolism. For example, administration of a relatively large amount of cortisol may lead to suppression of ACTH release (278). This, in turn, may influence the output of other steroids by the adrenal (279). In this connection, it may be recalled that cortisol is not a major adrenal steroid of the rat. The chief steroids produced by the adrenal of this animal are corticosterone, 11-deoxycorticosterone, 18-hydroxycorticosterone and with small amounts of 18-hydroxycorticosterone and aldosterone (280). Thus, administration of cortisone or cortisol to the rat virtually amounts to a 'pharmacological' experiment. Furthermore, the mineralocorticoid properties of adrenal steroids affect the salt and water composition of connective tissue (281) and thus can influence the binding of MPS (which behave essentially as polyanions) with the cations of salts. That cations greatly influence the configuration and physical properties of MPS <u>in vitro</u> has been amply demonstrated (282). Finally, the administration of steroids with predominantly min-

eralocorticoid properties to experimental animals, may produce chronic elevation of the blood pressure associated with pronounced changes in the MPS of blood vessels (283).

(2) Effect of Cortisone on the Concentration of Hexosamines, Hexuronic Acids and Hydroxyproline in Connective Tissue.

The hexosamine concentration in a tissue reflects the total MPS and glycoprotein content (284). The most commonly used procedure for estimation of the hexosamine content of tissues involves hydrolysis of the tissues with 3 or 4N HCl for 10-12 hours at 100°C, and determination of the hexosamines in the hydrolyzate, with a colorimetric procedure, usually based on the Elson-Morgan reaction (285). However, it is not possible to estimate accurately the hexuronic acid content of tissues in this manner as these compounds are readily destroyed by 3 or 4N HCl during the hydrolytic treatment. For this reason, it is common practice to isolate and at least partially purify the MPS before proceeding with the chemical estimation of hexuronic acid in the unhydrolyzed material (286). An alternative procedure (287) that allows direct estimation of hexuronic acid as well as hexosamines in tissues is described under the 'Experimental Section'.

The effect of glucocorticoids on the hexosamine concentration of skin has been most extensively studied. This topic recently has been reviewed by Pearce (172). The following Table, summarizing the findings of a number of investigators, has been taken from the review.

TABLE III

THE EFFECT OF ADRENOCORTICAL HORMONES ON CUTANEOUS HEXOSAMINE (192)

Species; weight; sex	Hormone	Dose (mg per kg)	Administration	Tissue sample	Hexosamine (μmoles/g)*		References
					Treated animals	Control animals	
Mouse;20 to 30 g;male	Cortisone acetate	100	Intraperitoneal,daily for 5 days	Dry	30.5	29.8	(288)
Rat;200 g; male	Cortisol acetate	25	Intracutaneous once, 24 hr before	Dry	29.8	30.7	(288)
Rat;150 to 200 g	Cortisone acetate	10	Subcutaneous, daily 7 or 14 days	"Tanned"	17.1	15.8	(289)
Rat;300 g; male	Cortisone acetate	30	Subcutaneous, daily for 7 days	Dry	13.2	12.9	(290)
Rat;220 to 250 g;male	Cortisol	2.8	Subcutaneous, daily for 2 days	Fresh	6.47	9.99	(291)
Rat;80 to 500 g;male	Cortisone acetate	10	Intramuscular,daily, 1 to 21 days	Fresh	5.0	5.0	(292)
Rabbit;2.0 to 2.5 kg	Cortisone acetate	10	Subcutaneous,daily, 7 to 25 days	Acetone-dried	38.9	49.1	(289)
Rat;256 g; male	Cortisone acetate	2	Subcutaneous,daily for 14 days	Whole dry, defatted	139.5**	195.4**	(293)

* The values have been calculated in terms of μmoles/g to facilitate comparison with the author's results.

** μmoles hexosamine in the whole skin.

It is apparent that the administration of various doses of cortisone or cortisol caused little change in the concentration of hexosamines in the skin of the rat or mouse. It is noteworthy, however, that the values are expressed in terms of the dry or the 'tanned' weight. Some workers consider that the foregoing criteria do not take into account the possible weight (tissue) loss that may result from cortisone administration. They prefer to calculate the results on the basis of the length of the femur since this remains unchanged regardless of the changes in body (tissue) weight (294). Calculated on this basis they found that cortisone treatment did cause a decrease in the hexosamine content of the skin.

The hexosamine content of bone can be reduced by cortisone treatment (295). Kowaleski (269) found that the concentration of hexosamines in bone of rats and cockerels was significantly decreased in terms of dry weight, after the administration of cortisone as compared to normally fed animals. It is noteworthy, however, that the concentration of hexosamines also decreased when the animals were fed a protein deficient diet. A further, but smaller drop was observed when the protein depleted animals were treated with cortisone.

For reasons pointed out above (p.58), information on the effect of cortisone on the hexuronic acid content of tissues comes mostly from experiments in which MPS were isolated from the tissues. These experiments are therefore reviewed under 'Effect of Cortisone on MPS Metabolism' (p. 62).

The hydroxyproline content of tissues is considered to reflect the collagen content (296). Collagen is synthesized intracellularly in the

fibroblasts as tropocollagen (297, 298). Extracellularly, fibrillar structures are formed (299), which are modified by the surrounding MPS (300). The total collagen content in granulomas (301) is diminished after cortisone administration. Sethi et al. (302) reported that the neutral-saline-soluble collagen fraction is decreased, while the citrate-soluble fraction is increased by cortisone. Siuko et al. (303) found that the alkali-soluble collagen fraction in the skin decreases after cortisone treatment. Houck (304) found a decrease in the sodium chloride extractable, insoluble and total hydroxyproline in rat skin, but an increase in 0.5M citrate extractable hydroxyproline. Kowaleski (305) recently has reported also that the saline extractable, the insoluble and the total hydroxyproline concentration in skin and liver are decreased in cortisone-treated rats. However, he noted an increase in the acid extractable hydroxyproline.

(3) Effect of Cortisone on MPS Metabolism.

Since the early experiments of Ragan (241) and Layton (244), previously described, several kinds of approaches have been used to study the effect of cortisone on MPS metabolism. In some of the in vivo procedures cortisone was administered to animals by injection; in in vitro studies it was added to the medium of tissue slices or to tissue cultures. The MPS in some cases were then isolated and purified. Still other workers have made use of radioisotopes such as $^{35}SO_4$ or ^{14}C-acetate, and studied the incorporation of the label into the sulfated and nonsulfated MPS by determining their specific activity. It is important to realize that the degree of incorporation of the label cannot be equated with rate of synthesis, although some authors seem to have made this assumption.

Boström and Odeblad (306), in 1953, studied the incorporation of $^{35}SO_4$ into the skin of cortisone-treated and of normal animals by means of radioautography. They found that the incorporation of the label in the treated animals was diminished and they attributed this to a decrease in the rate of exchange of the ester sulfate group of the chondroitin sulfate. Clark and Umbreit (307) studied the effect of cortisone and other steroids on the incorporation of ^{35}S by cartilage slices, and also in vivo experiments with rats. They observed that cortisone or hydrocortisone and their acetates, administered in vivo in large doses, inhibited the ^{35}S-incorporation into the chondroitin sulfate of cartilage. Desoxycorticosterone and 11-desoxy-17 hydroxycorticosterone, administered in vivo, were without effect. However, the addition of cort-

isone, cortisone acetate and desoxycorticosterone to cartilage slices had inhibited the ^{35}S-uptake, while hydrocortisone and hydrocortisone acetate stimulated the uptake. No difference was observed in the amount of sulfate uptake by cartilage between normal and adrenalectomized rats. These workers concluded that their results, *in vitro*, were of no significance in the interpretation of the *in vivo* results. Similar findings were obtained with granulation tissue. Both cortisone and cortisol suppress the ^{35}S uptake by rat granulomas, *in vivo* (308). However, studies on tissue slices from granulomas in the guinea pig showed that cortisone acetate had no effect, while free cortisone increased the ^{35}S uptake (309). The explanation for this apparently contradictory behaviour in some of the *in vitro* systems is not clear.

The results of *in vivo* studies with respect to uptake of sulfate appear to be more consistent. Kowaleski (310) studied the uptake of ^{35}S in normal and fractured bone of rats. Cortisone administration to the animals caused a pronounced decrease in the ^{35}S uptake in the fractured bone, while 17-ethyl-19-nortestosterone caused a significant increase. The latter compound counteracted the suppression effect of cortisone when the two steroids were administered together. Szigiti *et al.* (311) also found that cortisol inhibited the $^{35}SO_4$ uptake by bone in the chick. In addition, the ratio of the inhibitory potency with cortisol, prednisolone, dexamethazone was 1 : 2.3 : 5.4. Cortisone inhibits also the $^{35}SO_4$ uptake by the submaxillary salivary glands in the rat (312).

Rice (313) noted a decrease in the concentration of chondroitin sulfate (on a wet-weight basis) in syphilomas of rabbits that were treated with cortisone compared to that in the controls. He attributed the de-

crease in sulfation of chondroitin sulfate to the action of cortisone. However, the concentration of hyaluronic acid was not appreciably changed. Hence, the ratio of chondroitin sulfate to hyaluronic acid was decreased in the syphilomas of the cortisone-treated animals compared to that of the controls.

With tissue cultures also, Castor (314) observed a decrease in the MPS content, after addition of hydrocortisone. He found that 0.1-40 µg/ml of hydrocortisone, either as the alcohol or the succinate derivative caused a suppression in the hexuronic acid content in the cultures without apparent toxicity to the cells. Addition of 17-hydroxy-11 desoxycorticosterone was without effect.

Schiller and Dorfman (315) performed some of the most definitive studies on this problem. They administered cortisone acetate, 5 mg/day for four days, to rats. On the fourth day the animals received also ^{14}C-acetate and ^{35}S-sulfate. The animals were sacrificed 24 hours later and the hyaluronic acid and chondroitin sulfate isolated from the pelts. The incorporation of the labels into chondroitin sulfate were counted separately as $Ba^{14}CO_3$ and $Ba^{35}SO_4$. It was demonstrated that both the incorporation of ^{14}C into the hyaluronic acid, and the ^{14}C and ^{35}S into chondroitin sulfate, was diminished in the cortisone-treated animals. However, this conclusion appears to be based on the observed decrease in the specific activity of the hyaluronic acid and the chondroitin sulfate, after cortisone treatment. The turnover of hyaluronic acid and chondroitin sulfate also was studied by injection of the labelled compounds before the treatment with hydrocortisone acetate (5 mg/day), and sacrificing groups of animals at various intervals. The turnover of hyaluronic

acid was found to be 3 days, and that of chondroitin sulfate about 7 and 5 days, respectively, for the ^{14}C- and ^{35}S-labels. After 4 days of treatment with hydrocortisone acetate the turnover of the MPS in the skin progressively decreased, to the end of the 8th day period. The authors suggest that the synthesis of the entire MPS molecule, rather than that of the incorporation of sulfate alone, was decreased, since the incorporation of both the ^{14}C and ^{35}S label were suppressed to a comparable degree.

These results, however, do not prove that the rate of synthesis of the MPS of the skin was decreased. For example, similar results could arise if the pool size of the MPS were increased, thus lowering the specific activity of the MPS of the cortisone-treated group. In order to settle this question, Schiller *et al.*(316) isolated and purified the MPS from control and hydrocortisone-treated rats. They found that the concentration (micrograms of uronic acid/g dry skin) of both the hyaluronic acid and the chondroitin sulfate was decreased after hydrocortisone administration, thus ruling out the likelihood of pool dilution.

Two further problems arise in the interpretation of results from administration of steroids *in vivo*. First, there is little direct evidence on the effect of corticosteroids on the rate of catabolism of MPS. Schiller and Dorfman (315) report only the rates of decrease of the specific activity from experiments in which the MPS were 'pre-labelled' before commencing the hydrocortisone treatment. The rate of decrease with regard to the total counts unfortunately is not given. Pearce (172) reports an experiment in which uniformly labelled glucose

was administered to rabbits after pretreatment with cortisone. The MPS isolated from the skin at various intervals showed a more rapid decline in the specific activity than that of the controls. The observation was considered to indicate an enhancement of the catabolism of cutaneous MPS by cortisone although clearly there are other possible interpretations.

The second problem is that not all glucocorticoids with 'anti-inflammatory' activity have an identical effect on MPS metabolism. Kaplan and Fisher (317) report that methylprednisolone, administered to rabbits in small doses caused an increase in the hyaluronate concentration in the vitreous humour and probably also in the keratosulfate concentration of costal cartilage. These authors suggest that both the synthesis and the breakdown of MPS are decreased. The process of degradation was predominant over that of synthesis. Some support for this type of action with prednisolone was obtained by Schiller et al. (316) who observed that prednisolone causes an increase in the concentration of hyaluronic acid and a decrease in the concentration of chondroitin sulfate in the skin of the rat. At an equivalent dose (with respect to anti-inflammatory activity) hydrocortisone caused a decrease in the concentration of both the substances (316).

The problem of the in vitro effect of steroids on MPS metabolism has been studied in greater detail by Whitehouse and Boström (318). These workers found that hydrocortisone and cortisone at 10^{-4}M concentration, suppressed the $^{35}SO_4$ uptake of slices of cartilage from the skin of the calf and the cow. The suppression with the free

alcohol forms of these steroids was greater than with the acetate and the suppression was more pronounced in the tissues from older animals. Desoxycorticosterone produced only a slight suppression, but the addition of high concentrations of salicylates (5×10^{-3} M), and iodoacetate (2.5×10^{-3} M) caused a significant suppression. The same authors (319) studied also the action of a number of steroids and other anti-inflammatory agents, *in vitro*, on (i) the incorporation of glucose-^{14}C, acetate-^{14}C and $^{35}SO_4$ into sulfated MPS, and (ii) the oxidation of glucose-^{14}C, acetate-^{14}C, pyruvate-^{14}C and octanoate-^{14}C. In the latter study the incorporation of ^{14}C into $^{14}CO_2$ was measured. They found that hydrocortisone, 11-deoxycorticosterone, compound-'S' and prednisolone (all added in 0.25×10^{-3} M concentration) inhibited the incorporation of ^{14}C-glucose and ^{14}C-acetate into the sulfated MPS as well as the oxidation of the compounds mentioned in (ii) above. Hydrocortisone suppressed the $^{35}SO_4$ incorporation, but only after a time lag of two hours. The authors concluded that a number of anti-inflammatory drugs exert their effects on connective tissue by inhibiting exergonic reactions.

Not all connective tissues, *in vitro*, show the suppressive effect on MPS metabolism with hydrocortisone. Boström *et al.* (320) working with bovine heart valves, *in vitro*, found that although salicylates, chloroquine and phenylbutazone at a concentration of $1\text{-}5 \times 10^{-3}$ M, reduced the incorporation of $^{35}SO_4$ into glycosaminoglycans, cortisol was without effect in a concentration of 2.5×10^{-4} M. Thus again there is a discrepancy in the response of different *in vitro* systems after the addition of glucocorticoids. No conclusive explanation was advanced

for this discrepancy. The finding that corticotropin increases the sulfate uptake of rat aorta *in vitro* (321), further complicates the picture and raises doubt about the specificity of glucocorticoid effects on MPS metabolism *in vitro*.

Thus, while a large amount of study has been devoted to the action of cortisone on MPS metabolism, many of the ramifications of this topic have not yet been elucidated. Nevertheless, tentative generalizations can be made. There is substantial evidence that cortisone and cortisol, when administered *in vivo*, diminish the incorporation of $^{35}SO_4$ into the sulfated MPS in a number of tissues. It would appear that this effect arises from decreased synthesis of MPS, at least in the rat. The status of the catabolism of MPS *in vivo* after administration of cortisone or cortisol is less clear. The *in vitro* effects in some instances appear to be contradictory, although inhibition of the incorporation of $^{35}SO_4$ after cortisone or cortisol, was consistently observed with a number of preparations.

In view of the known close association of protein and MPS (P-P complexes) in cartilage and other tissues (p. 36), it may be expected that glucocorticoids can influence MPS metabolism by their effect on the protein metabolism of the tissues. Some experimental evidence supports this concept. For example, intra-articular injection of cortisol caused a pronounced inhibition of the incorporation of ^{14}C-glycine into rabbit articular cartilage, thus suggesting a decrease in the rate of protein synthesis (322). Furthermore, puromycin reversibly inhibits the synthesis of chondroitin sulfate by vertebral chondrocytes from chick embryo (323). The treatment does not affect the viability of the cells or

their capacity to multiply on subsequent release from the cartilage matrix. The authors infer from this observation that chondroitin sulfate exists in the tissues coupled with protein in the form of a conjugate. However plausible this may be, it is still necessary to disprove the possibility that puromycin may have inhibited the enzymes involved in chondroitin sulfate synthesis.

Relatively little is known about the effect of glucocorticoids on the biosynthesis of the connective tissue glycoproteins. However, it has been demonstrated that both cortisone, administered in vivo, as well as stress can inhibit the increase known to occur in the concentration of certain glycoproteins in wounds during healing process (324). Cortisone inhibits also the accumulation of saline extractable glycoproteins, which contain hexosamine and are rich in fucose and poor in sialic acid (324).

(4) Effect of Cortisone on Specific Enzymes Involved in MPS Metabolism.

The activity of a number of enzymes involved in MPS and glycoprotein metabolism is known to be altered after administration of glucocorticoids, adrenalectomy and exposure to stress. Some investigations have been carried out on the enzymes involved in the pathways of glucosamine and galactosamine synthesis and in the sulfation process.

Bollet et al. (325) demonstrated that a number of enzymes involved in MPS metabolism, could be obtained from extracts of connective tissue grown in polyvinyl sponges which were inserted under the skin of guinea pigs. They (326) found that the administration of 5 mg of cortisol per day for one week decreased the activity of certain enzymes. In the treated animals the activity of fructose-6-P-glutamine amidotransferase was found to be diminished about 25% in connective tissue homogenates as well as whole liver homogenates. However, the values of the activities were quite low and the differences were not statistically significant. As previously indicated (p.11) the low activities may be attributable to the use of whole homogenates, rather than supernatant fractions (21, 23).

Foster (327) studied the effect of adrenalectomy and steroid replacement therapy on the enzymatic activity of the supernatants from the liver and connective tissue of the rats. He observed that adrenalectomy caused a decrease of about 35% in the activity of amidotransferase in liver supernatants but had no effect on the activity of prepar-

ations from the connective tissue, compared to the values from the sham operated controls. Administration of corticosterone, deoxycorticosterone, hydrocortisone, progesterone or Reichstein's compound-S, at a dosage of 2 mg/day for 14 days, to the adrenalectomized animals, did not restore the activity of the amidotransferase in the liver supernatants to the levels of the controls. Moreover, the mean activity was not significantly altered from that of the adrenalectomized group. Administration of the same dosage of cortisone to the adrenalectomized animals caused a further decrease in transamidase activity in liver supernatants. All the results were expressed on the basis of protein content of the supernatants. On the other hand, neither adrenalectomy nor administration of cortisone or corticosterone to adrenalectomized animals had any effect on the level of activity in the connective tissue. The remaining steroids produced variable effects on the amidotransferase activity in connective tissue. While deoxycorticosterone, hydrocortisone and progesterone decreased the activities in the adrenalectomized animals, Reichstein's compound-S elicited an increase in the activity of the enzyme. The author offers no explanation for the difference in response of the amidotransferase of the liver and the connective tissue to the various adrenal steroids. No mention is made in the paper of studies on intact animals.

Subsequent studies (328) have indicated that adrenalectomy causes a significant decrease in the activity of phosphoglucomutase activity and a significant increase in that of hexokinase, uridine diphosphoglucose dehydrogenase and β-glucuronidase in the connective tissue of rats. However, adrenalectomy had no effect upon the activity of UDPG-

pyrophosphorylase or glucuronosyltransferase.

Varma and Backhawat (329) have reported that the activity of glucosamine-6-phosphate deaminase in rat granulomata is decreased on administration of massive doses (200 mg/kg/day) of cortisone. The activity of the 3'-phosphoadenosine-5'-phosphosulfate synthesizing enzyme in cartilage was not affected by cortisone *in vitro* (330),but was inhibited in granulomata, *in vivo*, on cortisone administration (331).

In a recent publication, Foster (332) reports on the activities of several enzymes that are involved in the synthesis of glucuronic acid. Among these are hexokinase, phosphoglucomutase, uridine diphosphoglucose pyrophosphorylase and uridine diphosphoglucose dehydrogenase. Administration of cortisone, corticosterone or hydrocortisone to sham-operated rats, produced an increase in the activity of all these enzymes in the connective tissue. Adrenalectomy caused a variable response in the activity of all these enzymes. In general, replacement therapy by corticosterone, cortisone or hydrocortisone restored the activity of the enzymes to normal.

(5) Effect of Cortisone on Metabolism of Carbohydrates, Proteins and Lipids.

It is evident that the biosynthesis and the catabolism of MPS and glycoproteins are closely associated with the metabolism of carbohydrates and proteins. Cortisone is known to influence the metabolism of these substances and of lipids as well. The influence of glucocorticoids on carbohydrate and protein metabolism has received extensive study and is the subject of several recent comprehensive reviews (333-335). It is beyond the scope of this presentation to deal in any detail with these topics. However, certain points pertinent to hexosamine and MPS metabolism will be discussed.

(a) The Influence of Glucocorticoids on some Interrelationships among Carbohydrates, Proteins and Lipids.

In studying the action of glucocorticoids in the intact animal, one must consider both the action of the steroid on particular tissues and the glucose balance of the whole animal.

In the rat, increased glucose production occurs after prolonged administration of cortisone (336). The dog responds in a similar manner (337). The impaired glucose utilization by a number of tissues tends to complicate the picture and contributes to the appearance of increased glucose production by the whole animal (338). However, in the sheep, at least, characteristic hyperglycemia tends to counteract the decreased rate of utilization by peripheral tissues and can ultim-

ately lead to restoration of the normal rate of glucose utilization (339).

Glycogen accumulates in the liver after cortisone treatment (340). However, the source of the carbon atoms of the glycogen is not unequivocally established. Some evidence suggests that after glucocorticoid administration, the carbon atoms are derived directly from the blood glucose (341). Other studies indicate that only a small percentage of the glycogen atoms is derived from the circulating glucose (342). In fact, a net increase in the synthesis of carbohydrate after glucocorticoid administration can arise only from a change in the other two principal metabolic materials - fat and protein. However, a net synthesis of carbohydrate from fatty acids cannot occur by way of the presently accepted pathways (except propionic acid which fixes CO_2 to form succinic acid), and the two carbons of acetyl CoA formed from fatty acid must transverse the Krebs cycle before they are converted into phosphoenolpyruvate. Two of the remaining carbons would then be oxidized for each round of the cycle (343). It is clear, nevertheless, that the carbon label in fatty acid will appear in carbohydrate. Indeed, increased incorporation of the ^{14}C from palmitate-^{14}C and butyrate-^{14}C, has been demonstrated (344) after cortisol administration. It is likely, therefore, that net carbohydrate synthesis and gluconeogenesis can occur from amino acids, and there is good evidence to support this (338. 342). Furthermore, there is a close correlation between the increase in the carbohydrate content of tissues and in the nitrogen excretion (345). However, the objection

has been raised to this view, that the magnitude of the excretion of nitrogen, after prolonged glucocorticoid administration, is inadequate to account for the quantity of glucose formed when hyperglycemia and glycosuria occur (346). This objection may be met, by taking into account that the increased glucose content of the animal is due to diminished glucose utilization by the peripheral tissues, and that it is not attributable to increased glucose formation by the tissues.

Individual tissues have been studied, in vitro, either after adrenalectomy or administration of glucocorticoids to animals, or by the addition of the steroid to the medium and the normal tissue slices. The liver and kidney slices from adrenalectomized animals show a decrease in net carbohydrate formation (347). An increase in the carbohydrate content and in the degree of incorporation of ^{14}C from alanine-^{14}C and pyruvate-^{14}C by the liver slices has been noted after addition of glucocorticoids in vitro. The increase is greater with alanine-^{14}C (348, 349). There is evidence of decreased utilization of glucose on the addition of steroids in vitro, to muscle, adipose tissue, skin, lymphoid tissue and leucocytes (350-354).

The action of glucocorticoids on protein metabolism merits special comment. It is generally held that glucocorticoids have an 'anti - anabolic' action on the proteins in most tissues. For example, the incorporation of ^{14}C-labelled glucose, carboxylic acids and bicarbonate into muscle protein is enhanced by adrenalectomy and diminished by cortisone treatment (355). Similarly, the synthesis of collagen (which constitutes a large proportion of the body's protein pool) is decreased

by cortisone (356). However, once collagen is laid down it is not influenced by cortisone administration to an appreciable degree (357). Glucocorticoids tend to inhibit protein synthesis in lymphatic and reticuloendothelial tissues (358). Exposure of thymocytes to cortisol, *in vivo* or *in vitro*, results in an inhibition of RNA, DNA and protein synthesis, and to decreased transport of amino acid and protein precursors into the thymocytes (359). These effects are accompanied by a decrease in the activity of the DNA-dependent RNA polymerase, followed by a diminished degree of synthesis of ribosomal protein (360). The liver, however, presents a more complex picture. In this tissue a number of enzymes involved in gluconeogenesis, including enzymes associated with amino acid metabolism, are 'induced' after cortisol administration, thus greatly complicating studies on the protein metabolism of this tissue.

The key question arising from the above discussion is: by what mechanisms are the acceleration of gluconeogenesis, increased deposition of liver glycogen, and the anti-anabolic effect of protein metabolism brought about? In an attempt to answer this question it is pertinent to review the behaviour of some of the enzymes involved in these processes, after administration of glucocorticoids. These enzymes have been more extensively studied in the liver.

(b) Effects of Glucocorticoids on Enzymes Involved in Gluconeogenesis and Protein Metabolism.

A number of enzymes which catalyze the reactions of the glycolytic system between lactate, glucose and glycogen have been reported to be 'induced' after glucocorticoid administration. A number of the hexose-phosphate phosphatases have been extensively studied by Weber and his coworkers (361). The activity of glucose-6-P phosphatase, fructose-6-P phosphatase and fructose-1,6-diphosphate phosphatase all have been shown to be increased in activity within a few hours after administration of a massive injection of glucocorticoid (e.g., triamcinolone, 25 mg/100 g body weight). It is important to note that these enzymes are associated with particulate cell fractions and no activity is found in the liver supernatants either before or after cortisol treatment (361,389).

The second group of enzymes, whose activity is increased, comprises a number of the transaminases associated with amino acid metabolism. The increase occurs generally after several days of moderately large doses of glucocorticoids. For example, alanine-α-ketoglutarate transaminase, tryosine transaminase and tryptophan pyrrolase, all have been reported to be induced after cortisol administration (335). Muscle amino peptidase also has been reported to be increased (362).

Glycogen synthetase is said to undergo a moderate increase after cortisol administration, according to some reports (363), but not to others (364).

In addition to the transaminases and the phosphatases, which have been extensively studied, a number of other enzymes, involved in the carbohydrate metabolism of the liver, are said to be increased in activity after glucocorticoid administration. Some of these are summarized in the following Table:

TABLE IV

Enzymes Influenced by Administration of Glucocorticoids

Type of Response		
Increased activity	Decreased activity	Unchanged activity
Glyceraldehyde phosphate dehydrogenase (365)[1]	Hexokinase (adult rat) (368)	Hexokinase (embryonic rat) (368)
Phosphorylase (366)[2]	Phosphofructokinase (369)	Phosphoglycerate kinase (370)
6-Phosphogluconate dehydrogenase (365)[3]		Aldolase (370)
Aldolase (367)[4]		Phosphohexose isomerase (370)
Phosphohexose isomerase (367)[4]		Phosphoglycerate kinase (370)
Phosphoglucomutase (367)[4]		Lactic dehydrogenase (370)
Lactic dehydrogenase (367)[4]		Malic enzyme (371)
Malic dehydrogenase (367)[4]		
Phosphoenol pyruvate Carboxykinase (392)[4]		

1. Increased within 8 hours after cortisol administration.
2. Increased within 12 hours after cortisol administration.
3. Increased between 24 - 96 hours after cortisol administration.
4. Increased after several days of cortisol administration.

It is apparent that not in all cases has there been agreement as to observed changes in activity after glucocorticoid administration. It is important to note that in most cases, where enzymatic activity has been found to be increased, the increase occurred several hours or a number of days after the cortisol administration. The enzymes listed are involved in gluconeogenesis, and where increased activity is reported, the observations appear to be in accord with the concept of increased gluconeogenesis probably from amino acids.

The decreased phosphofructokinase activity is of interest as this enzyme is thought to be rate-limiting for glycolysis in several tissues (372).

The increased activity of these enzymes in the liver is preceded by a sharp increase in the turnover of RNA and an increase in amino acid nitrogen within the first 8 hours after cortisol administration (373). Stimulation of the synthesis of messenger RNA has been reported to take place within a few hours (373).

Thus it is evident that changes in the carbohydrate and amino acid metabolism are related, at least in part, to the synthesis of enzymes which catalyze these processes. However, there are two further points to be considered. First, the possibility exists that a number of the enzymes, whose activities are known to be increased, may be 'induced' only secondarily to increase in the substrate concentration (333). This could be so, particularly with enzymes whose activity is altered only at some time after the administration of cortisol. Secondly, there

are reports to the effect (374) that gluconeogenesis, after cortisol, occurs even when actinomycin-D has been administered in sufficient quantities to block 'induced' synthesis of enzymes operative in gluconeogenesis. These writers suggest that the primary action of glucocorticoids is on the existing enzyme systems, and that the induction of enzyme synthesis is secondary.

Clearly then, the mechanism of action of glucocorticoids is not as yet precisely defined. The possibility does exist that glucocorticoids may influence more than one regulatory mechanism in the cell, prominent among which are enzyme induction and membrane permeability (333).

Some of the better defined effects of glucocorticoids on membrane systems have been demonstrated with lysozomes. Reference to this is made in the subsequent section.

(c) The Action of Glucocorticoids on Lysozomes.

In 1952, Fell and Mellanby (375) reported that an excess of vitamin-A, added to chick embryo cultures, caused depletion of the chondroitin sulfate of the chick cartilage. In 1956, Thomas (376) found that intravenous injection of papain into rabbits caused collapse of their ears and depletion of the metachromatic cartilage matrix. In a later collaborative study, Fell and Thomas (377) demonstrated that intravenous injection of vitamin-A to rabbits, also collapsed the ears and caused changes indistinguishable from those with papain. It was postulated (378) that vitamin-A causes release of a proteolytic enzyme. The presence of such an enzyme later was demonstrated in embryonic cartilage (379). The enzyme was identified as an acid protease with optimum activity at pH 3.0. Furthermore, it was shown that the enzyme is contained in the subcellular particulate fractions, and that it is activated in hypotonic medium or on acidification of the medium. In the meantime, de Duve has shown that acid proteases are contained in the lysozomal structures (380). It was found (381) that an acid protease, which was observed to cause rapid degradation of cartilage, was released after incubating liver lysozomes with vitamin-A, <u>in vitro</u>.

It was later discovered (382) that pretreatment of rabbits with cortisone, before the administration of vitamin-A, completely prevented the lytic effect on cartilage matrix. Similarly, cortisone was found to be protective against the action of vitamin-A in embryonic tissue culture. It was postulated, therefore, that cortisone may stabilize

the lysozomes, *in vivo*, and prevent the release of the acid hydrolases after administration of vitamin-A. Furthermore, treatment of animals with cortisone tended to stabilize the lysozomes isolated from the liver (383). Also, addition of cortisol to lysozomes from normal animals protected them against the lytic actions of vitamin-A, ultraviolet light and Streptolysin-O.

Lysozomes are known to contain a number of enzymes directly involved in MPS and glycoprotein metabolism; among these are β-glucuronidase, β-N-acetylglucosaminidase, arylsulfatases-A and -B, lysozyme and β-galactosidase. In addition to the proteases they contain acid phosphatases, acid lipases and acid DNAase and RNAase (384). The protection afforded by cortisol against release of a number of these enzymes has now been demonstrated (385). There is evidence that glucocorticoids stabilize the lipoprotein membrane of the lysozomes, thus decreasing the likelihood of release of these various enzymes (386). Thus it would appear that glucocorticoids can affect the properties of the membranes of at least certain subcellular particles, both *in vivo* and *in vitro*.

EXPERIMENTAL

The effect of cortisone on MPS and hexosamine metabolism in the rat, has been studied along four lines.

First, we have studied, with the aid of radioautography, the incorporation of labelled sulfate into the sulfated MPS in a number of tissues with active MPS metabolism in normal and cortisone-treated animals. As an adjunct to the investigation histochemical studies also have been included.

Second, under comparable conditions of cortisone treatment, we have estimated the tissue content of hexosamines and hexuronic acids as an index of the total MPS content of the tissues.

Third, as the results obtained in the experiments with the cortisone-treated animals indicated findings compatible with a decrease in the synthesis of MPS, we proceeded to investigate the influence of cortisone on the activity of some of the enzymes involved in the synthesis of the hexosamine intermediaries. A decrease in the activity of hexose-6-P amidotransferase, which is the enzyme which catalyzes the first step in the glucosamine synthetic pathway, was observed with rat liver fractions from the cortisone-treated animals, ('in vivo' effect). A similar decrease in activity was observed on the addition of a soluble derivative of cortisol to hexose-6-P amidotransferase preparations from control rat liver, ('in vitro' effect).

Fourth, the incorporation of glucosamine-^{14}C into various intermediaries of the glucosamine synthetic pathway and into liver glyco-

proteins and the MPS was studied to ascertain whether other steps also in the sequence toward MPS synthesis may be suppressed by the action of cortisone.

It is proposed to present, in this section, the data for each of these approaches, preceded in each case, by pertinent methodology. The sub-sections have thus been arranged so that the overall effects of cortisone on MPS metabolism are described first, and the investigations as to a possible site of action follow.

1. RADIOAUTOGRAPHIC AND HISTOCHEMICAL STUDIES OF TISSUES FROM NORMAL AND CORTISONE-TREATED RATS.

(1) Methods:

Six male Sprague-Dawley rats, twenty-two days old, were given 5 mg cortisone acetate subcutaneously for 10 days. Six other males of comparable age and weight, received saline and served as the controls. At the end of the 10-day period each animal received an intraperitoneal injection of 0.2 μC/g body weight of ^{35}S-sulfate (carrier free). The animals were killed by decapitation 2 hours after the injection. The appropriate tissues were removed immediately and dropped into a precooled (-20°C) mixture (9:1 v/v) of ethanol and formalin. A portion was taken of each of the following tissues: the anterior edge of the liver, the skin from the lower dorsal area, the aorta, adrenals and the proximal halves of both tibiae. The adrenals were weighed and, along with the other soft tissues (liver, skin, aorta), were fixed in individual containers in the ethanol-formalin mixture for 24 hours at 5°C. The bone specimens likewise were placed in the fixative. Twenty-four hours later, the latter specimens were split lengthwise and most of the shaft was trimmed off leaving essentially the epiphysis and diaphysis. They then were fixed for an additional 24 hours. The fixation and all subsequent steps were designed so as to minimize any solubilization of the MPS. For this reason the bones purposely were not subjected to decalcification treatment. The tissues were passed three times through absolute ethanol, and then, four times

through xylene to ensure clearing of the specimens. They were next impregnated with paraffin, and set in paraffin blocks, with each tissue oriented in the same fashion. Finally, sections (5-6 μ) were cut. The sections were mounted and the paraffin removed. Contact radioautograms were prepared in a dark-room with the photographic plate (Kodak contrast process ortho safety film, 1 x 3 inches) placed between the mounted tissue section and a glass slide, and the 'sandwich' held in place with rubber bands. Each set was wrapped in black paper and stored in light-proof wrapping in the dark-room until due for development. Exposure trials revealed that a satisfactory image was produced on exposure of the bones for 4 weeks, and the soft tissues, for 8 weeks. These exposure times were used with all of the radioautograms.

Coated radioautograms also were prepared in the dark-room. The mounted and deparaffinized sections were dipped in a liquid photographic emulsion (Kodak NTB) and the coating of emulsion permitted to dry. The coated slides of the bone sections were then stored for 3 weeks, and those of the soft tissues, about 6 weeks, and then developed. The sections were passed through graded concentrations of ethanol and xylene to remove inorganic ^{35}S-sulfate, and finally prepared for light microscopy.

Altogether, thirty-six contact radioautograms were prepared from the bones (3 from each control and cortisone-treated animal), and 24 each of the specimens (2 from each animal) of skin and aorta. One coated radioautogram was prepared from each of the tibiae, skin, aorta and liver, and from each of the controls and the cortisone-treated animals.

In addition to the radioautograms, sections were prepared from each tissue for histochemical study. Three staining procedures were used: Hematoxylin and eosin staining for observation of morphological changes; toluidine blue for staining the MPS (387); and periodic acid-Schiff for glycoproteins and polysaccharides with free vicinal hydroxyl groups (388).

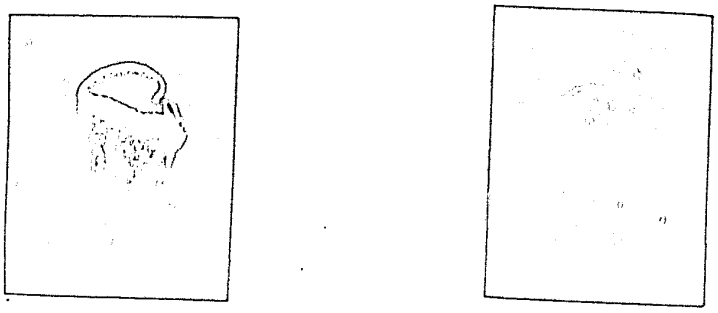
(2) Results:

(a) Radioautography.

Figure 1 illustrates a typical contact radioautogram from the epiphysial-metaphysial region of the tibia from a 'control' and a cortisone-treated animal.

FIGURE 1

CONTACT RADIOAUTOGRAMS FROM THE TIBIAE OF 'CONTROL' AND CORTISONE-TREATED RATS.



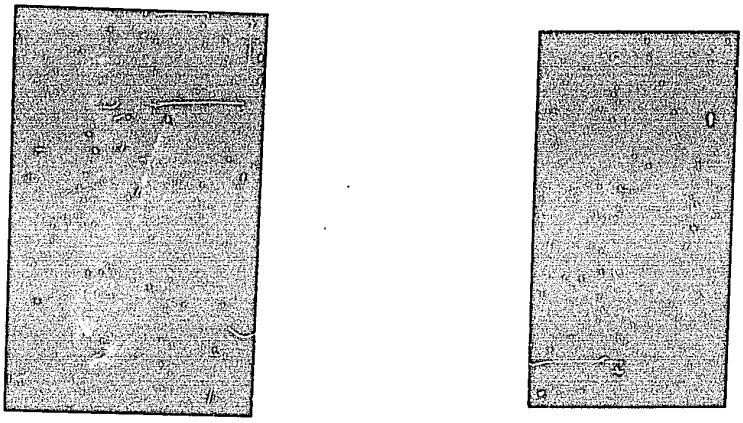
Control Cortisone Treated

It is apparent that in the control group there was a considerable uptake of ^{35}S by the epiphysial cartilage, and to a lesser extent, by the articular cartilage of the tibia. In the cortisone-treated group, on the other hand, the ^{35}S uptake by all regions of the bone was relatively very small.

Figure 2 shows representative contact radioautograms from the skin of control and cortisone-treated animals.

FIGURE 2

CONTACT RADIOAUTOGRAMS FROM THE SKIN OF 'CONTROL' AND CORTISONE-TREATED RATS.



Control Cortisone Treated

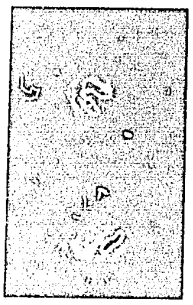
Again, it is plain that the ^{35}S uptake was relatively much less in the skin of the animals treated with cortisone than in that of the controls.

Figures 3 and 4 represent the contact radioautograms from the aorta and liver, respectively.

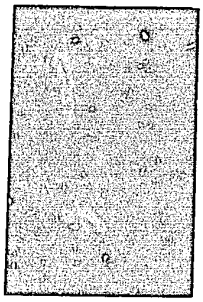
FIGURES 3 and 4

CONTACT RADIOAUTOGRAMS FROM THE AORTA AND THE LIVER OF CONTROL AND CORTISONE-TREATED ANIMALS.

AORTA

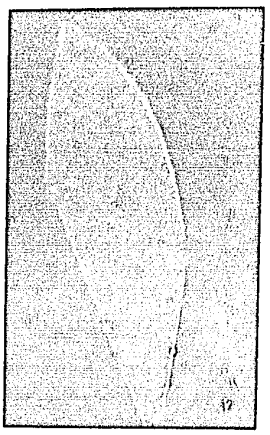


Control

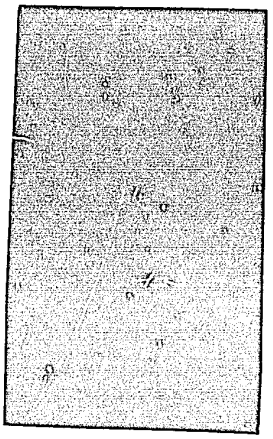


Cortisone Treated

LIVER



Control



Cortisone Treated

It is apparent that the uptake of ^{35}S by the control aorta and liver was less than that of the cartilage and the skin. In the case of the liver the uptake was greater in the capsular area than in the parenchyma. Again it would appear that there was a diminished ^{35}S uptake by the cortisone-treated animals, although the difference was not as pronounced as with the cartilage and skin.

Figures 5 A and 5 B show microphotographs (phase-contrast optics) from representative coated radioautograms of tibial epiphysial cartilage from control and cortisone-treated animals.

It is evident that there was a considerable uptake of ^{35}S by the cartilage plate cells, within two hours after the administration of the radioisotope. It may be observed further that within this time interval very little of the ^{35}S had been secreted by the cartilage cells into the adjacent matrix. It may be recalled from the 'Introduction' (p.39), that it was shown by other workers that in similar radioautograms nearly all of the ^{35}S is present as ^{35}S-sulfate incorporated into the sulfated MPS of the cartilage.

Very little of the ^{35}S was taken up by the cartilage plate cells of the cortisone-treated animals. Furthermore, practically no ^{35}S label appears in the adjacent matrix, thus suggesting that very little sulfated MPS had been synthesized by the cartilage cells of the cortisone-treated animals, during the two-hour period.

FIGURE 5 A

COATED RADIOAUTOGRAM FROM CONTROL EPIPHYSIAL AREA.

(non-decalcified section, phase contrast optics x 100)

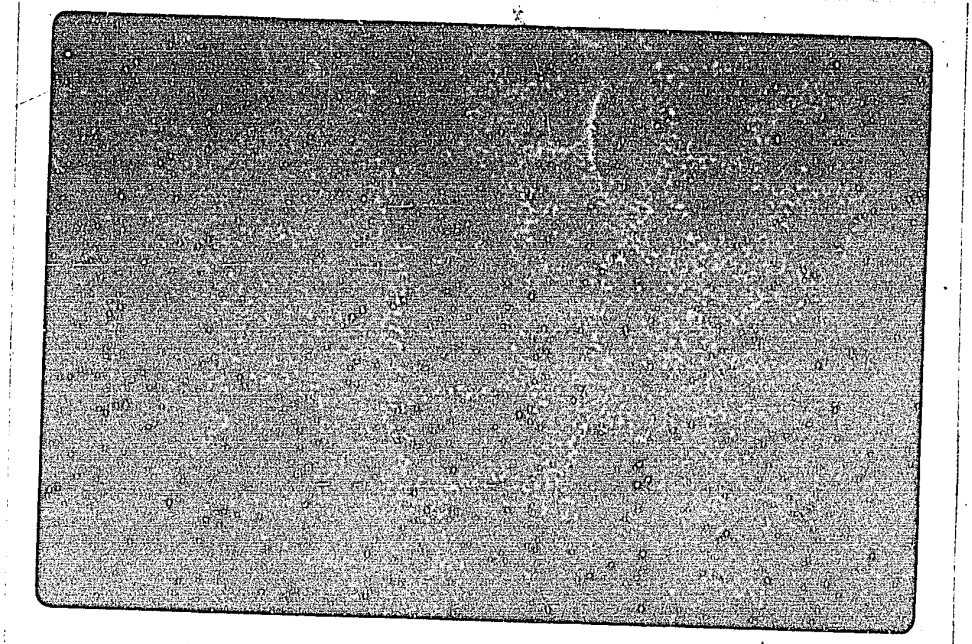
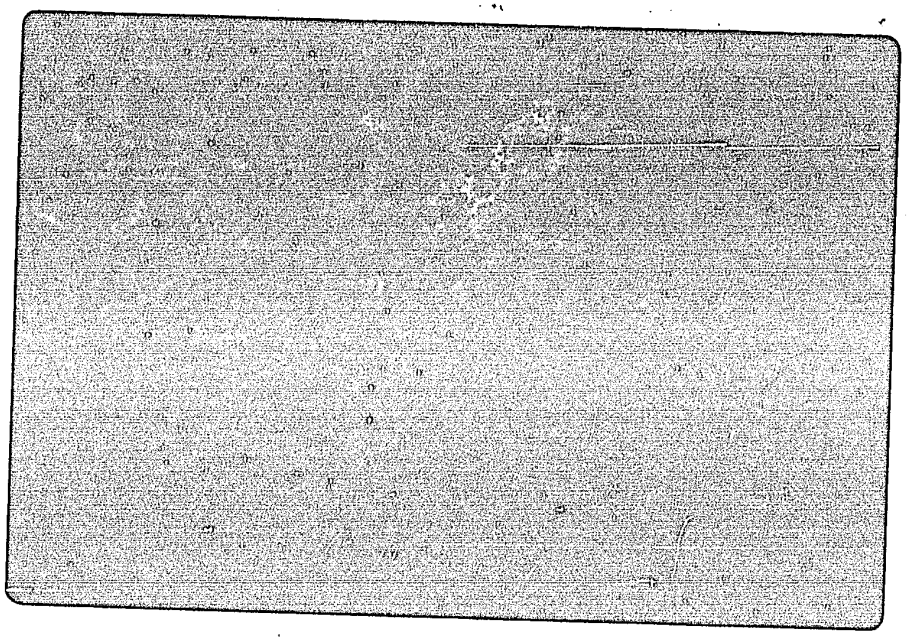


FIGURE 5 B

COATED RADIOAUTOGRAM FROM EPIPHYSIAL AREA OF CORTISONE-TREATED ANIMAL



Figures 6 A and 6 B show a representative micrograph from a contact radioautogram of skin from control and cortisone-treated animals.

It is evident that there was a considerable uptake of ^{35}S especially by the skin follicle cells. Again little ^{35}S was present outside the cells after two hours.

There was a striking diminution in the amount of ^{35}S taken up by the cells of the skin. Again, the fact that there is no evidence of secretion of ^{35}S in the adjacent skin region, suggests that very little of the sulfated MPS had been synthesized during the two-hour period.

The aorta and liver showed changes qualitatively similar to those of the cartilage and the skin, after cortisone administration. However, the ^{35}S uptake was less, especially in the case of the liver and most of the label was distributed in the region of the cells of the liver capsule.

FIGURE 6 A

COATED RADIOAUTOGRAM FROM CONTROL SKIN.

(phase contrast optics x 100)

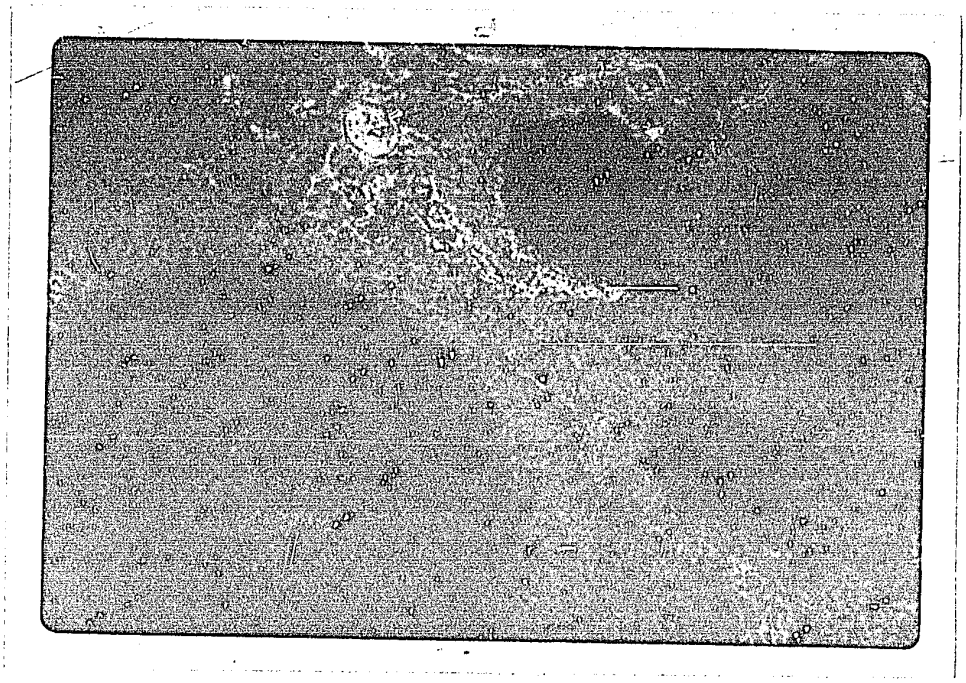
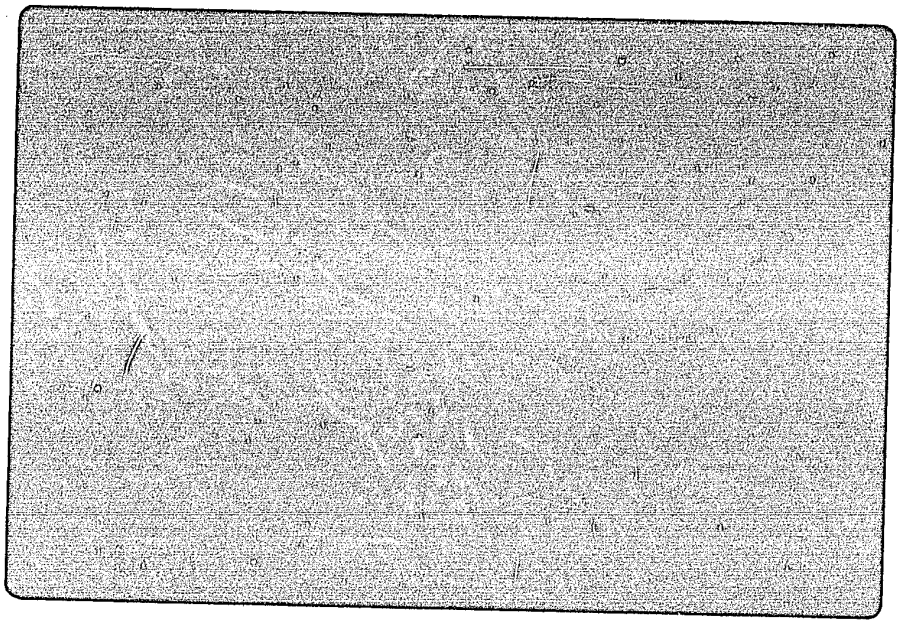


FIGURE 6 B

COATED RADIOAUTOGRAM FROM THE SKIN OF A CORTISONE-TREATED ANIMAL

(phase contrast optics x 100)



(b) Histochemical studies.

Figures 7 A and 7 B demonstrate the morphology of the epiphysial cartilage region in control and cortisone-treated animals, respectively. These sections have been stained with hematoxylin and eosin (H & E).

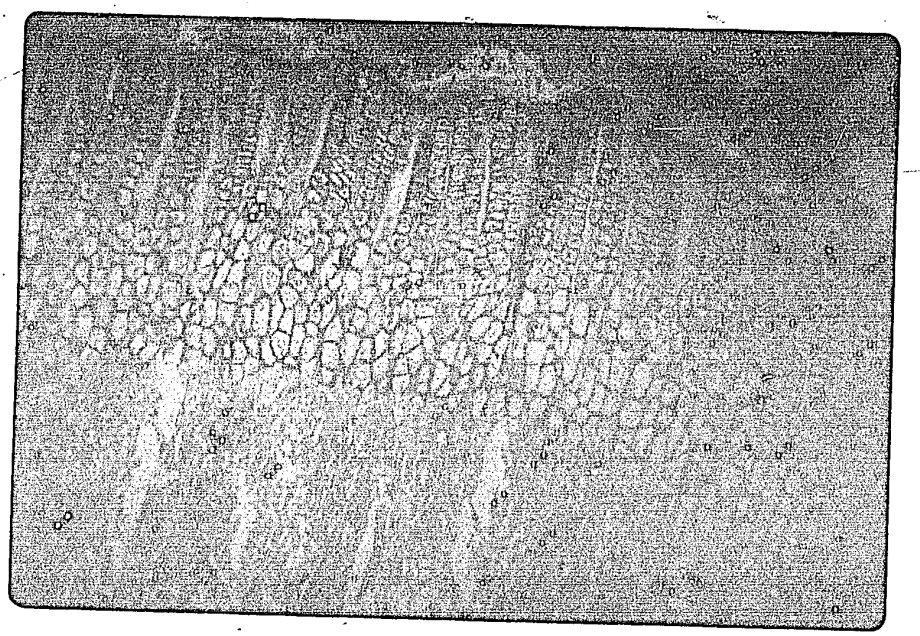
In the control tissue it is apparent that the epiphysial cartilage cells are arranged in well defined rows. The hypertrophic zone cells (close to the primary spongiosa) are well developed and demonstrate large lacunae. It may be recalled that the rat develops a secondary centre of ossification within the epiphysis itself, as is evident in the epiphysial plate in the above figure.

By contrast, the epiphysial cartilage plate in the cortisone-treated animals is much narrower and the cells appear under-developed. The difference is especially prominent in the cells of the hypertrophic zone where the lacunae are abnormally small. Furthermore, the trabeculae of the primary spongiosa are narrower and more irregular, and the ossification, in the secondary centre in the epiphysis, is less advanced in the cortisone-treated animals.

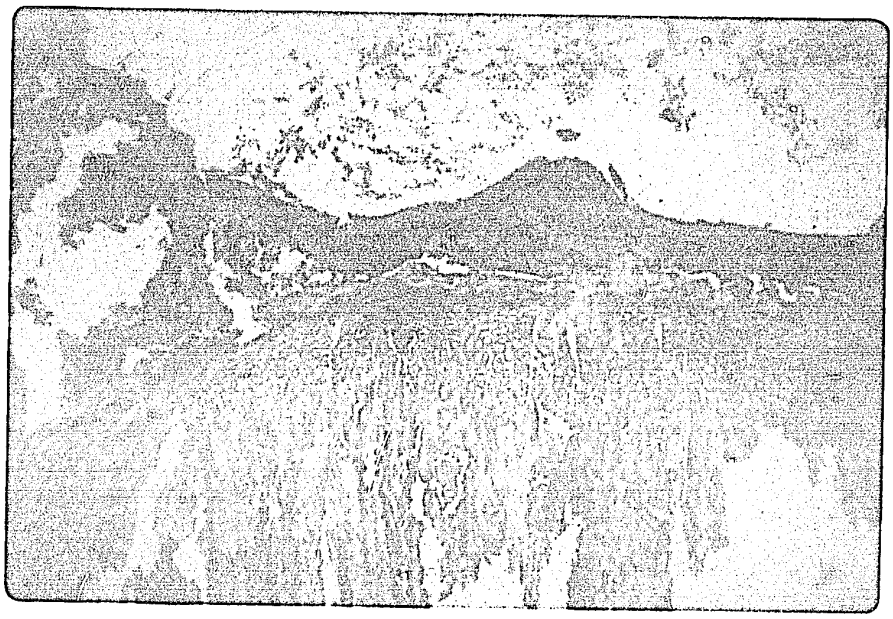
FIGURE 7 A and B)

HEMATOXYLIN AND EOSIN STAINING OF EPIPHYSIAL CARTILAGE REGION FROM CONTROL AND CORTISONE-TREATED ANIMALS.

A. CONTROL



B. CORTISONE-TREATED

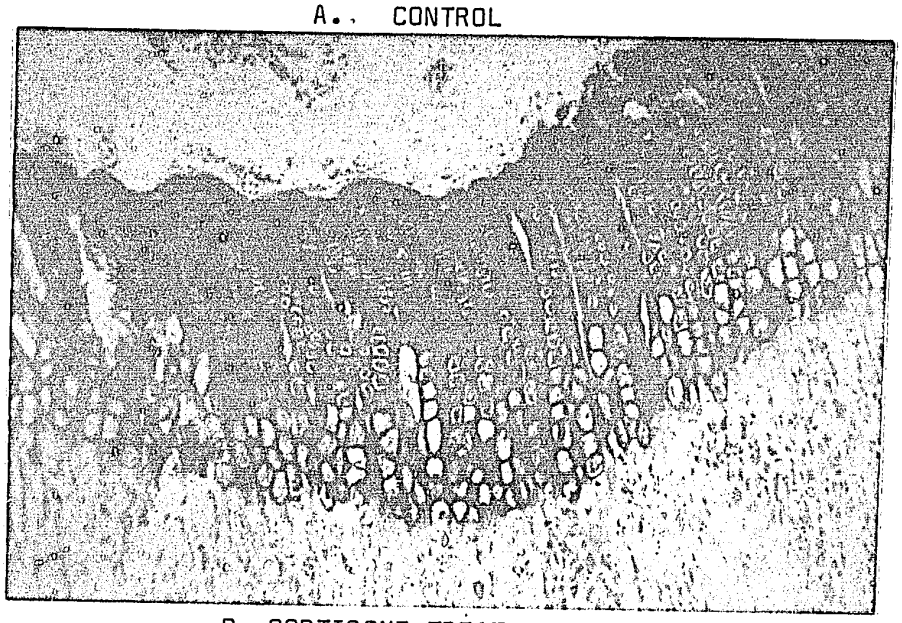


Figures 8 A and B illustrate typical sections, from the epiphysial cartilage region of control and cortisone-treated rats. The sections were stained with the metachromatic dye, toluidine blue

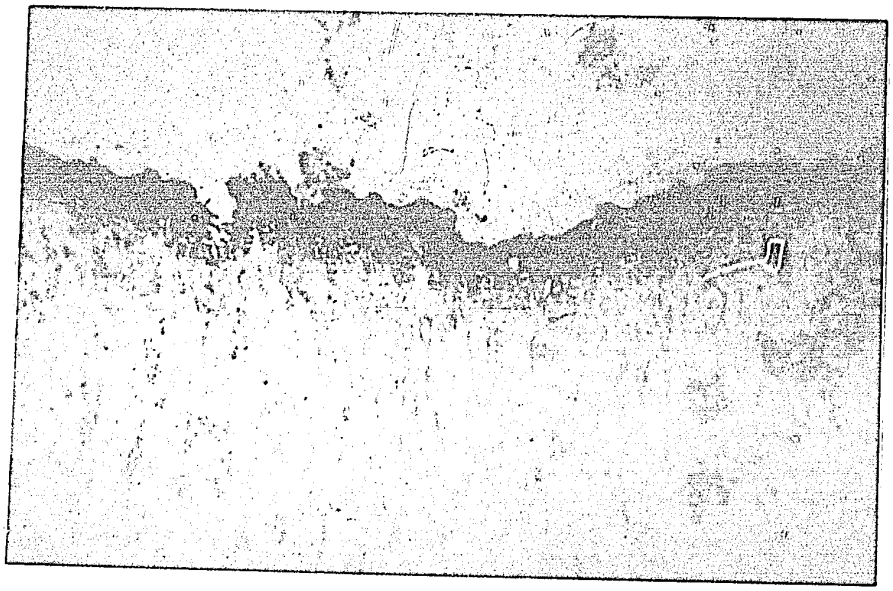
FIGURE 8 (A and B)

TOLUIDINE BLUE STAINS OF EPIPHYSIAL CARTILAGE REGIONS OF CONTROL AND CORTISONE-TREATED ANIMALS.

A. CONTROL



B. CORTISONE-TREATED



THE EFFECT OF CORTISONE ON

HEXOSAMINE AND MUCOPOLYSACCHARIDE METABOLISM

by

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The matrix and many of the cells of the control cartilage had taken up a considerable amount of dye. This illustrates a normal metachromatic reaction. In the case of the cortisone-treated animals, on the other hand, the narrow epiphysial plate has reacted with the metachromatic dye in an abnormal manner, as indicated by the densely appearing dye-cartilage complex with no true metachromasia.

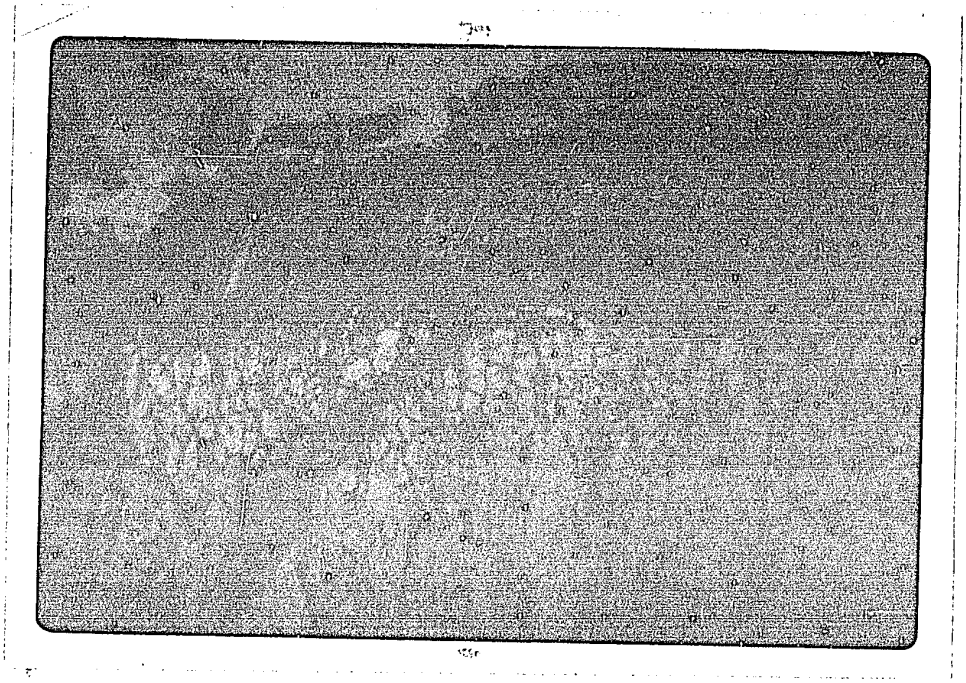
Figure 9 (A and B) illustrates the PAS reaction of epiphysial cartilage from control and cortisone-treated animals.

It is apparent that the cartilage plate of the cortisone-treated animals gave a generally weaker PAS reaction than did that of the controls.

FIGURE 9 (A and B)

PERIODIC ACID-SCHIFF STAINS OF EPIPHYSIAL CARTILAGE FROM CONTROL AND CORTISONE-TREATED RATS.

A. CONTROL



B. CORTISONE-TREATED

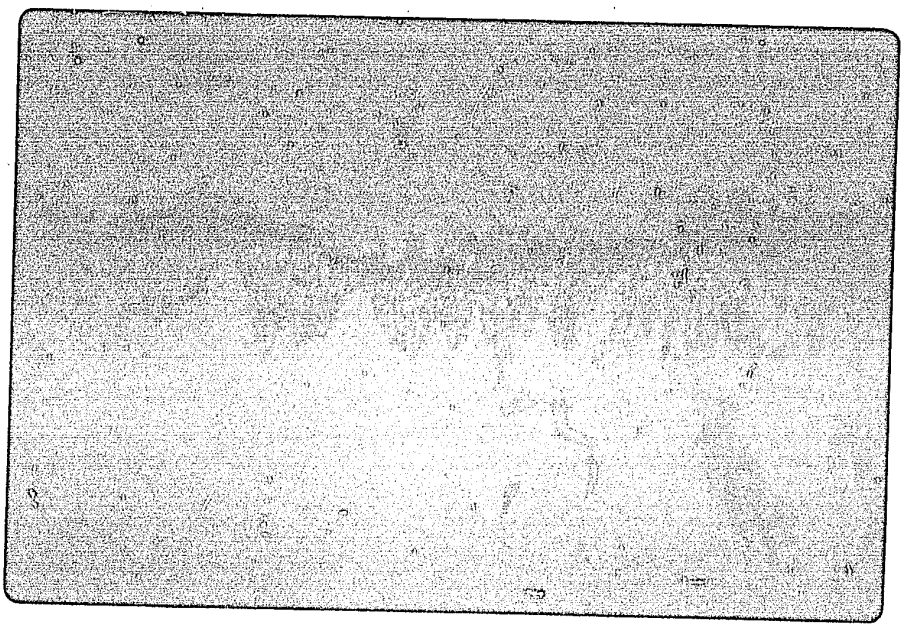


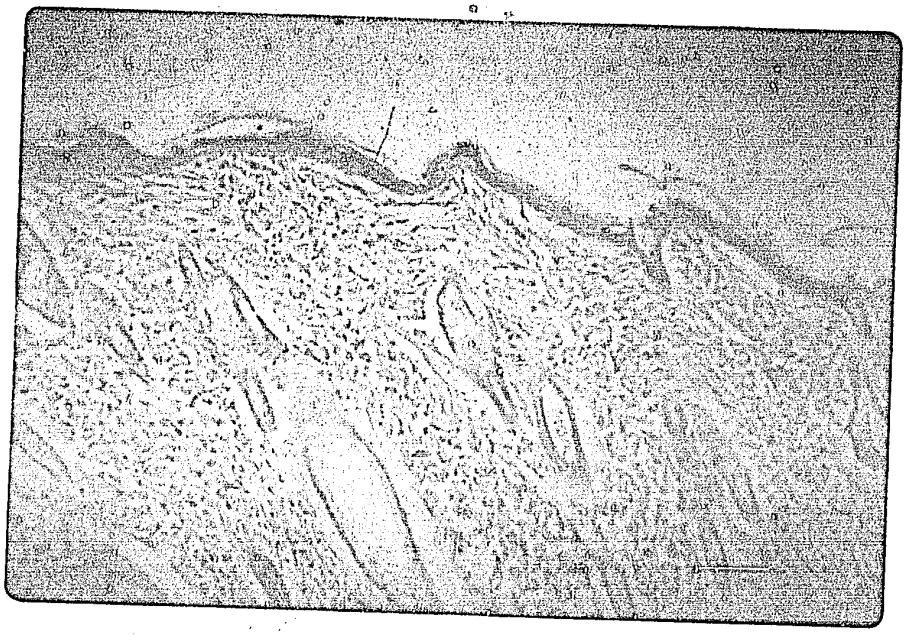
Figure 10 (A and B) shows representative sections of skin stained with hematoxylin and eosin, from control and cortisone-treated animals.

It is apparent that the skin of the cortisone-treated animals exhibits several morphological changes as compared to that of the controls. Thus, the skin follicles and glands were under-developed and the thickness of the epidermis was considerably reduced in the cortisone-treated animals. The staining characteristics also are altered, in that in general, the eosinophilic reaction was predominant in the skin of the cortisone-treated rats compared to the controls.

FIGURE 10 (A and B)

HEMATOXYLIN AND EOSIN STAINED SECTIONS OF SKIN FROM CONTROL AND CORTISONE-TREATED ANIMALS.

A. CONTROL



B. CORTISONE-TREATED

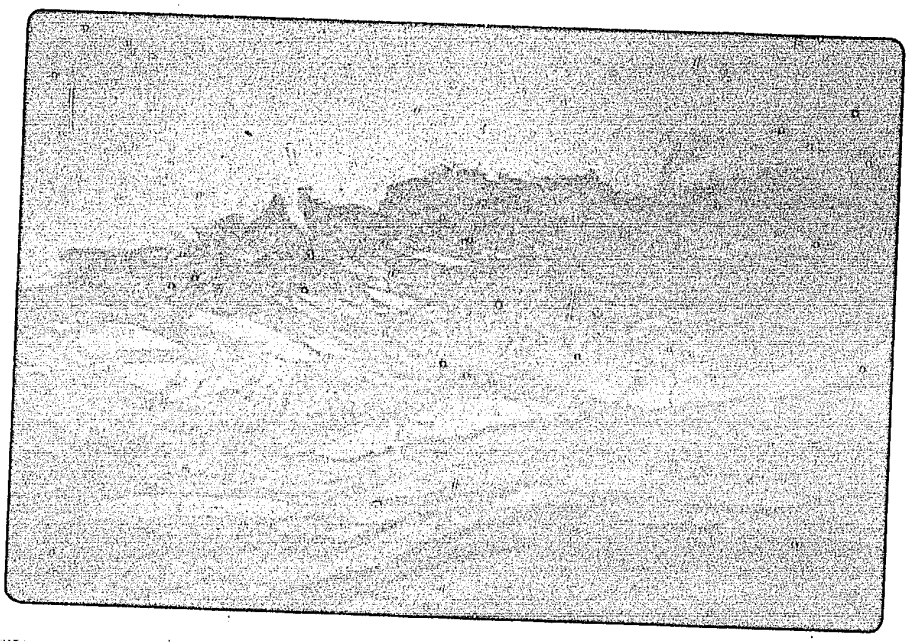


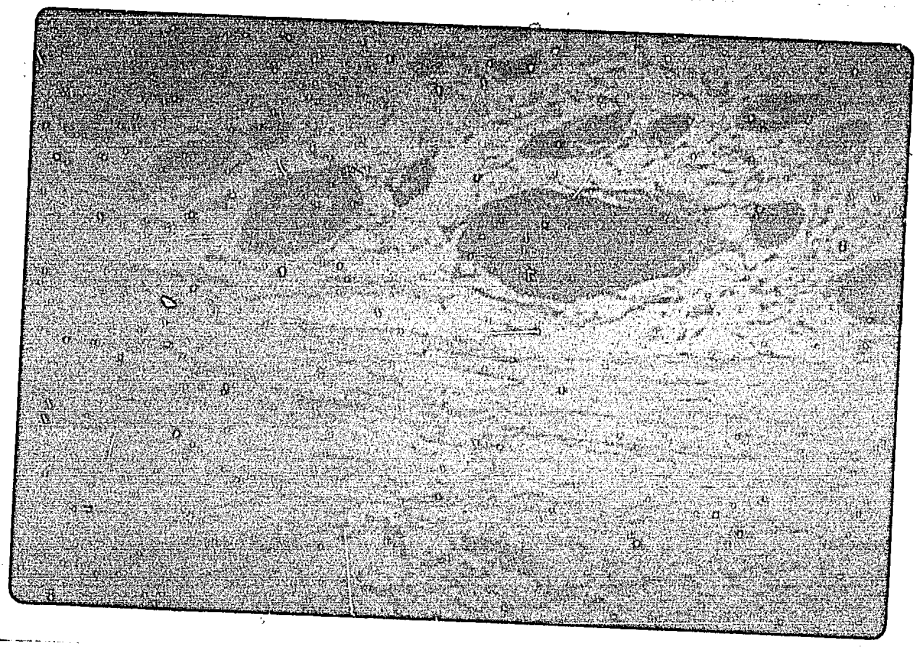
Figure 11 (A and B) gives a typical example of toluidene blue staining of the skin from control and cortisone-treated rats.

It is evident that in the control animal there was considerable dye-uptake by several of the skin structures, especially the follicles. Further, a metachromatic reaction is evident in the follicular area. By contrast, the skin from the cortisone-treated animals showed very weak dye uptake with no metachromasia.

FIGURE 11 (A and B)

TOLUIDINE BLUE STAINS OF SKIN FROM CONTROL AND CORTISONE-TREATED ANIMALS.

A. CONTROL



B. CORTISONE-TREATED

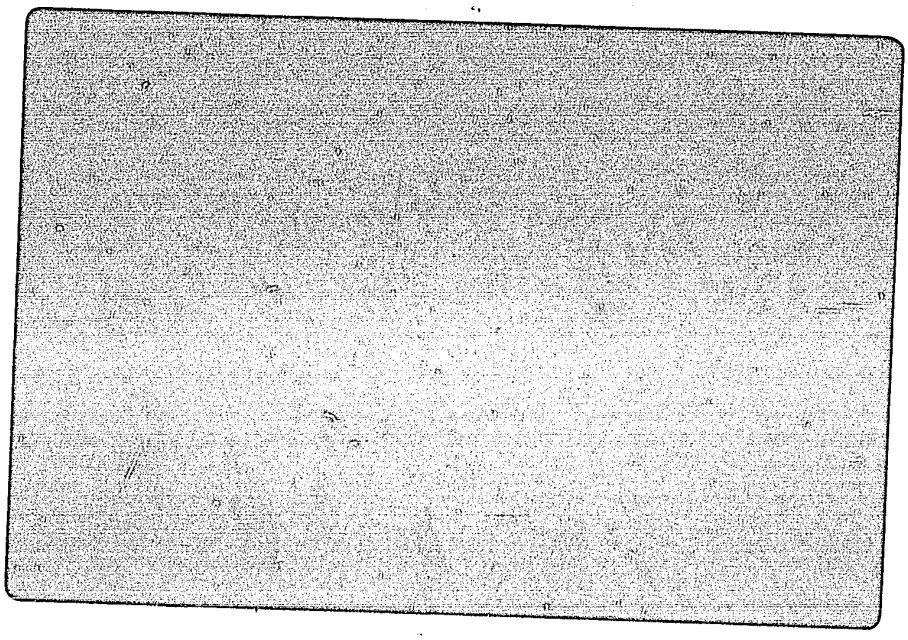


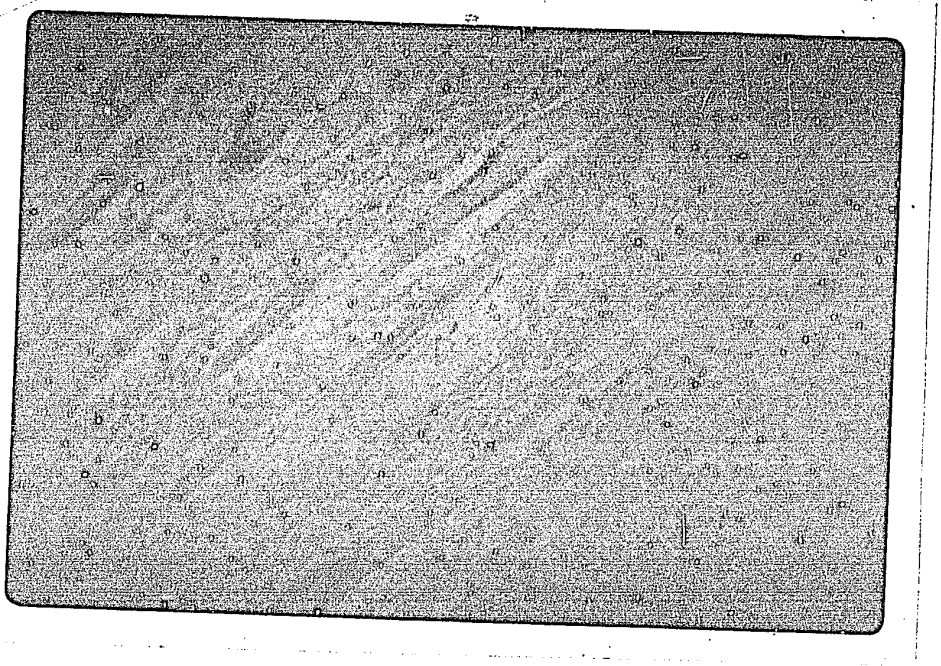
Figure 12 (A and B) shows PAS stained sections of skin from control and cortisone-treated animals.

There is a difference in the intensity of reaction between the control and the cortisone-treated groups. For example, the follicular areas reacted more strongly in the skin of the controls. However, in general, the difference was not as great as with toluidine blue.

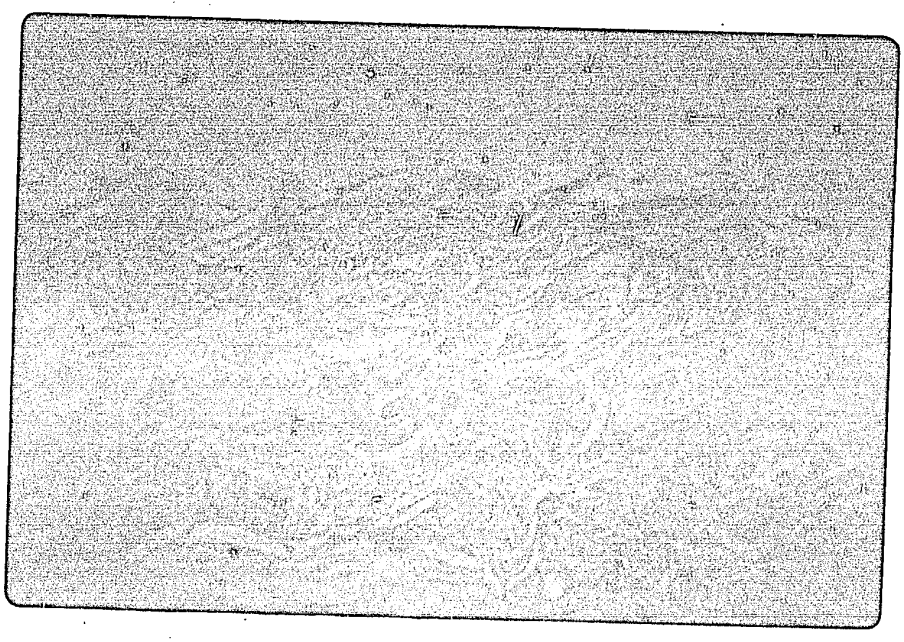
FIGURE 12 (A abd B)

PERIODIC ACID-SCHIFF STAINING OF SKIN FROM CONTROL AND CORTISONE-TREATED RATS.

A. CONTROL



B. CORTISONE-TREATED



Staining with hematoxylin and eosin, toluidine blue and periodic acid-Schiff was carried out also with sections from the aorta and liver of the control and cortisone-treated animals. Again, however, in these tissues the differences between the two groups were not as striking as in the case of the cartilage and the skin. In the liver, the more prominent metachromatic reaction occurred in the region of the capsule, while the more prominent PAS reaction was observed in the cytoplasm of the parenchymal cells.

The mean weights of the adrenals from normal and cortisone-treated rats were 13.8 $\pm$ 0.5 and 4.5 $\pm$ 0.4 mg, respectively. The cortical regions appeared hypodeveloped in the treated animals as compared to controls (H and E sections).

In summary, the above findings indicate that the administration of cortisone to rats decreases the $^{35}SO_4$ uptake in a number of tissues but especially in cartilage and skin. Coated radioautograms suggest a decrease in synthesis of sulfated MPS (or at least a decrease in sulfation), since individual cells show decreased ^{35}S uptake, without any evidence of loss of label from the cell by secretion. The weaker density of the staining with toluidine blue would appear to be consistent with a decreased synthesis of MPS in tissues, while the comparison obtained with PAS-Schiff stains is not as clear-cut.

2. HEXOSAMINE, HEXURONIC ACID AND HYDROXYPROLINE CONTENT OF TISSUES FROM CONTROL AND CORTISONE-TREATED ANIMALS

In view of the radioautographic findings described in the previous section, it was considered desirable to obtain an estimate of the total MPS and glycoprotein content of the tissues in the control and the cortisone-treated animal.

The most direct approach is to estimate the total hexosamine and hexuronic acid content of tissue hydrolyzates. However, two analytical problems present themselves. First, the usual methods of tissue hydrolysis for amino sugar estimation, with 3 or 4N HCl, largely destroy hexuronic acids. To overcome this problem, hydrolysis was carried out with a polysulfonated resin, under very mild acidic conditions. Second, the estimation and identification of the hexosamines in livers of normal and cortisone-treated rats is complicated by the high protein content of this organ. The methodology in this section is concerned with an evaluation of hydrolysis procedures and the identification of hexosamines in liver hydrolyzates.

(1) Methods.

(a) Comparison of the procedures for tissue hydrolysis.

(i) Procedure for resin hydrolysis and for 3N HCl hydrolysis.

Dowex-50 polysulfonated resin (200-400 mesh) was repeatedly washed with water. The resin was then transferred to a large fritted glass funnel, washed with 2N NaOH four times, and washed with distilled

water until neutral. The same procedure was then repeated, starting with 2N HCl, and followed by washing with water until the washings were found to be neutral. The resin was then suspended in 0.05N HCl (1:2 weight/volume).

The samples to be hydrolyzed were placed in pyrex test tubes and 5.0 ml of resin suspension were added. The tubes were sealed in an oxygen flame and fixed with clamps on a rotating disc in a specially constructed oven, maintained at 100 $\pm$ 2°C. The hydrolysis was continued for the stated time intervals.

At the end of the period, the tubes were opened and the contents transferred quantitatively to short glass columns. The resin was allowed to drain and then eluted with about 20 ml of distilled water(202,287). The collected liquid may be designated the 'water eluates'. This fraction contained most of the hexuronic acids and neutral sugars. The resin then was eluted with 4 changes of 2N HCl (4 x 5 ml), and the 'acid eluates' collected in 100 ml beakers. This fraction contained most of the hexosamines and most of the amino acids. In order to estimate the amino sugars, it is necessary to desiccate the 'acid eluates', *in vacuo*, over solid sodium hydroxide, to remove the HCl, The residue is then taken up in a known volume of water, and the amino sugars estimated with a modification of the Elson-Morgan procedure (23). Similarly, the water eluates, if necessary, were reduced to an appropriate volume for estimation of the hexuronic acids with the Dische procedure (286).

For hydrolysis treatment with stronger acid, 5 ml of 3N HCl were added to the sample, in pyrex test tubes. The latter were sealed and placed in the oven maintained at 100°C for 4 hours. The contents of

the tubes then were transferred quantitatively to beakers and desiccated *in vacuo*. The respective residues were taken up in an appropriate volume of water and an aliquot of each taken for estimation of the hexosamines and hexuronic acids.

(ii) 'Recovery' tests with standard solutions of hexosamine and hexuronic acids after resin hydrolysis and 3N HCl hydrolysis.

Several sealed test tubes, each containing 500 µg of D-glucosamine and 500 µg glucuronic acid, were heated at 100°C either with the resin suspension for 13, 21 or 26 hours, respectively, or with 3N HCl for 4 hours, as described above. At the end of the period, the contents of each tube were appropriately processed, depending on the method of hydrolysis. The results are summarized in Table V.

TABLE V

Recovery of Glucosamine and Hexuronic Acid Standards After Hydrolysis with the 'Resin' or 3N HCl

Hydrolytic agent	Duration of hydrolysis (hours)	Hexosamine recovered (%)	Hexuronic acid recovered (%)
RESIN	13	92	106
RESIN	13	90	98
RESIN	21	88	76
RESIN	21	94	74
RESIN	26	87	81
3N HCl	4	90	28
3N HCl	4	92	25

It is evident that the glucosamine was satisfactorily recovered, either after 'resin'hydrolysis or the '3N HCl treatment'. However, while hexuronic acid largely survives up to 26 hours of 'resin' hydrolysis it is extensively destroyed even after hydrolysis for 4 hours with 3N HCl.

The capabilities of the two methods of hydrolysis were evaluated next with MPS isolated from tissues.

(iii) Recovery of hexosamines and hexuronic acids from mucopolysaccharides after hydrolysis.

The MPS used were: (1) chondroitin sulfate (C.S.) sodium salt of mixed isomers Grade III, Sigma Chemical Co., prepared from whale and shark cartilage. (2) Protein-polysaccharide, light component (PP-L, see also p.37) isolated from calf rib cartilage by Dr.Vincent Hascal of Rockefeller University, Sample No.66A. This MPS was chosen in order to obtain experience with the estimation and separation of hexosamines in the presence of large amounts of protein.

Two mg of each of the MPS, in 0.4 ml of water, were placed in each test tube for hydrolysis as described in the preceding section. However, the hexuronic acid concentration was estimated in the 'water eluates' as well as in the 'acid eluates' in order to ascertain the degree of loss in the latter fraction.

For purposes of comparison, the hexuronic acid content of the isolated MPS was estimated also without hydrolysis as is commonly done in order to avoid destruction. A quantity (2 mg) of each of the MPS was dissolved in 25 ml water and the hexuronic acid content estimated (286).

The results for hexuronic acid are given in Table VI A and for hexosamine, in Table VI B.

TABLE VI A

Recovery of Hexuronic Acid from CS and PP-L after 'Resin' or 3N HCl Hydrolysis and without Hydrolysis

Material	Hydrolytic agent	Duration of hydrolysis	Hexuronic acid found in water eluate	Hexuronic acid found in acid eluate	Total	Hexuronic acid content of MPS
		(hours)	(µg/fraction)			(%)
CS	RESIN	10	475.0	202.5	677.5	33.8
CS	RESIN	24	420.0	52.5	472.5	23.6
CS	RESIN	30	370.0	50.0	420.0	21.0
CS	3N HCl	10	-	-	172.5	8.6
CS	WITHOUT HYDROLYSIS	-	-	-	582.5	29.1
PP-L	RESIN	10	402.5	165.0	567.5	28.2
PP-L	RESIN	24	315.0	55.0	370.0	18.5
PP-L	RESIN	30	320.0	45.0	365.0	18.2
PP-L	3N HCl	10	-	-	152.5	7.6
PP-L	WITHOUT HYDROLYSIS	-	-	-	462.5	23.0

Several considerations arise from the results in the Table. First, there was little loss of hexuronic acid from the resin treatment, while with 3N HCl most of it was destroyed compared to the results with the unhydrolyzed specimens. Second, the values for hexuronic acid obtained on the unhydrolyzed materials (the partially purified, commercial CS and the purified PP-L), agree with those reported in the literature for these materials (127, 130, 141). As might be expected, the value obtained with CS is lower than the theoretical. CS theoretically contains about 35% uronic acid. No theoretical value for the hexuronic acid content of PP-L can be given as the structure has not been completely established.

Third, while the free hexuronic acid is readily released from the resin by elution with water, any that remains bound in the incompletely hydrolyzed fragments may not be released but would appear in the acid (2N HCl) eluate along with hexosamine. It is evident from the Table that the acid eluate from the 10-hour resin hydrolyzate still contained a large proportion of the hexuronic acid, and that the quantity was greatly lowered in the 24-hour hydrolyzate. This suggests that hydrolysis was incomplete at the 10th hour. The yield of hexuronic acid in the water eluate of the 24-hour and the 30-hour hydrolyzates were lower than in the 10-hour hydrolyzate, thus suggesting some destruction of the hexuronic acid on prolonged hydrolysis.

However, the reliability of the colorimetric estimation of hexuronic acids in the acid eluate is questionable since the fraction from the PP-L will contain hexosamine and amino acids which may give coloured products with the Dische reagent and hence give erroneous

values for hexuronic acid. For this reason, the 10-hour period of hydrolysis cannot be considered reliable for the estimation of hexuronic acid. Among the hydrolysis times tried with the resin, the 24-hour period appears to give a closer approximation to the true hexuronic acid content of the isolated mucopolysaccharides than the 30-hour hydrolysis, although it is evident that some destruction of hexuronic acid has occurred even during 24 hours of resin hydrolysis.

Table VI B indicates the results of the hexosamine analysis with a modified Elson-Morgan procedure (23). The values are for the same experiments as shown in Table VI A, except that no hexosamine determination on the nonhydrolyzed material has been carried out.

It is evident from the Table that the total hexosamine recovered (as estimated by the Elson-Morgan reaction) was not appreciably increased by extending the duration of hydrolysis beyond 10 hours, for the case of the two isolated MPS studies. However, it is of interest that although the Elson-Morgan colour density from the two was comparable after 10 hours and 24 hours of hydrolysis, this does not necessarily mean that the hydrolysis had reached completion at the end of 10 hours.

The completeness of hydrolysis can be established by chromatographically separating and estimating the amounts of individual hexosamines in hydrolyzates, as is described in the subsequent section.

TABLE VI B

Recovery of Hexosamine from CS and PP-L after 'Resin'

or 3N HCl Hydrolysis

Material	Hydrolytic agent	Duration of hydrolysis	Hexosamine* found in acid eluate	Hexosamine content of MPS
		(hours)	(µg/fraction)	(%)
CS	RESIN	10	740	37.0
CS	RESIN	24	735	36.7
CS	RESIN	30	650	32.5
CS	3N HCl	10	705	35.2
PP-L	RESIN	10	755	37.7
PP-L	RESIN	24	670	33.2
PP-L	RESIN	30	685	34.2
PP-L	3N HCl	10	640	32.0

* Estimated by Elson-Morgan procedure, acetic anhydride modification (23).

(b) Methods for separation and identification of hexosamines in tissue hydrolyzates.

(i) Separation and identification of hexosamines from isolated MPS.

The separation of the hexosamines was carried out by means of an ion exchange column coupled with an automated ninhydrin detection system. The apparatus was constructed in Prof.Dziewiatkowski's laboratory at Rockefeller University, and was an adaptation of features of the amino acid analyzer designed by Stein and Moore (390). The principal difference was that borate buffer, 0.35M, pH 7.60 was used for elution from a 'Technicon chromobead, type A' 50 x 1 cm resin column. The flow-rate was 0.50 ml/min and the water jacket temperature, 60°C. An automatic recording apparatus recorded the optical density of the ninhydrin-reacting substances in the outflow. Work in Prof. Dziewiatkowsky's laboratory had shown that with these conditions of elution, various hexosamines in a mixture are readily separated from one another and from amino acids.

As in the previous section (where total hexosamines were estimated with the Elson-Morgan reaction), samples of CS and PP-L were hydrolyzed with the resin for 10, 24 or 30 hours at 100°C. Again, for purpose of comparison, the commonly used hydrolytic procedure for the release of hexosamines (hydrolysis with 3N HCl for 10 hours at 100°C) also was used. The hydrolyzates from the 3N HCl method and the 'acid eluates' from the resin method were taken to dryness and

then dissolved in 2.0 ml of borate buffer, pH 7.6. An aliquot(of known volume) of the solution (see Figures 13 and 14) was then applied to the column and chromatographed as above described.

The upper parts of Figures 13 and 14 illustrate the separation of hexosamines obtained from the hydrolyzates of CS and PP-L, respectively, after 10 and 24 hours of resin hydrolysis. The lower parts of the Figures illustrate the separations between the aminosugar standards.

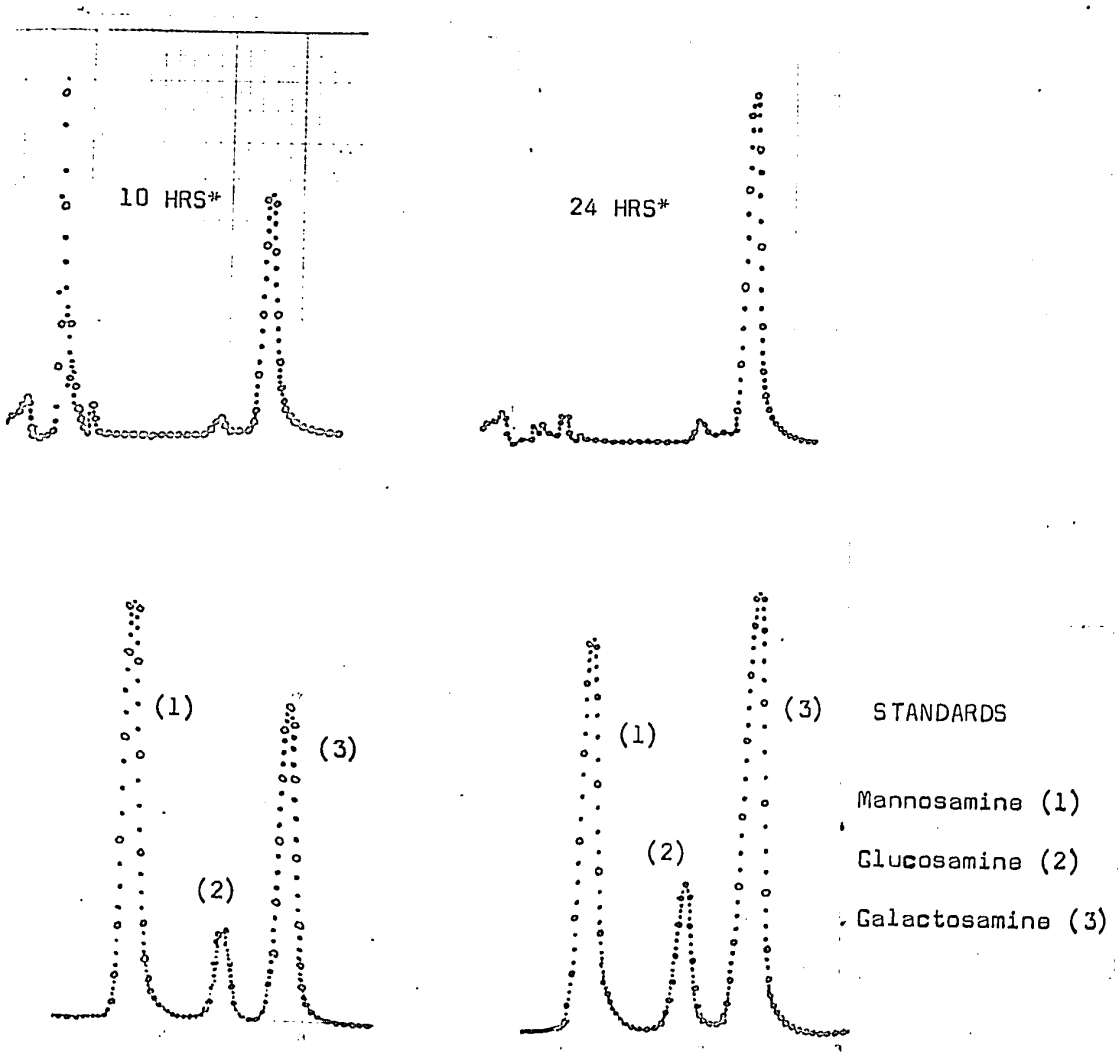
From Figure 13 it is apparent that the commercial sample of chondroitin sulfate contained two hexosamines. The main peak corresponds to the galactosamine, and the small peak, to the glucosamine standard. Since chondroitin sulfate is a polymer of galactosamine and glucuronic acid, the small amount of glucosamine indicates the presence of impurities in the commercial preparation.

Figure 14 shows that the PP-L sample contains peaks corresponding to galactosamine and glucosamine. These aminosugars are known to be present in PP-L and represent chondroitin sulfate and keratan sulfate, respectively, (127, 129, 130).

FIGURE 13

ION EXCHANGE CHROMATOGRAPHY

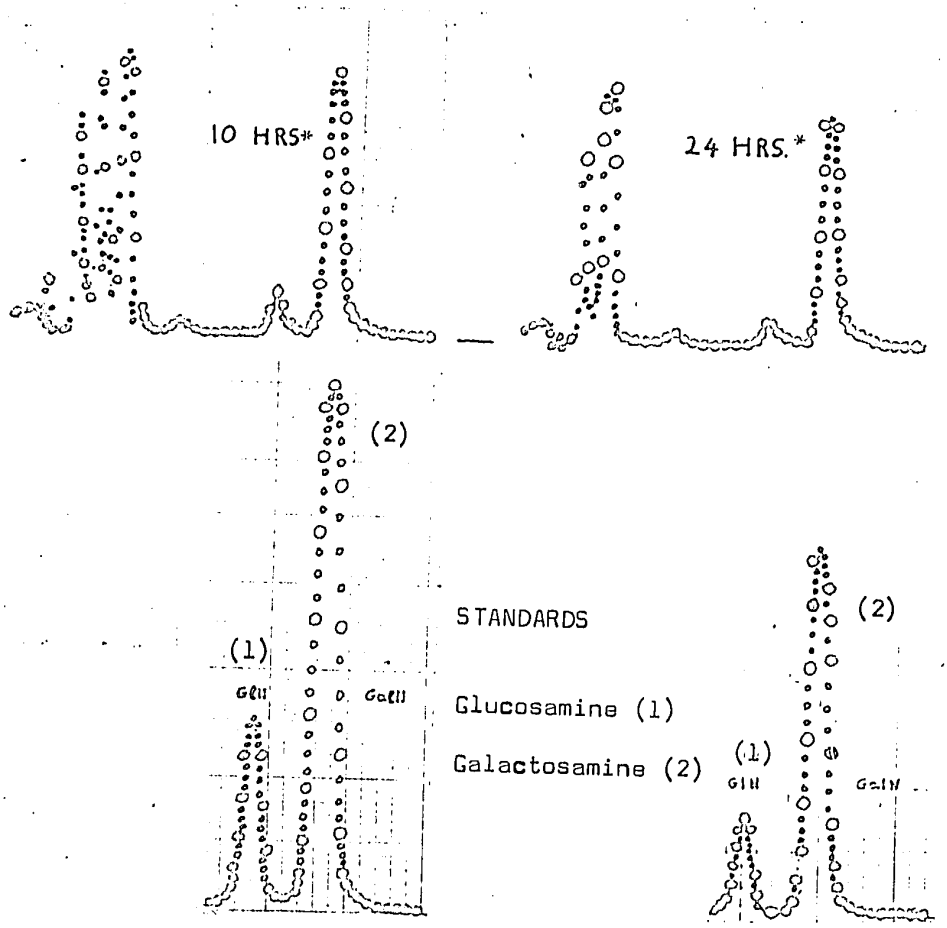
OF CS HYDROLYZATES



* Time of resin hydrolysis

FIGURE 14

ION EXCHANGE CHROMATOGRAPHY OF PP-L
AFTER RESIN HYDROLYSIS AND FRACTIONATION



* Time of resin hydrolysis

It is interesting to note, also, from the Figures that in the case of both the CS and the PP-L there was considerably more ninhydrin-positive material in front of the hexosamine eluting area, in the 10-hour hydrolysis runs than in the 24-hour runs. Chromatography of the 30-hour resin hydrolyzates and the 3N HCl hydrolyzates of CS and PP-L gave patterns idential with those of the corresponding 24-hour resin hydrolyzates. Furthermore, the amount of the hexosamines (computed from the elution patterns and the standards) obtained from the 24 and 30-hour resin hydrolysis and the 3N HCl hydrolysis was comparable and greater than the amount of hexosamines obtained from the 10-hour resin hydrolysis. The results are summarized in Tables VII A and VII B.

TABLE VII A

Recovery of Hexosamines from Commercial CS after Resin and 3N HCl Hydrolysis and Ion Exchange Chromatography

(Results obtained with the automated ninhydrin detection system)

Type of hydrolysis	Duration of hydrolysis	Hexosamine Content	
		Glucosamine*	Galactosamine
	(hours)	(%)	(%)
Resin	10	1.3	22.4
Resin	24	1.4	33.8
Resin	30	1.4	32.4
3N HCl	10	1.7	34.1

* Presence of glucosamine in the commercial CS indicates that the sample was not pure.

TABLE VII B

Recovery of Hexosamines from PP-L after Resin and 3N HCl Hydrolysis and Ion Exchange Chromatography

(Results obtained with the automated ninhydrin detection system)

Type of hydrolysis	Time	Hexosamine Content		Total
		Glucosamine	Galactosamine	
	(hours)	(%)	(%)	
Resin	10	2.4	19.4	21.8
Resin	24	2.5	27.4	29.9
Resin	30	2.7	27.4	30.1
3N HCl	10	2.5	28.4	31.9

The values presented in Table VII A for the galactosamine content of CS after 24 and 30 hours of resin hydrolysis or after 3N HCl hydrolysis, agree with those reported in the literature that have been obtained by other methods (126). The values (Table VII B) for the glucosamine and galactosamine content of PP-L after 24 or 30 hours resin or 3N HCl hydrolysis also agree with values in the literature (127, 129, 130). However, the 10-hour resin hydrolysis values for both CS and PP-L are considerably lower. The chromatograms obtained after 10 hours of resin hydrolysis (Figures 13 and 14) indicate the presence of additional ninhydrin-positive material ahead of the amino-sugar region, thus suggesting that hydrolysis was incomplete at that time. However, resin hydrolysis appears to be complete by 24 hours. This interpretation is supported also by the data that have been presented for glucuronic acid (Table VI A).

(ii) Separation and identification of hexosamines in resin hydrolyzates of rat liver.

The estimation of hexosamines in the liver presents special analytical difficulties. Boas (407) has pointed out that unless a preliminary resin fractionation of the liver hydrolyzates is carried out, erroneously high values are obtained for the total hexosamines. The resin hydrolysis procedure is suitable for analysis of liver as it affords also a fractionation of the hydrolyzate. However, since the author was interested primarily in the effect of cortisone on the

glucosamine synthetic pathway in rat liver, (see 'Experimental', sections 3 - 6), it was desirable to identify the hexosamines present in hydrolyzates of liver of the normal and the cortisone-treated animals. To this end, in addition to the ninhydrin detection system already described, an automated Elson-Morgan detection system also was used.

Ion exchange chromatography of liver hydrolyzates with automated ninhydrin detection systems

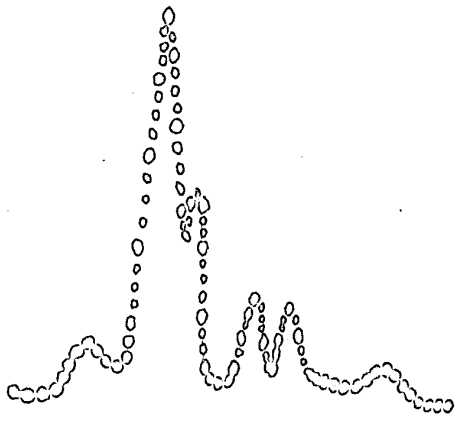
Fifty mg of desiccated liver from normal animals was hydrolyzed by the 'resin' procedure for 24 hours at 100°C. The hydrolyzates were fractionated with water and 2N HCl. The 'acid eluates' were desiccated, dissolved in a known volume of borate buffer, pH 7.6, and aliquots were chromatographed and analyzed with the ninhydrin automated system (p.116). Figure 15 shows typical elution patterns obtained with the hydrolyzates from livers of normal animals. The main peaks in the chromatograms correspond with those of the glucosamine standard. One of the smaller peaks corresponds with that of the galactosamine standard. The identity of the materials represented by the other small peaks is unknown. It is noteworthy, however, that the elution pattern of the unknown peaks was the same in all the experiments. Furthermore, the extraneous peaks do not correspond to the elution times of any of the commonly occurring hexosamines.

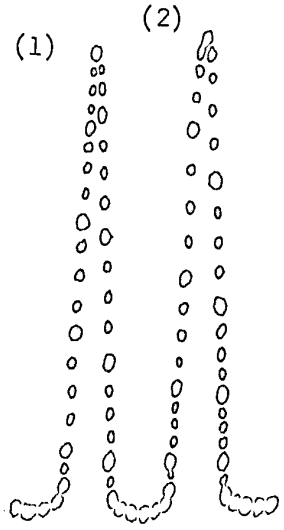
In order to substantiate further the identity of the peaks that correspond to glucosamine and galactosamine, internal standards of these substances were added to the hydrolyzates of normal liver. The results are shown in Figure 16.

FIGURE 15

ION EXCHANGE CHROMATOGRAPHY OF NORMAL LIVER HYDROLYZATES

(Ninhydrin detection system)





Standards

Glucosamine (1)

Galactosamine (2)

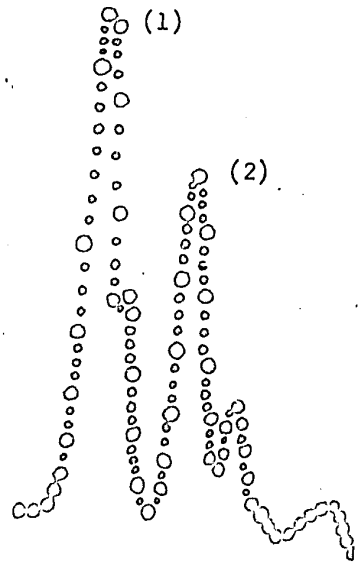
FIGURE 16

ADDITION OF HEXOSAMINE INTERNAL STANDARDS TO LIVER HYDROLYZATES

INTERNAL STANDARDS

75 µg glucosamine (1)

75 µg galactosamine (2)



It is apparent that the addition of glucosamine and galactosamine to the hydrolyzate increased the height of the peaks which were ascribed to glucosamine and galactosamine.

A sample of resin hydrolyzate was analyzed also with the Beckman model 120BB amino acid analyzer, on the long column and under the conditions commonly used for amino acid elution, i.e., with 250 ml of 0.2 N Na citrate buffer, pH 3.25, followed by 250 ml of 0.2 N Na citrate buffer, pH 4.25. The hexosamines were released after tyrosine and phenylalanine in the sequence of elution. The pattern on the chart again was similar to that described with the borate buffer system, in that there were two peaks corresponding, in elution times, to those of the glucosamine and galactosamine standards as well as the extraneous peaks.

Ion exchange chromatography of liver hydrolyzates with the automated Elson-Morgan detection system

To establish unequivocally that the peaks corresponding to glucosamine and galactosamine, from the liver hydrolyzates, represent these amino sugars, and to exclude the possibility that some of the unknown peaks may represent hexosamine-containing material as well, samples of the resin hydrolyzate were chromatographed with the ion-exchange column, equipped with an automated system adapted to the Elson-Morgan reaction. The use of this equipment was kindly made available to the writer by the designer of the adaptation, Prof. John Gregory in Rockefeller University. The apparatus is similar to and the conditions of elution the same as those previously described (p.116) for the ninhydrin adaptation for

chromatography of hexosamines. The detection thus was specific for hexosamines and ignored the presence of non-hexosamine material.

Figure 17 illustrates the results of the separation of hexosamines from a resin hydrolyzate of normal liver after preliminary resin fractionation with the Gregory apparatus.

It is evident that only two hexosamine peaks occurred, namely, those of glucosamine and galactosamine. The quantities of glucosamine and galactosamine expressed as mg per 100 g dry weight of liver, were, respectively, 187.5 and 36.3, or a ratio of about 5 : 1.

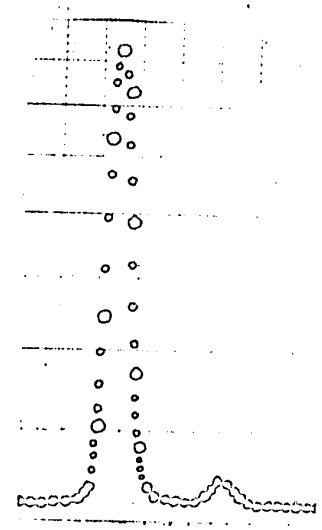
The above mentioned findings, based on both of the ninhydrin and Elson-Morgan reactions, strongly suggest that glucosamine is the main hexosamine present in the liver of normal rats. Galactosamine also is present, but in much smaller concentration. It is apparent, therefore, that the estimation of the total hexosamines in the liver hydrolyzates reflects primarily the amount of glucosamine present. The distribution of thesehexosamines in the livers of cortisone-treated rats is given on page 135.

FIGURE 17

ION EXCHANGE CHROMATOGRAPHY

OF LIVER HYDROLYZATES

(AUTOMATED ELSON-MORGAN)



STANDARDS

Glucosamine (1)

Galactosamine (2)

(c) Methods for estimation of total hexosamines, hexuronic acids and hydroxyproline

Young,male, Sprague Dawley rats were injected subcutaneously daily with 3 mg of cortisone acetate or saline as described previously (page 85). At the end of the ten-day period of injection six of the controls and six of the cortisone-treated animals were killed by exsanguination. The liver, aorta, tibiae and the skin (shaved) were excised and quick frozen in containers immersed in an acetone-dry-ice mixture. The tissues were then lyophilized and the desiccated specimens stored at -20°C until required for analysis.

Weighed portions of the desiccated residues were taken for resin hydrolysis and fractionation. Resin hydrolysis was carried out for 24 hours at 100°C with all the samples, except the skin. It had been observed with the skin specimens which included the whole of the dermis, that after 24 hours resin hydrolysis globules of fatty material were still present. For this reason, the period of hydrolysis was extended to 32 hours. In a subsequent experiment (p.136) portions of the skin were extracted three times with chloroform-methanol (2:1) over a period of 24 hours, and the defatted material then hydrolyzed with the resin for 24 hours at 100°C.

The total content of hexosamines in the acid eluates was estimated with the acetic anhydride modification of the Elson-Morgan procedure (23). Hexuronic acids were estimated in the water eluates according to the method of Dische (286). Hydroxyproline was determined in the acid eluates according to the method of Neuman and Logan (404).

DNA was estimated with a modification (202) of the procedure of Kissane and Robbins (405). The total lipid of the skin from the control and the cortisone-treated animals was estimated gravimetrically on the residue from the chloroform methanol extract. Thin-layer chromatography of the lipids extracted from the skins was carried out on silica gel with a petroleum ether : diethyl ether : acetic acid = 190:55:5 system (406).

The results of these investigations are presented in the following section.

(2) Results.

(a) Hexosamine content of tissues from control and cortisone-treated animals.

(i) Liver.

Total hexosamine.

Since the liver in the rat is known to undergo complex changes on administration of cortisone, the value of the total hexosamines is expressed on the basis of both the dry weight and the DNA content of the tissue. The mean DNA content of the livers from control and cortisone-treated rats (6 animals in each group) respectively, was 5.7 $\pm$ I.2 and 7.2 $\pm$ 0.9 mg/g dry tissue. The difference is significant ($p = 0.01$).

The following Table presents the total hexosamine content of livers, estimated by the modification of the Elson-Morgan procedure (23).

It is evident, when the results are expressed on a dry weight basis, that the livers of the cortisone-treated animals contained significantly less total hexosamine than did those of the controls. Expressed on the basis of the DNA concentration, the total hexosamine concentration in the liver of the cortisone-treated animals was only 63% of that of the controls, and this difference is highly significant.

TABLE VIII

Hexosamine Content of Livers from Normal and Cortisone-Treated Animals

Group *	Average Hexosamine Content (µg/100 mg dry weight)	(µg/mg DNA)
CONTROLS	120 ± 4.4 **	21 ± 0.9
CORTISONE-TREATED	97 ± 3.8	13 ± 0.6
	p = 0.01	p = 0.005

* Six animals in each group

** Standard errors are given henceforth, unless otherwise noted.

Glucosamine and galactosamine content

Four quantitative runs with ion exchange chromatography were carried out with the liver hydrolyzates from normal and cortisone-treated rats, with the aid of the automated ninhydrin detection system (p.116). Typical elution patterns are illustrated in Figure 18, shown on the next page. The resin hydrolyzates from livers of cortisone-treated animals demonstrate an idential elution pattern to the controls. The peaks that correspond in elution times to glucosamine and galactosamine are present in both cases.

The amounts of the two hexosamines were computed on the basis of glucosamine and galactosamine standards which were chromatographed after each hydrolyzate. Table IX summarizes the results.

TABLE IX

Glucosamine and Galactosamine Content of

Livers of Normal and Cortisone-Treated Rats

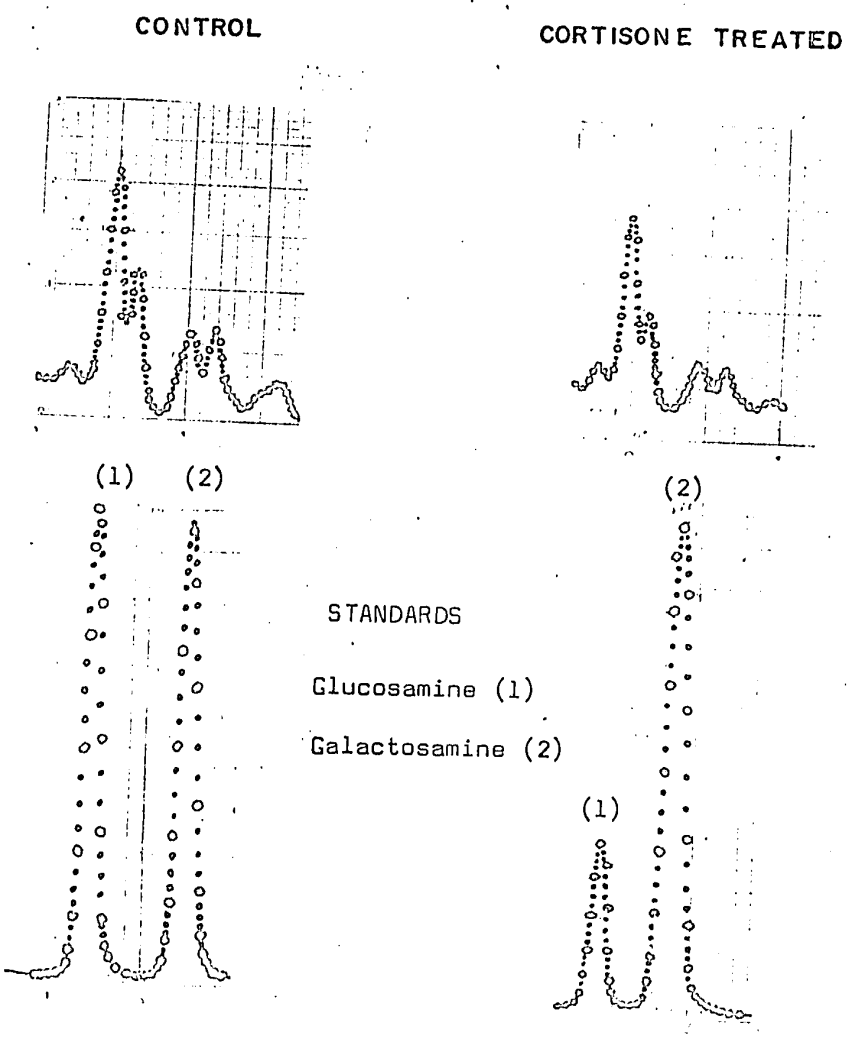
Group	Glucosamine (μg/100 mg dry liver)	Galactosamine (μg/100 mg dry liver)
CONTROL	189	69
	154	44
CORTISONE-TREATED	100	37
	134	50

These two experiments appear to support the data in Table VIII and indicate that the glucosamine content in the liver of the cortisone-treated

FIGURE 18

ION EXCHANGE CHROMATOGRAPHY OF LIVER HYDROLYZATES FROM CONTROL AND CORTISONE-TREATED ANIMALS

(NINHYDRIN DETECTION SYSTEM)



animals was decreased compared to that of the controls. However, no conclusion can be drawn about the galactosamine content of the liver in the two groups.

(ii) Hexosamine content of the skin.

In this study the whole dermis-epidermis area was used for analysis. In view of possible complication of the estimation of hexosamines from the large amount of lipid present in the dermis, two experiments were carried out with untreated and defatted skin from the control and cortisone-treated rats (see also p.130). In the first experiment, the unextracted skin was subjected to a 32-hour period of resin hydrolysis; in the second, the skin, previously defatted by extraction with methanol-chloroform, was subjected to a 24-hour period of the hydrolytic treatment.

Table X gives the results of the experiment with nondefatted skin. There was little difference in the hexosamine content of the skin between the control and cortisone-treated group on a dry-weight basis. It is apparent, however, that the dermal -epidermal region of the control animals contained,on the average, approximately twice as much methanol-chloroform extractable lipid as that of the cortisone-treated animals. When the amount of hexosamine released on hydrolysis is expressed in terms of fat-free dry weight, as shown in Table XI, the results are considerably different from those in the preceding Table.

TABLE X

Total Hexosamine and Total Lipid Concentration of Skin from Control and Cortisone-Treated Rats

Group *	Total Hexosamine content** (μmoles/g dry weight)	Total lipid content (% of dry weight)
CONTROL	8.1 ± 0.9	50.0 ± 4.5
CORTISONE-TREATED	8.8 ± 0.8	22.8 ± 3.1
STATISTICAL TREATMENT	N.S. ***	p = 0.01

* Six animals in each group.

** Hexosamine released after 32 hours of resin hydrolysis.

*** Difference between control and treated group is not significant.

TABLE XI

Total Hexosamine Content of Skin from Control and Cortisone-Treated Rats **

Group *	Total Hexosamine Content (μmoles/g dry defatted weight)	
	24 Hour Hydrolysis	36 Hour Hydrolysis
CONTROL	14.0 ± 1.3	16.4 ± 1.2
CORTISONE-TREATED	10.0 ± 0.8	11.4 ± 0.9
STATISTICAL TREATMENT	$p = 0.05$	$p = 0.05$

* Six animals in each group.

** Results corrected for lipid content of dry tissue.

Thus, calculated on the basis of the dry weight of the defatted tissue, significantly less hexosamine was released from the skin of the cortisone-treated animals than from the controls, in both the 24 or 36-hour resin hydrolysis. It is apparent also, that about 15% less hexosamine was released from the defatted samples after 24 hours of hydrolysis than from the untreated skin specimens after 36 hours of hydrolysis . It is not evident whether this difference is attributable to the longer hydrolytic treatment or to loss of hexosamine during the defatting process. Nevertheless, the values for the control and cortisone-treated groups are comparable in the two experiments, i.e., the ratio of hexosamine (control) : hexosamine (cortisone) is about 1 : 0.8 in both cases.

Thin-layer chromatography was carried out with samples of the chloroform-methanol extractable lipid from the skin of control and cortisone-treated animals, as referred to previously (p.130). Among the substances identified from comparison of the Rf values with those reported in the literature (406) and from data acquired in our own laboratory were: cholesterol esters, triglycerides, 1,2-diglycerides and phospholipids. These substances were among the lipids extracted from the skin of both the control and cortisone-treated groups. A quantitative study of the lipids was not undertaken.

(iii) Hexosamine content of tibial epiphyses and the aorta.

To obtain sufficient material for analysis with these tissues, it was necessary to include the entire sample from the animal. Furthermore, the weight of the specimens from the cortisone-treated animals

was usually about half that from the controls.

The following Table gives the total hexosamine content of the epiphyses (including articular cartilage) of the proximal part of the tibiae and of the aorta from the control and the cortisone-treated animals.

TABLE XII

Hexosamine Content of Epiphyses and Aorta from Control and Cortisone-Treated Rats

Group*	Hexosamine Content	
	Epiphyses	Aorta
	(μmoles/g dry tissue)	
CONTROL	82.9 ± 1.4	26.1 ± 1.2
CORTISONE-TREATED	83.0 ± 1.4	20.4 ± 1.1
STATISTICAL TREATMENT	N.S.	p = 0.005

* 6 animals in each group.

While there was no difference in the hexosamine content of the epiphyses, the aorta of the cortisone-treated animals contained significantly less hexosamine than that of the controls.

(b) Hexuronic acid content of skin and aorta from control and cortisone-treated animals.

With the liver hydrolyzates, an atypical, dark colour was obtained with the Dische reaction (286). A similar colour was produced on the addition of the sulfuric acid alone. Spectral analysis or the coloured products obtained with the Dische reaction and the liver hydrolyzates, indicated the presence of an additional peak at 410 mμ, along with the typical hexuronic acid peak at 530 mμ. The writer concluded, therefore, that the values for hexuronic acid content of the livers, with this method, were unreliable, and accordingly were rejected. The following two Tables give the analytical results obtained with the skin, aorta and the epiphysial specimens.

TABLE XIII

Hexuronic Acid Content of Skin from Control and Cortisone-Treated Rats

Group *	Hexuronic Acid Content	
	Dry tissue	Dry defatted tissue
	(μmoles/g)	
CONTROL	7.4 ± 0.8	11.0 ± 0.5
CORTISONE-TREATED	7.8 ± 0.7	7.8 ± 0.4
STATISTICAL TREATMENT	N.S.	p = 0.005

* 6 animals in each group.

TABLE XIV

Hexuronic Acid Content of Aorta and Epiphyses from Control and Cortisone-Treated Rats

Group *	Hexuronic Acid Content	
	Aorta	Epiphyses
	(μmoles/g dry tissue)	
CONTROL	14.7 ± 1.1	75.5 ± 0.8
CORTISONE-TREATED	10.5 ± 0.6	85.0 ± 1.6
STATISTICAL TREATMENT	p = 0.005	N.S.

* 6 animals in each group.

As with the hexosamines, the hexuronic acid content of the skin from the cortisone-treated animals was significantly less than that of the controls. This is so, only when the results are expressed in terms of the dry weight of the defatted tissues. Expressed on the basis of the unadjusted dry weight, there is no significant difference between the values for the two groups.

The aorta from the cortisone-treated animals, on the other hand, contained significantly less hexuronic acid than that of the controls on a dry weight basis. As with the hexosamines, there was no significant difference in the hexuronic acid content of the epiphysis.

(c) Hydroxyproline content of liver, aorta, skin and epiphysis from control and cortisone-treated rats.

The results of these analyses are summarized in Table XI.

TABLE XV

Hydroxyproline Content of Tissues from Normal and Cortisone-Treated Animals

Group*	Hydroxyproline Content				
	Liver	Skin (A)	Skin (B)**	Aorta	Epiphysis
	(mg/g dry tissue)				
CONTROL	0.24 ± 0.005	21.9 ± 0.3	33.6 ± 0.3	50.6 ± 0.9	22.7 ± 0.5
CORTISONE-TREATED	0.28 ± 0.005	39.5 ± 0.6	43.9 ± 0.3	40.6 ± 1.2	27.8 ± 0.5
STATISTICAL TREATMENT	p = 0.05	p = 0.01	p = 0.01	N.S.	p = 0.05

* 6 animals in each group

** Results expressed on the basis of dry defatted tissue.

The hydroxyproline content of liver, skin and epiphysis is significantly increased in the cortisone-treated animals as compared to controls. The hydroxyproline content of the aortas from the treated animals was less than the controls but the difference was not statistically significant ($p = 0.1$).

3. THE EFFECT OF DAILY CORTISONE ADMINISTRATION ON THE CAPACITY OF RAT LIVER FRACTIONS TO SYNTHESIZE GLUCOSAMINE-6-PHOSPHATE.

Early in this investigation the writer observed that daily administration (by injection) of relatively large doses of cortisone to young Sprague Dawley rats, resulted in suppression of the capacity of subcellular fractions of the livers of the animals, to synthesize glucosamine-6-P from glucose-6-P (or fructose-6-P) and glutamine. Thus cortisone inhibited the initial step of the dominant pathway of the synthesis of the intermediaries leading to the formation of the hexosamines contained in MPS and glycoproteins (p. 9).

The observed inhibition of glucosamine-6-P synthesis by cortisone suggested that this reaction, which is catalyzed by amidotransferase, may represent a site of inhibitory action of cortisone on MPS synthesis. The phenomenon, therefore, was studied in fair detail.

(1) Methods.

(a) Preparation of liver fractions and procedure of assay of 'amidotransferase' activity from control and cortisone-treated rats.

Young rats were injected with cortisone as previously described (p. 85). At various time intervals, some of the rats of the cortisone-treated and the control groups were anesthetized with ether and killed by exsanguination, through an incision in the ventricle. The livers

were quickly removed and placed in tared beakers containing about 20 ml of ice-cold 'homogenization medium' prepared according to the method of Ghosh et al. (23). The homogenization medium was composed of 0.154M KCl, 0.001M EDTA and mercapto-ethanol 0.2 mg/ml. The beakers and contents were reweighed and the wet weight of the livers determined within 0.01 grams. The liver then was transferred to a cooled glass homogenizer and a quantity of the ice-cold homogenization medium was added to bring the tissue to medium proportion to 0.33% (w/v). The liver was lightly blotted with tissue paper, cut into small pieces and quickly dropped into the homogenizer, and the homogenizer was surrounded with ice. Homogenization was carried out manually with a teflon plunger. About 12 up and down movements of the plunger were sufficient to give free movement and adequate homogenization. The subsequent steps of fractionation and assay were performed according to the procedures of Pogell and Gryder (21) and of Roseman and coworkers (23), as indicated below.

In some of the experiments, 0.2 ml of liver homogenates were incubated at 37°C for 1 hour, with 1.2 ml of incubation mixture, composed of the following substances in μmoles: G-6-P(Na).$3H_2O$*15.00, l-glutamine 18.75, EDTA 2.50, phosphate buffer (Na), pH 7.4, 45.00. The incubation was carried out at 37°C for 1 hour and the reaction stopped by immersing the tubes in boiling water for 3 minutes. Zero-time samples always were included. This incubation system will henceforth be referred to as the 'ordinary' incubation system.

In most of the experiments, the homogenate was centrifuged at 18,000 x g for 1 hour, either in a Lourds refrigerated centrifuge or

*No difference in assay values was observed whether G-6-P or F-6-P were used as substrates in this system as has been previously reported (21, 23).

in a Spinco, model L, preparative ultracentrifuge. The clear supernatant represented a preparation of soluble enzymes (see below). An 0.2 ml aliquot of the supernatant was taken for incubation with 1.2 ml of the 'incubation' mixture, under conditions identical to those described above for whole homogenates.

After immersion of the tubes in boiling water for 3 minutes stop reaction, the samples were centrifuged at 2,000 r.p.m. for 15 minutes. Portions (0.4 ml) of the supernatant were then analyzed for glucosamine-6-P content with the 'acetic anhydride' modification of the Elson-Morgan procedure (23). Standards of 0.04 and 0.08 μmoles of crystalline glucosamine HCl were included in each run. Glucosamine-6-P gives about 85% the absorption of glucosamine (on a molar basis) with the Elson-Morgan reaction, but both substances were found to have an identical absorption spectrum with a maximum at 585 mμ. No correction was made for the difference in the absorption.

In all cases the livers from the control and cortisone-treated rats were processed simultaneously. The incubations and assays for hexosamine also were carried out simultaneously. The results of these assays are summarized in Figures 23 and 24, and Tables i, ii and iii of the Appendix. Each value represents the mean from two incubations and each incubation mixture was assayed in duplicate.

(b) Subcellular distribution of amidotransferase and glucose-6-P phosphatase in the liver of control and cortisone-treated rats.

Liver homogenates from control and cortisone-treated animals were prepared in 0.154M KCl, EDTA and mercapto-ethanol as described above, (p.145). A quick and simplified fractionation scheme was fol-

lowed as the amidotransferase was relatively unstable (p. 160). The experiments of amidotransferase assay (p. 145) were done either with this fraction or the whole homogenate. The subcellular fractionation was prepared as follows: The whole homogenate was centrifuged at 1,200 x g for 15 minutes to sediment the nuclei and the debris ('N'-fraction). The supernatant was removed and centrifuged at 18,000 x g for 1 hour to sediment other subcellular particles ('P'-fraction). The clear supernatant ('S'-fraction) was removed. The 'N'-and the 'P'-fractions were suspended in a volume of 'homogenization mixture' equal to that of the 'S'-fraction, and portions of each fraction were assayed for amidotransferase activity as described above (p.145). The glucose-6-P phosphatase activity in the various fractions was assayed under the conditions used in the assay of the amidotransferase activity. In this assay, glucose-6-P and glutamine were added in phosphate buffer and the activity of the phosphatase was assayed by measuring the amount of glucose released from the glucose-6-P. Glucose was measured with the glucose-oxidase reagent (392). The results of this experiment are presented in Table XVII.

(c) Isolation and identification of the Elson-Morgan-positive product in the incubation mixture.

Other authors (21) indicate that the Elson-Morgan-positive substance, formed on incubation of hexose-6-phosphates with rat liver extracts is, Gm-6-P. Nevertheless, it was important to establish the identity of the product in the liver supernatants from the normal and

the cortisone-treated rats. Furthermore, it was necessary to investigate whether other Elson-Morgan-positive products also might be formed in the course of the incubation, as for example, glucosamine from the action of a phosphatase on glucosamine-6-phosphate (389) The presence of such products would lead to erroneous interpretation of the assay results. To obtain quantitative results, the ion-exchange chromatographic procedure of Pogell and Gryder (21) was modified as follows:

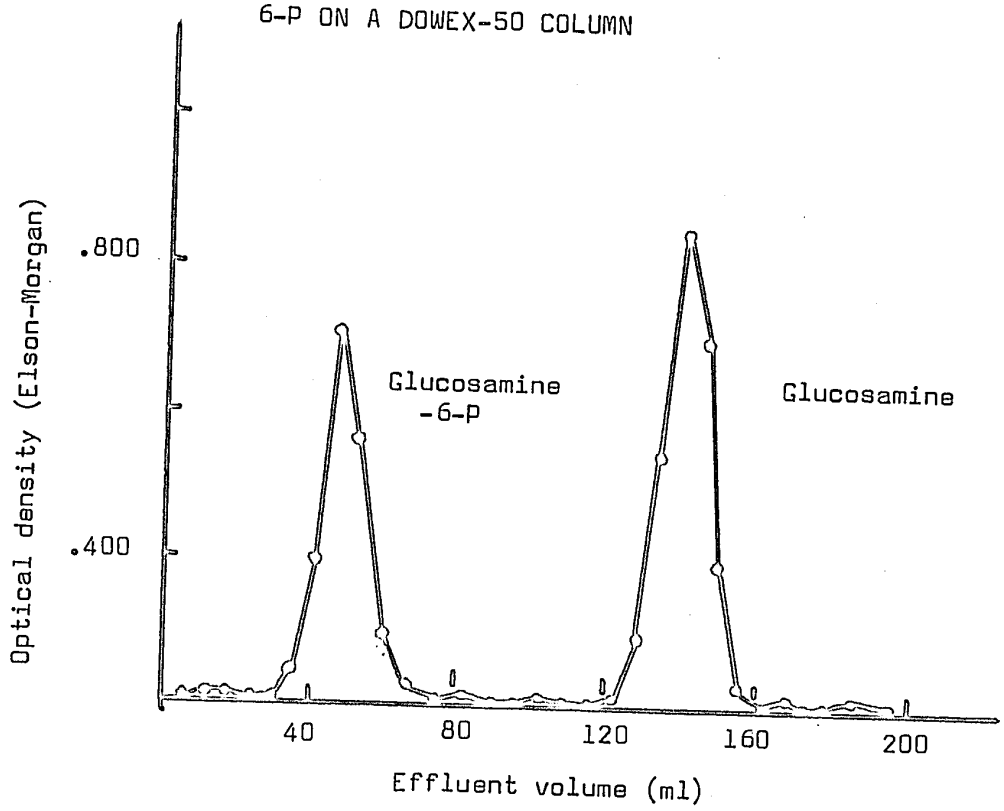
A 15 x 1 cm. column of Dowex-50, 200-400 mesh (H form) was equilibrated with distilled water. The lower, closed chamber of the reservoir system, which contained 150 ml of distilled water, was connected to an open, upper chamber containing about 250 ml of 1.0N HCl. The contents of the lower chamber were kept stirred with a magnetic stirrer and the outflow tube was connected with a Beckman pump and flowmeter, adjusted to permit the flow of the liquid at 0.8 ml/minute. The pump outflow, in turn, was connected, by ground glass fittings, with the column in a closed system. The effluent from the column was collected in 3.0 ml fractions, in test tubes with an automatic fraction collector, controlled by the drop-counting method. Portions (0.5 ml) of each of the fractions were analyzed for hexosamine content according to the method of Gardel (390).

(i) Ion-exchange chromatography of glucosamine-6-P standards.

Figure 19 illustrates the separation obtained with a mixture of glucosamine and a sample of glucosamine-6-P (crystalline, Nutritional Biochemicals Corp.), with the above-described system.

FIGURE 19

ION-EXCHANGE CHROMATOGRAPHY OF GLUCOSAMINE AND GLUCOSAMINE-6-P ON A DOWEX-50 COLUMN



The glucosamine-6-P and glucosamine peaks, respectively, were obtained at about the 50th and the 150th ml of effluent. These substances were eluted consistently in this manner and with a recovery higher than 90%.

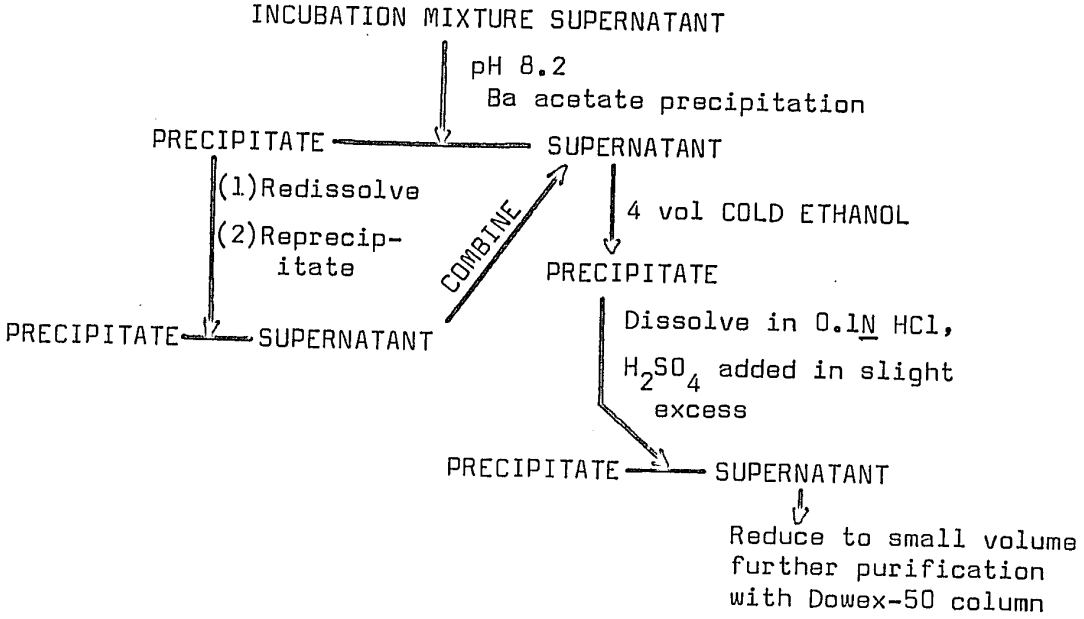
Amother means used to obtain information concerning the synthesis of glucosamine-6-P by liver supernatants, was measurement of the incorporation of radioactive label precursors into the hexosamine product of the reaction. The most convenient radioactive precursor for use as the substrate was glucose-6-p-U-^{14}C. Ion-exchange chromatography of the labelled hexosamine product would provide information as to the specific activity of the product and the identity of the

hexosamine. The specific activity might indicate any difference in the pool size of either the glucose-6-P or the hexosamine product between the liver supernatants from control and cortisone-treated animals.

For this experiment it was necessary to prepare a substrate. Glucose-6-P-U-^{14}C was prepared from glucose-U-^{14}C and ATP as follows: 100 ml of 0.1M tris buffer, pH 7.5, containing 50 μC glucose-U-^{14}C, 5.0 millimoles of 'cold' D-glucose, 2.0 millimoles $MgCl_2$ and 10 mg hexokinase (Sigma, Grade II), was incubated at 37°C for one hour. The reaction was stopped by heating at 100°C for 1 minute and the preparation was cooled and centrifuged. The glucose-6-P-U-^{14}C was isolated and partially purified by means of the 'barium-alcohol procedure' (391), as outlined below:

FIGURE 20

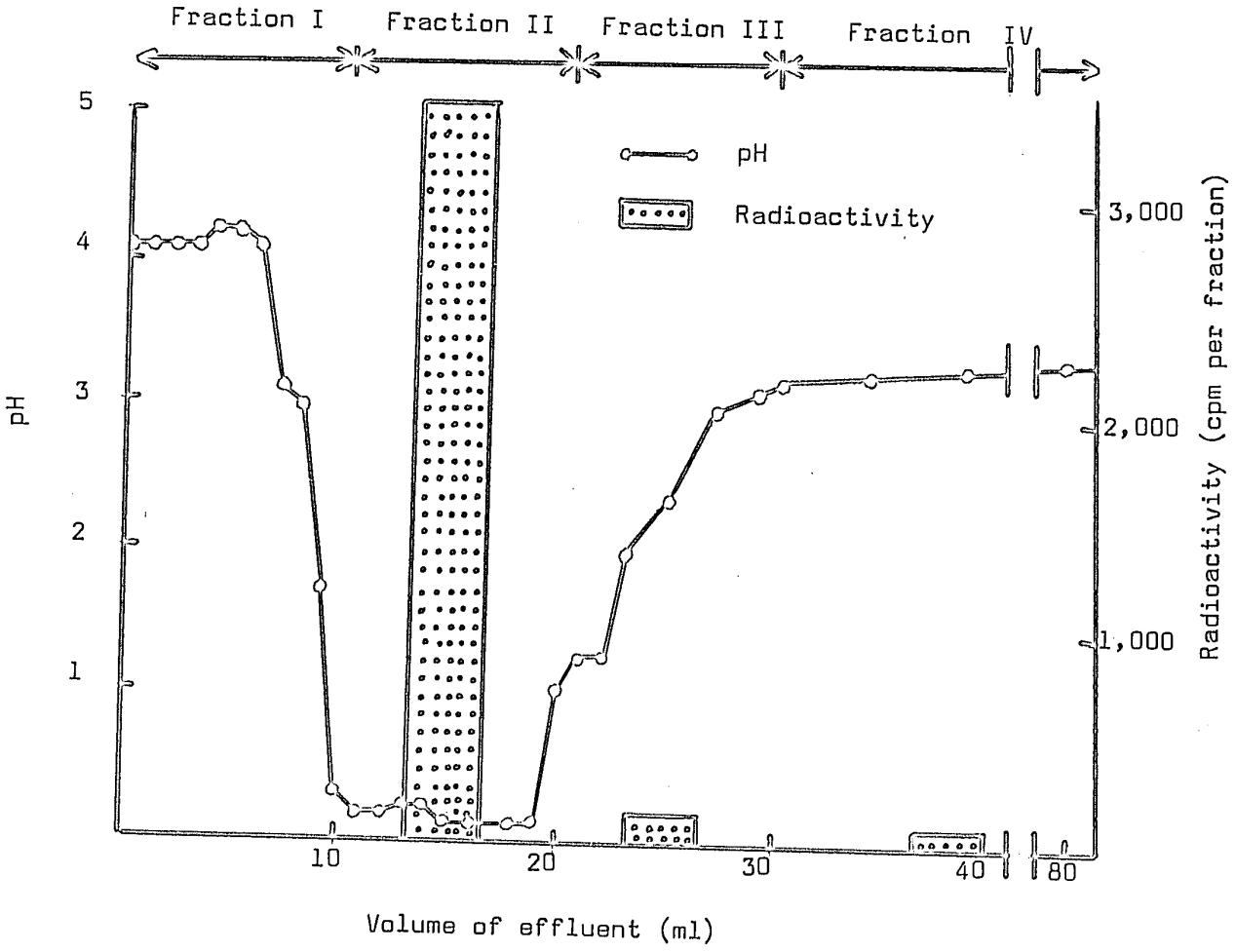
ISOLATION OF GLUCOSE-6-P-^{14}C FROM AN INCUBATION MIXTURE WITH THE 'BARIUM-ALCOHOL PROCEDURE'



The final supernatant was further purified, by concentration under reduced pressure, to a volume of about 4 ml. The pH was adjusted to 6.0 and the sample chromatographed on a Dowex-50 column, 200-400 mesh (H^+) 40 x 1 cm. Elution was carried out with slightly acidified distilled water (pH 5.0). The pH of the column effluent was recorded periodically. Four fractions were collected and the radioactivity in each fraction, measured. The results are indicated in Figure 21.

FIGURE 21

PURIFICATION OF GLUCOSE-6-P-U-^{14}C ON DOWEX-50 COLUMN



The fraction II, which was eluted at the lowest pH, was found to contain most of the radioactivity. This fraction was neutralized with NaOH and lyophilized. The yield of the desiccated material was 600 mg, with a specific activity of 7,240 cpm/mg. This material, on analysis, gave 95% of the reducing equivalent of the authentic crystalline G-6-P (dry disodium salt). On assay with the 'ordinary' system (p.145) for glucosamine-6-P synthesis, the result was the same as with an equal quantity of crystalline G-6-P.

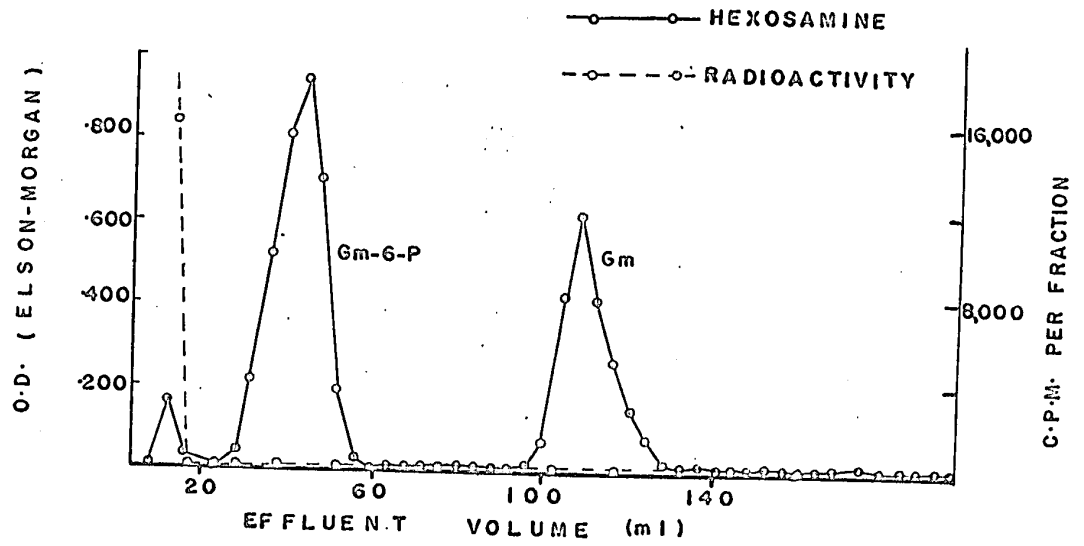
(ii) Separation of glucose-6-P-U-^{14}C and glucosamine-6-P with ion-exchange chromatography.

That relatively large (substrate) amounts of glucose-6-P-U-^{14}C can be separated satisfactorily from glucosamine-6-P, was demonstrated as follows: To a solution containing glucosamine-6-P and glucosamine standards and 30 mg of the prepared glucose-6-P-U-^{14}C was added. The material was chromatographed on the Dowex-50 column with the gradient elution system as described. Portions of each fraction were analyzed for hexosamines (p.146), and other portions were applied to filter paper and counted in a gas-flow system (Nuclear - Chicago). Figure 22 illustrates the separation achieved.

It is clear that the radioactive glucose-6-P came off the column at a relatively early stage at the 18th ml of effluent and that there was no overlap with the elution of the glucosamine-6-P. The first small peak with the Elson-Morgan procedure gave a nonspecific and atypical colour similar to the colour given by a large amount of glucose-6-P.

FIGURE 22

CHROMATOGRAPHIC SEPARATION OF GLUCOSE-6-P-U-^{14}C, GLUCOSAMINE-6-P AND GLUCOSAMINE



It would then appear that this method would be suitable for separating and identifying the hexosamine products obtained on incubation of liver supernatants with glucose-6-P-U-^{14}C and cold glutamine.

The actual experiments with normal and cortisone-treated animals were performed as follows: Three Sprague-Dawley, male rats, 23 days of age, were injected daily for 7 days with 5 mg cortisone acetate, as previously described (p. 85). The livers from the treated animals were pooled, as were the livers from the 3 control animals. The livers were

homogenized and the 18,000 x g supernatant was prepared (p.145). 'Ordinary' incubation mixtures (p.145) were prepared, except that glucose-6-P was replaced by the glucose-6-P-U-^{14}C at the same concentration. The concentration of the labelled glucose-6-P was calculated on the basis of glucose-6-P (disodium salt . $3H_2O$).

A quantity (3.3 ml) of the enzyme preparations from the control and the cortisone-treated rats, was added to a flask containing 20 ml of the incubation mixture containing the labelled glucose-6-P. Incubation of the samples was carried out for 1 hour at 37°C. The reaction was stopped by addition of 5 ml of 20% trichloroacetic acid. The mixture was kept in the cold for 15 minutes and then centrifuged. The supernatants were extracted 4 times with 10 ml of diethyl ether to remove the trichloroacetic acid, and the preparation was lyophilized. The dry residue in each tube was dissolved in 1.5 ml distilled water and 1.0 ml of the solution was chromatographed on the Dowex-50 column, as described. Measurement of the optical density of the Elson-Morgan colour (390), and of the radioactivity were done on aliquots of the 3.0 ml fractions eluted from the column. The results of a typical experiment are given in Figure 26.

(2) Results.

The results of the assays for amidotransferase activity in homogenates and supernatants from livers of normal and cortisone-treated rats are given first. The validity of the difference in activity observed in the liver supernatants from the two groups is then demonstrated by means of ion-exchange chromatography of the hexosamine product.

(a) Glucosamine-6-P synthesis in homogenates and supernatants from normal and cortisone-treated rats.

(i) Homogenates.

The results are indicated in Figure 23 and in Table i , of the Appendix.

It is apparent that after about the second day of cortisone administration the capacity of the liver homogenates to synthesize glucosamine-6-P decreased and remained at a low level during the remaining 24 days of the experimental period. Nevertheless, it will be observed that in addition to the relatively low yields of glucosamine-6-P, (compared to values obtained with supernatants, p.158), there was also considerable variability between experiments. These disadvantages were not observed in experiments with the liver supernatant fractions, the results of which are presented below.

FIGURE 23

EFFECT OF DAILY INJECTIONS OF CORTISONE ON THE AMIDOTRANSFERASE ACTIVITY IN WHOLE LIVER HOMOGENATES

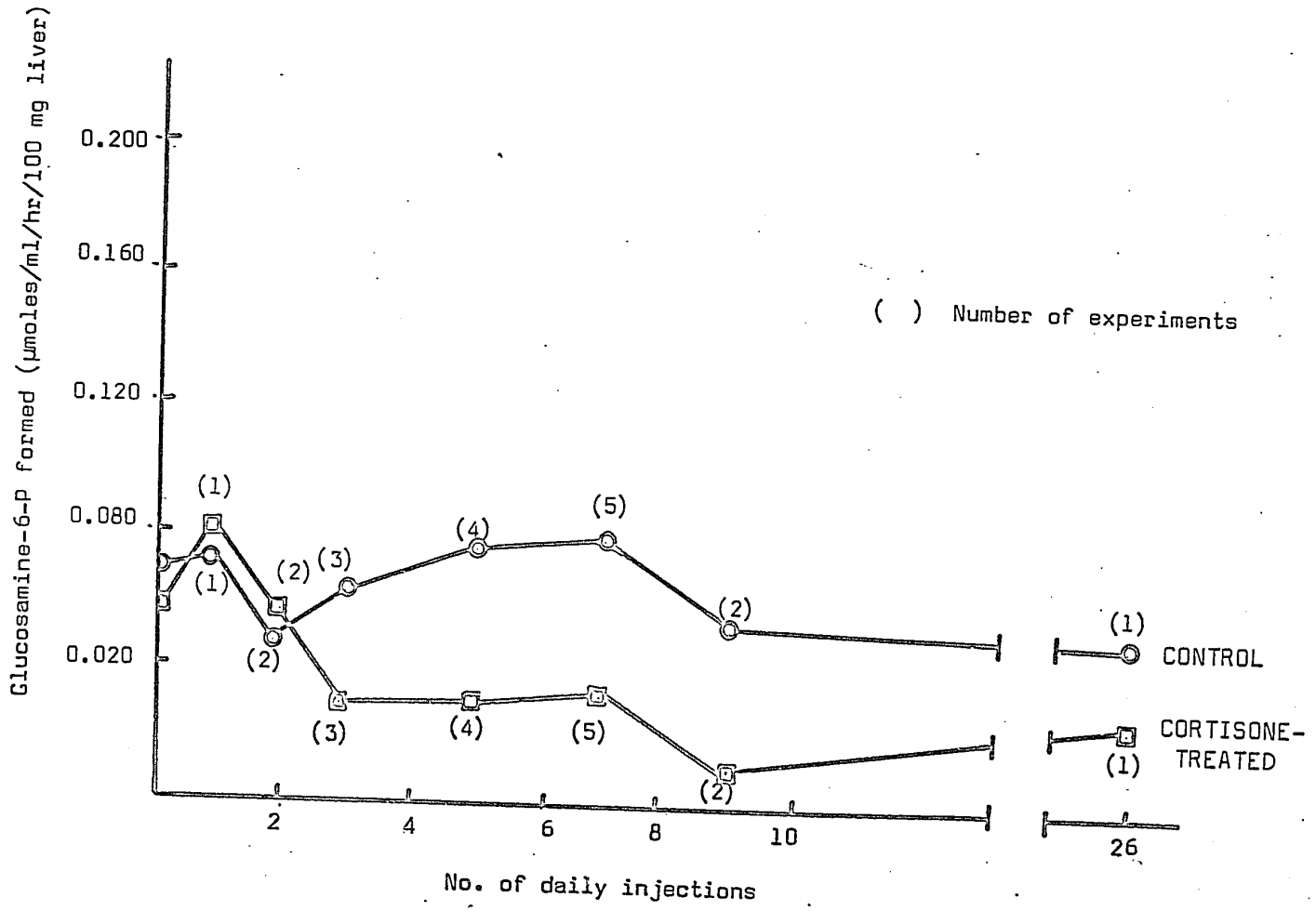
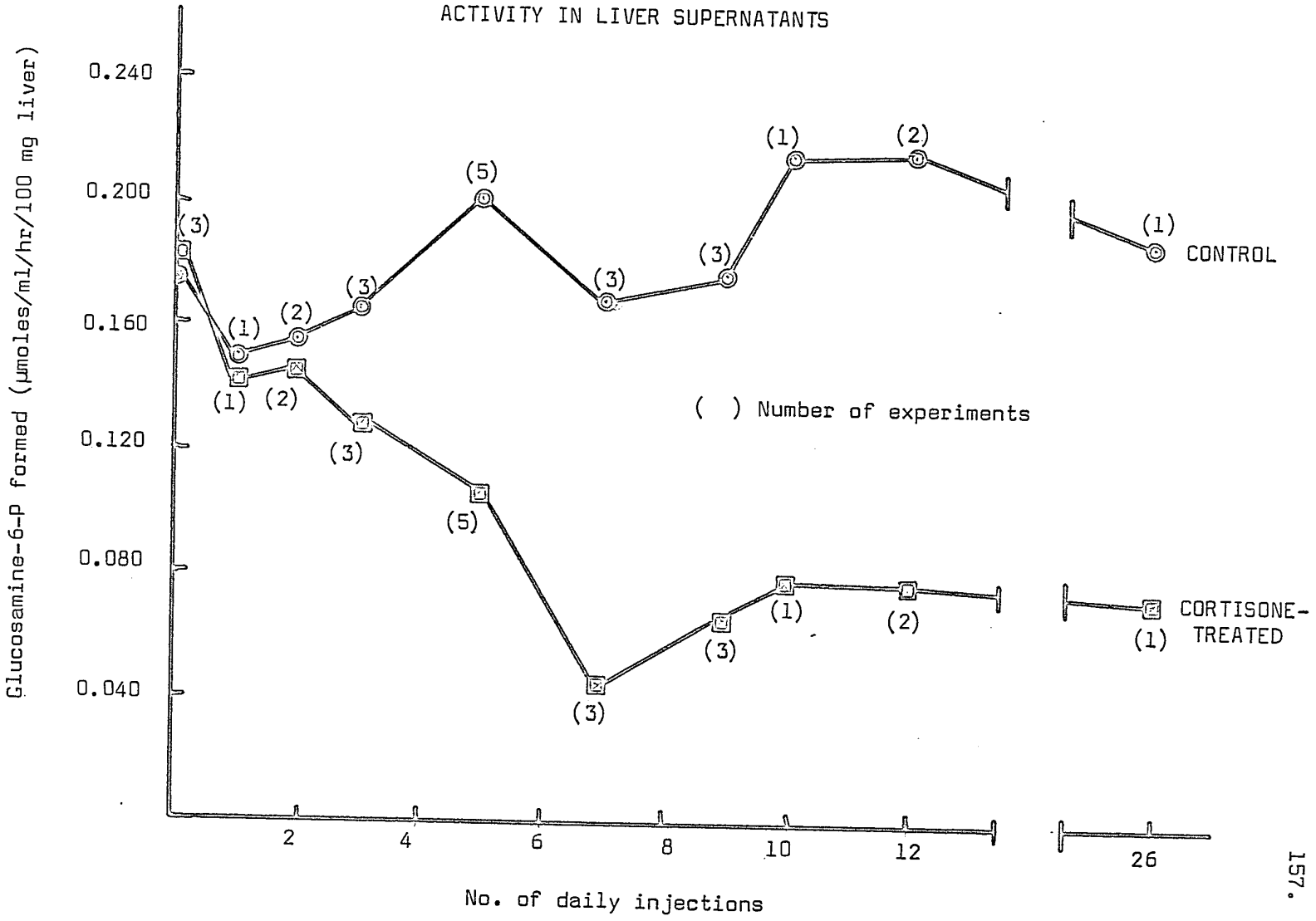


FIGURE 24

EFFECT OF DAILY INJECTIONS OF CORTISONE ON THE AMIDOTRANSFERASE ACTIVITY IN LIVER SUPERNATANTS



(ii) Supernatants (18,000 x g fraction)

Figure 24 and Table ii,in the Appendix, illustrate the results of assays of supernatants prepared from livers of control and cortisone-treated animals.

It is apparent that the amount of glucosamine synthesized per hour by the supernatants, from the cortisone-treated animals, fell off sharply between the 2nd and 7th day of injection, and remained at a constant level thereafter.

The results are expressed in terms of total (wet) liver weight. Several investigators have reported that the nitrogen content of the liver (393) and the liver supernatants (394), tends to be increased after glucocorticoid administration. In confirmation of these observations, the writer found the mean protein concentration (395) of the liver supernatants, after five days of cortisone administration, to be 2.1 mg % compared to the control mean of 1.6 mg %. After 10 days of cortisone administration the mean concentrations were 2.8 and 1.5 mg % for the experimental and the control groups, respectively. As observed previously also (p.132), the DNA content of the liver from animals that had been treated with cortisone was found to be higher than that of the controls. It is apparent, therefore, with regard to the capacity to synthesize glucosamine-6-P, that when the experimental results are expressed on the basis of protein, total nitrogen or DNA, an even greater difference would be observed between the capacity of the control and the cortisone-treated animals.

(iii) Influence of storage of liver supernatant preparations on the stability of the amidotransferase.

It may be recalled that Pogell and Gryder (21) and Ghosh et al. (23) found that the amidotransferase obtained from rat liver was relatively unstable in storage at 4°C, or even -20°C. The author also has studied the stability of the amidotransferase. Table XVI indicates typical results with regard to the synthesis of glucosamine-6-P from experiments with freshly prepared supernatant from the liver of normal animals and with supernatant that had been stored for 6 hours at 4°C.

TABLE XVI

Comparative Assay of the Glucosamine-6-P Formed by Freshly Prepared and from Stored Liver Supernatants

Experiment Number	Rat Number	State of Preparation	Glucosamine-6-P Produced (μmoles/ml incubation mixture/hour)
I	1	Fresh	0.13
	2	Fresh	0.14
II	1	Fresh	0.12
	2	Fresh	0.13
I	1	Stored 6 hours at 4°C	0.05
	2	Stored 6 hours at 4°C	0.06
II	1	Stored 6 hours at 4°C	0.05
	2	Stored 6 hours at 4°C	0.06

It is apparent that storage of the supernatant preparation for 6 hours at 4°C resulted in a loss of about 50% in the enzyme activity, i.e., the capacity to synthesize glucosamine-6-P. Furthermore, no stabilization was noted on storing at -20°C or on addition of glucose-6-P to the fresh supernatant before storage.

In view of the observed decrease in the synthetic activity in the supernatants on storage it was of importance to determine the rate of loss of activity so that correction might be made in cases where delay in performing the assay may be unavoidable. Accordingly, a further study was undertaken of the rate of loss of activity in the supernatants during storage at 4°C.

Supernatant fractions from control and cortisone-treated animals were kept at 4°C and assayed periodically for the capacity to synthesize glucosamine-6-P. The results of the assays are indicated in Figure 25-A.

It is apparent from Figure 25-A that the relationship between the synthetic activity and the duration of storage of the supernatant at 5°C is nonlinear. The curve for the activity of the supernatants from cortisone-treated animals still is nonlinear when plotted on a semilogarithmic scale. Thus, one would not be certain of the validity of a correction of amidotransferase activity in liver supernatants from normal or cortisone-treated animals in which a delay in assay was unavoidable. For these reasons the values obtained from such experiments have been set in a separate Figure (Fig. 25-B), and Table in the Appendix (Table iii). As noted with the fresh supernatants

FIG. 25A
LOSS OF ACTIVITY OF THE AMIDOTRANSFERASE WITH TIME IN LIVER SUPERNATANTS FROM NORMAL AND CORTISONE TREATED RATS

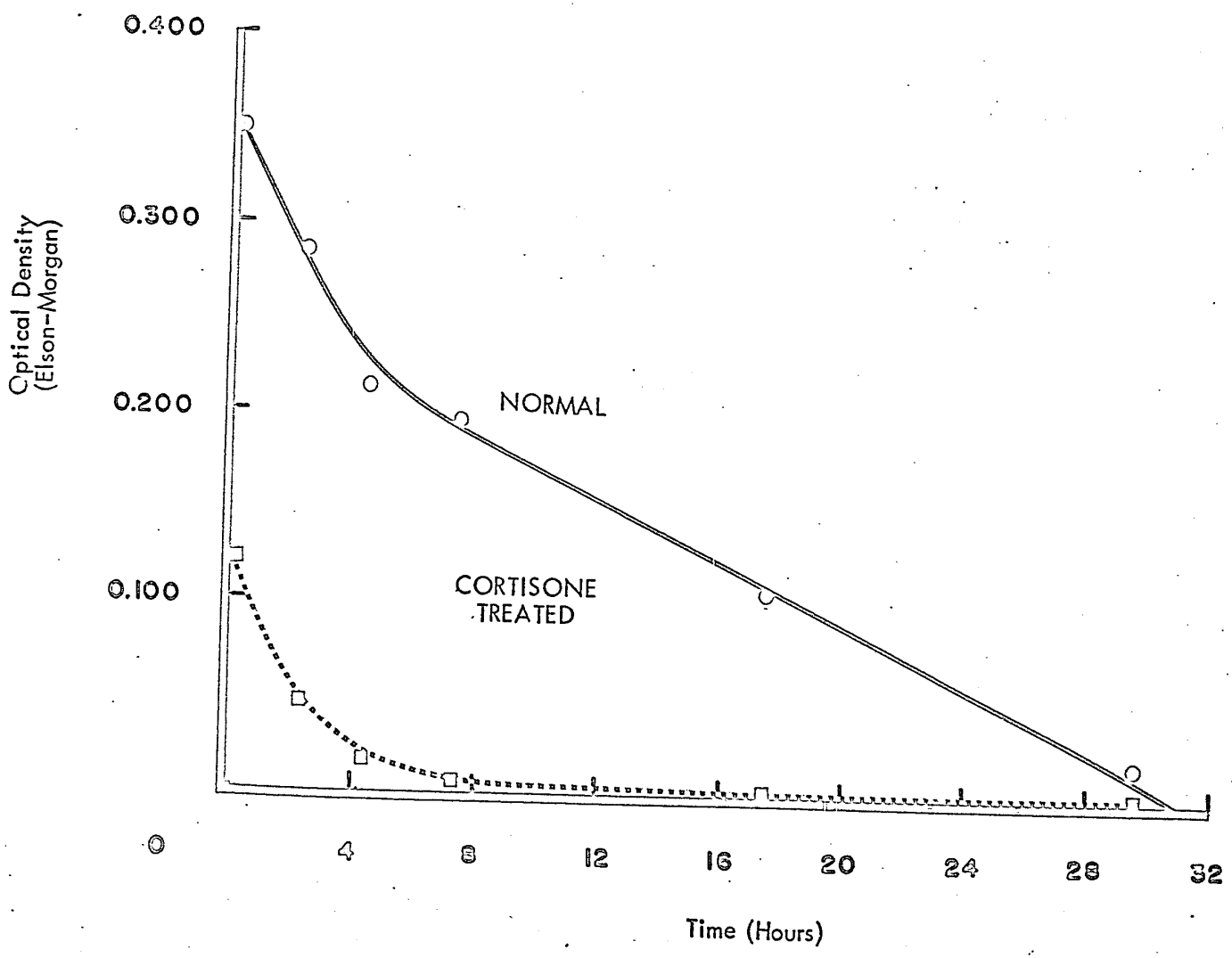
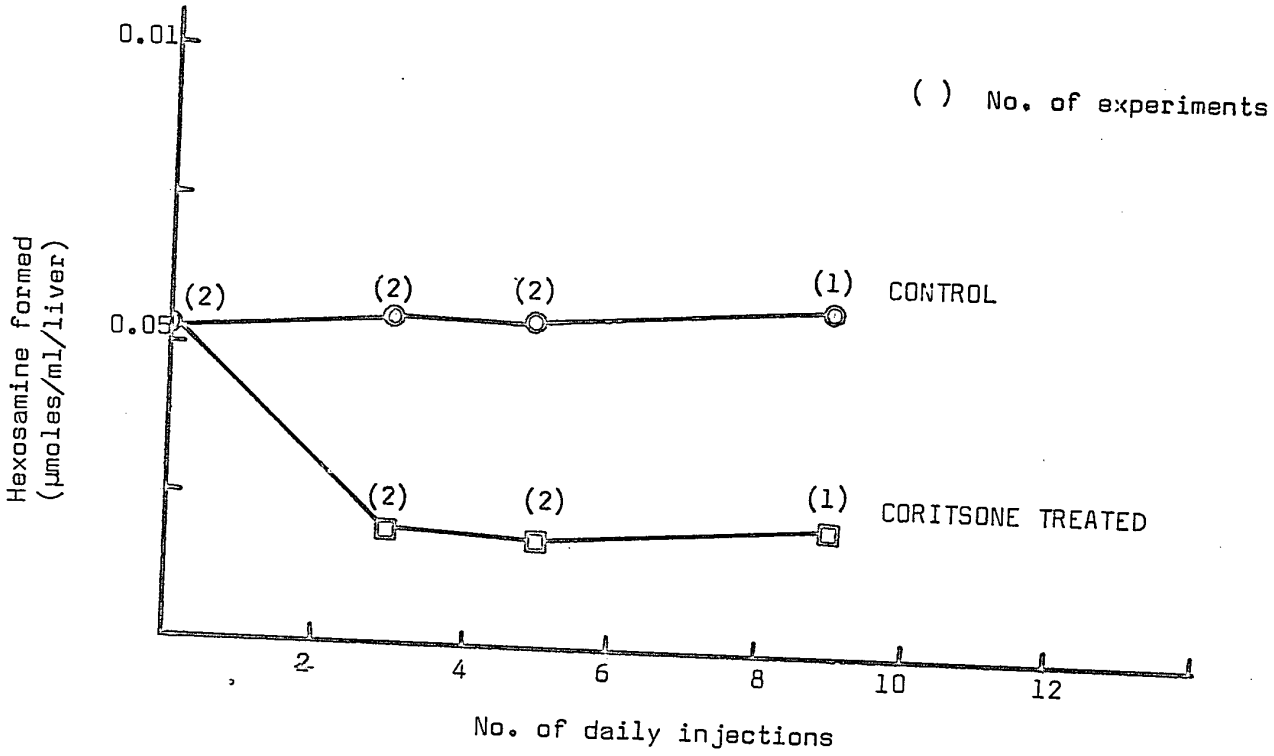


FIGURE 25 B

EFFECT OF DAILY INJECTIONS OF CORTISONE ON THE AMIDOTRANSFERASE ACTIVITY IN LIVER SUPERNATANTS

(Delayed Assay)



(Fig. 24), the activity of the amidotransferase decreased after about the third day of cortisone administration.

(b) Subcellular distribution of amidotransferase and glucose-6-P phosphatase in rat liver from control and cortisone-treated rats.

This experiment was undertaken to evaluate the effect of preparations from liver containing glucose-6-P phosphatase on the formation of glucosamine-6-P from glucose-6-P and glutamine. This evaluation was especially pertinent in a comparison between normal and cortisone-treated animals, as glucose-6-P phosphatase activity has been reported to be increased (389) after cortisone administration, and thus the amount of glucosamine-6-P formed would be decreased if the substrate, glucose-6-P, were to be broken down by the phosphatase.

Table XVII summarizes the distribution of the activities of the two enzymes in the subcellular fractions. It is clear that the supernatants prepared from the livers of the control and the cortisone-treated animals contained no detectable glucose-6-P phosphatase activity. The amidotransferase activity, on the other hand, is largely in the 18,000 x g supernatant fraction. It is reasonable to conclude, therefore, at least as far as supernatants are concerned, that the decrease in glucosamine-6-P synthesis observed after cortisone administration cannot be attributable to breakdown of the substrate glucose-6-P by phosphatase.

TABLE XVII

Distribution of Glucose-6-P Phosphatase and Amidotransferase in the Subcellular Liver Fractions from Control and Cortisone-Treated Rats

Group	Subcellular Fraction	Activity	
		Glucose-6-P phosphatase	Amidotransferase
		(% of total activity)	
CONTROL	Nuclear (N)	15	2
	Particulate (P)	75	6
	Soluble (S)	0	92
CORTISONE-TREATED ANIMALS	Nuclear (N)	15	1
	Particulate (P)	80	8
	Soluble (S)	0	91

As mentioned before, the above fractionation was carried out in a medium of 0.154M KCl and not 0.25M sucrose that is commonly used for subcellular fractionation. However, glucose-6-P phosphatase is known to be distributed in the particulate (microsomal and mitochondrial) fractions and has been used as a 'marker' for such fractions. The absence of glucose-6-P phosphatase activity from the supernatant would suggest that this fraction is free of mitochondria and microsomes.

(c) Separation and identification of the Elson-Morgan-positive product in the incubation mixture with supernatants from control and cortisone-treated animals.

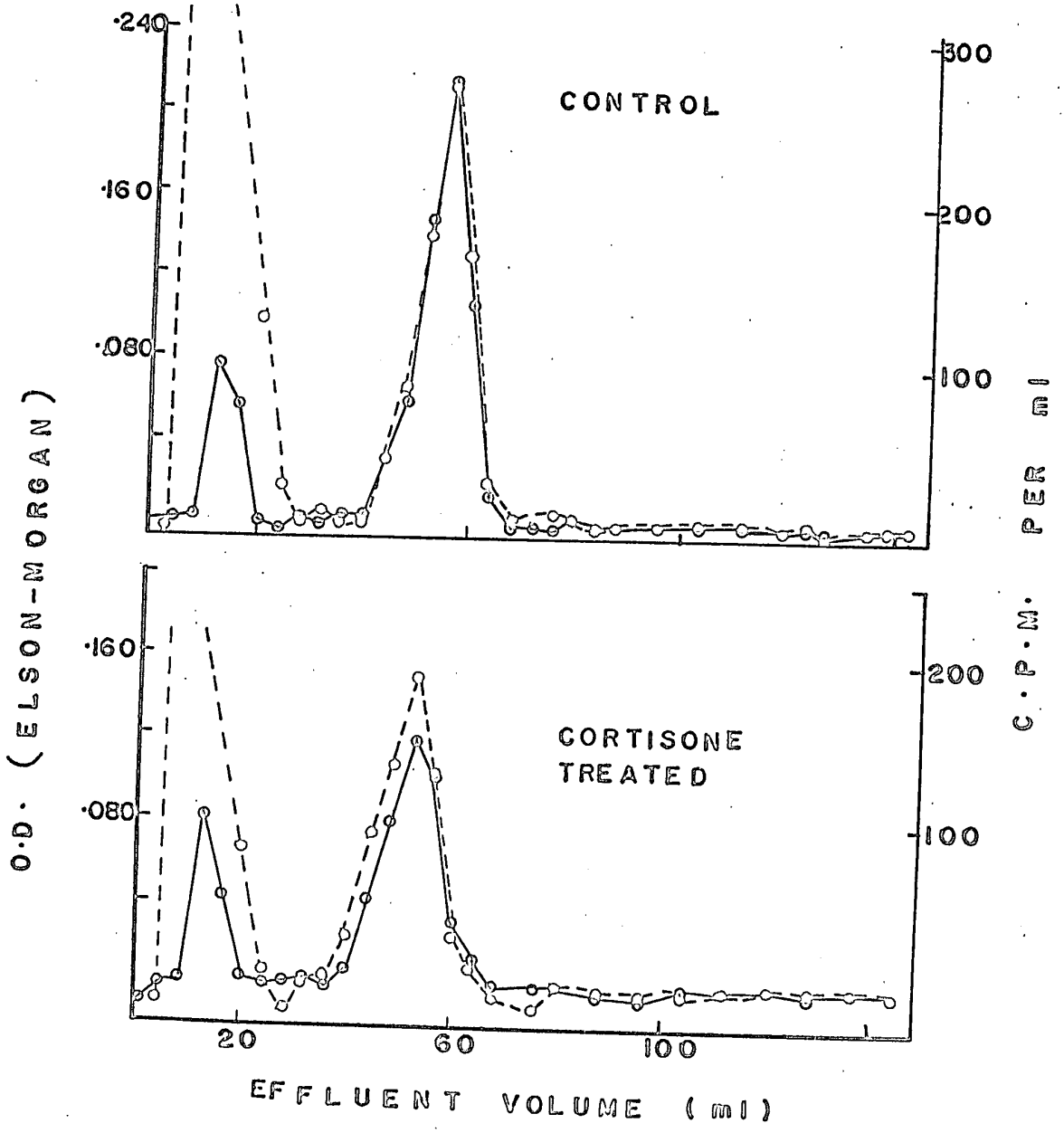
Liver supernatants from control and cortisone-treated rats were incubated with glucose-6-P-U-^{14}C, on a preparative scale, as previously described (p.154). Aliquots of the incubation mixture were chromatographed on the Dowex-50 column, as described on p.152. The following Figure indicates the results of a typical experiment .

Comparison of the curves with those of the glucosamine and glucosamine-6-P standards (p.153) indictes that the major optical density peak corresponds to the time of elution of glucosamine-6-P. The material represented by the initial small peak did not give the typical Elson-Morgan colour reaction. As concluded previously (p.152) the peak probably represents a nonspecific reaction with the relatively large concentration of hexose-6-P with the Elson-Morgan reagent. Furthermore, the curves obtained for the radioactivity and for the optical density of the Elson-Morgan reaction almost coincide. The concentration of glucosamine-6-P obtained with the liver supernatants from the cortisone-treated animals was about 40% less than that of the controls.

There was no evidence from the radioactivity or by optical density of the Elson-Morgan reaction that any glucosamine was formed (the glucosamine peak, in this system, appears at about the 120th ml (pp.149,153). It is unlikely, therefore, that glucosamine-6-P was dephosphorylated to any significant extent during the incubation.

FIGURE 26

GLUCOSAMINE-6-PHOSPHATE SYNTHESIS
BY RAT LIVER SUPERNATANTS



The values of the specific activities of the glucosamine-6-p (calculated from the average of the three uppermost plots on each curve), were nearly the same, namely, 1,335 and 1,385 cpm/μmoles of hexosamine, respectively, with the supernatants from the control and the cortisone-treated animals. It is unlikely, therefore, that the two supernatant preparations contained significantly different amounts of the intermediaries involved in the formation of glucosamine-6-p, as any such difference in the amounts of the intermediaries would have resulted in different specific activities.

4. INHIBITION OF GLUCOSAMINE-6-P SYNTHESIS *IN VITRO*.

The question arose whether the inhibition of hexose-6-P amidotransrerase activity in liver preparations, observed after the administration of cortisone to animals, could be produced also *in vitro*, on the addition of cortisone to liver preparations.

(1) Methods.

The low solubility of cortisone in water presented a problem in these experiments. It was found that when cortisone acetate was dissolved in ethanol (1.5 mg/ml) and this solution was added to normal rat liver supernatants (18,000 x *g* fraction) no change in activity of the amidotransferase was observed except with a large enough quantity of the alcoholic solution to bring the concentration of the steroid in the supernatant to 5×10^{-4} *M*. With still larger quantities of the solution deactivation of the amidotransferase occurred. However, deactivation occurred also when the equivalent amount of ethanol alone was added, thus suggesting that the deactivation may have been caused by the alcohol and not the cortisone.

In an effort to circumvent the difficulty of the limited solubility of cortisone, an effort was made to dissolve the steroid in normal rat plasma, by incubation of the latter with excess of finely divided cortisone. The incubated plasma then was added to the 'ordinary' assay mixture (p.145). No difference in the activity of the enzyme was observed between the assay mixture and the controls

prepared with normal plasma. Similarly, no difference in activity was observed on the addition of plasma from cortisone-treated animals to the 'ordinary' incubation mixture.

Finally, a water-soluble form of hydrocortisone - hydrocortisone-21-sodium succinate - was added, in relatively high concentration, to the 'ordinary' incubation system. In this case, an inhibitory effect on the activity of the amidotransferase was observed. The results are summarized below.

(2) Results.

The results of the experiments with hydrocortisone-21-sodium succinate are indicated in the following Figure. (Fig. 27).

It is apparent that the decrease in activity of the transferase was not caused by the succinate moiety, as sodium succinate by itself in an equivalent concentration was without effect. The inhibitory action thus must be attributable to the hydrocortisone.

The relation between the concentration of the steroid and the inhibition of the hexose-6-P transferase is indicated in Figure 28.

The inhibition of the activity of the amidotransferase in the supernatant fraction from normal rat liver thus was proportional to the logarithm of the concentration of the hydrocortisone succinate.

It is noteworthy, also, that the decrease in activity was obtained only with relatively large concentrations of the steroid, i.e., of the equivalent of approximately one-tenth of the concentration of the substrate.

FIGURE 27

EFFECT OF THE ADDITION OF HYDROCORTISONE SUCCINATE TO NORMAL RAT LIVER SUPERNATANTS

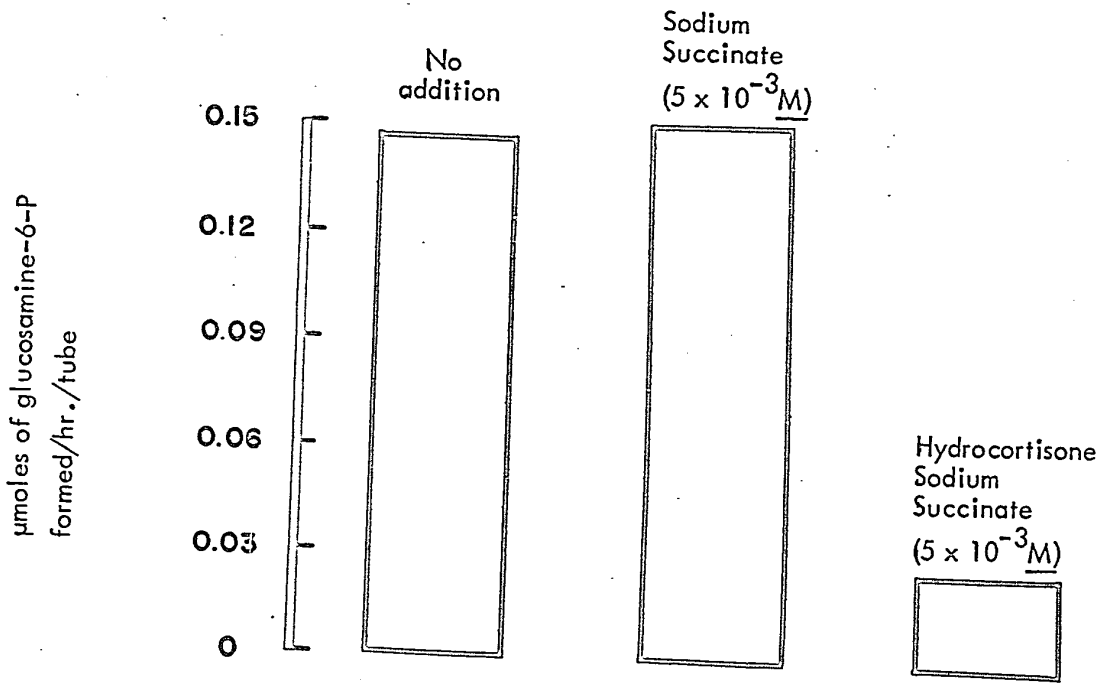
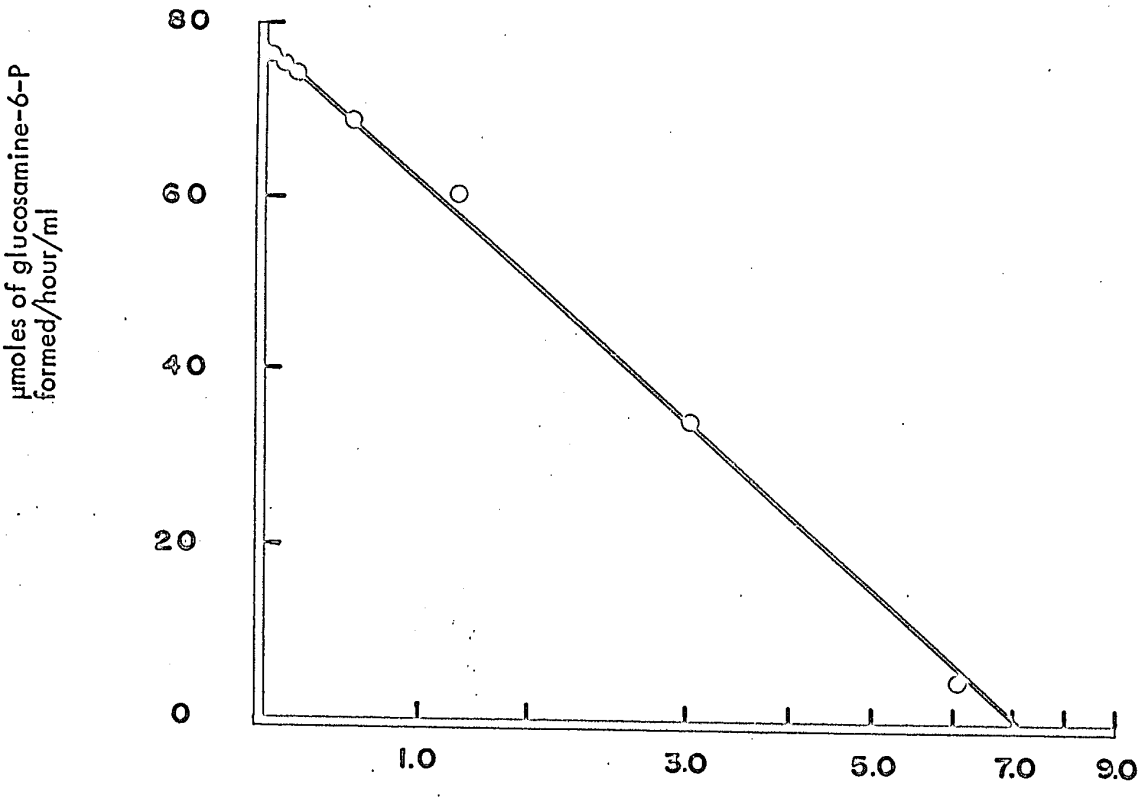


FIGURE 28

EFFECT OF HYDROCORTISONE SUCCINATE CONCENTRATION ON AMIDOTRANSFERASE ACTIVITY



5. RELATION OF *IN VIVO* AND *IN VITRO* EFFECTS.

Experiments were carried out to establish whether the mechanism of the decrease in the hexose-6-P amidotransferase activity *in vivo*, i.e., the decrease produced by administration of hydrocortisone, is related to the inhibition produced *in vitro*, i.e., decrease in enzyme activity after the addition of hydrocortisone succinate to the supernatant fractions from normal liver. Three types of experiments were performed in an attempt to answer this question:

Effect of hydrocortisone succinate administered in vivo, at peak concentration of the steroid.

Hydrocortisone succinate inhibits amidotransferase activity in normal liver supernatants immediately when added *in vitro*. It was of interest, therefore, to examine its effect on amidotransferase activity also when the steroid is given *in vivo* at a time when it is known to reach peak concentration in the liver.

Activity of dialyzed supernatants from control and cortisone-treated rats and of control supernatant to which hydrocortisone succinate has been added.

The object of these experiments was to ascertain whether either in the *in vivo* or the *in vitro* systems a dialyzable factor that could affect the activity of the amidotransferase could be removed.

Kinetic study of the behaviour of the in vivo and in vitro systems.

The activity of the amidotransferase was measured with respect to substrate (glucose-6-P) concentration, in liver supernatants from

normal and cortisone-treated animals as well as in normal supernatants to which hydrocortisone succinate had been added.

(1) Methods.

Assay of amidotransferase were as previously described (p.145). Cortisone acetate was administered subcutaneously to one group of rats daily for 10 days, as previously indicated (p.85).

Hydrocortisone-21-sodium succinate was injected as a water solution into the tail vein, at the indicated dosage.

The dialysis experiments were carried out with an apparatus designed to handle small volumes of solution. In most of the experiments a 'rocking' type dialyzer was used, with the membrane attached to the bottom of a glass cylinder (50 mm length). The tube with the membrane was fixed inside a 50 ml flask with the dialyzing fluid. The liver supernatant to be dialyzed (1 - 2 ml) was placed inside the tube in contact with the membrane. The flask was then attached to a rocking device oscillating at about 20 movements/minute.

Two dialysis media were used. The first, as recommended by Ghosh et al. (23), consisted of 0.005M K_2HPO_4, 0.005M EDTA and 0.5 mg/ml mercapto-ethanol. The pH of the solution was 7.2. This medium was hypotonic relative to the liver supernatant preparation (p.145). For purposes of comparison, the dialysis experiments were also carried out against this medium. The former medium will be referred to as the 'phosphate medium', and the latter, as the 'homogenization medium'.

(2) Results.

(a) The effect of hydrocortisone succinate administered *in vivo*, in high concentration.

Table XVIII indicates the results of very high concentrations of hydrocortisone succinate administered to normal rats on the activity of the amidotransferase reaction.

TABLE XVIII

Effect of Intravenously Administered Hydrocortisone Succinate on the Activity of the Hexose-6-P Amidotransferase

Time lapse from injection to removal of liver	Quantity of steroid injected (mg)	(μmoles/g body weight)	Activity of hexose-6-P transaminase (% of control)*
30 (min)	20	0.42	98
45 "	50	1.58	104
45 "	50	1.58	104
90 "	20	0.42	90
3 (hr)	15	0.31	110
4 "	15	0.31	102
6 "	20	0.42	115
8 "	20	0.42	87

* Controls received saline.

Clearly, with the large doses of hydrocortisone succinate there was no significant effect on the activity of hexose-6-P amidotransferase up to the 8th hour after the injection of very large doses of hydrocortisone succinate.

In the later discussion (p.196) the relationship between the maximum concentration of the steroid that may be attained in the liver in these experiments, and the concentration required to produce inhibition of the amidotransferase in vitro will be further considered.

(b) Influence of dialysis of liver supernatants from control and cortisone-treated rats, and from normal supernatants to which hydrocortisone succinate was added.

Preliminary experiments indicated that the hexose-6-P amidotransferase activity was lost relatively rapidly on dialysis of the liver supernatants from normal animals against either the 'homogenization medium' or the 'phosphate medium'. None of the amidotransferase activity could be recovered in the dialyzate when assayed with either glucose-6-P and glutamine, or with fructose-6-P and glutamine. Nor did addition of the dialyzate to the dialyzed enzyme restore the amidotransferase activity.

(i) Experiments with supernatants from normal liver.

In this experiment, shown in Table XIX , 2 ml of supernatant were dialyzed at 4°C against 50 ml of the homogenization medium (D-1), or 50 ml of the phosphate medium (D-2). The outer medium was replaced

with fresh medium three times during the 8-hour dialysis period. Three controls also were included (C-1), fresh undialyzed supernatant, assayed immediately after preparation; (C-2), undialyzed supernatant that had been kept at 4°C for 8 hours; (V-T), the undialyzed supernatant, in which a piece of cellulose acetate dialysis tubing ('Visking') had been placed, and the tube kept in the rocking dialysis apparatus for 8 hours at 4°C. All runs in this experiment were simultaneous and with the same enzyme preparation.

TABLE XIX

Influence of Dialysis of Normal Rat Liver Supernatant

(Experiment-B)

Specimen	Description	Time of Assay: Immediate	Time of Assay: Stored (8 hrs, 5°C)	Post Dialysis (8 hrs, 5°C)	Outer Dialyzing Medium	Gm-6-P Produced (µm/tube /hr)	% of Control
C-1	Undialyzed supernatant	+			-	0.18	-
C-2	"		+		-	0.13	71% of that of C-1
D-1	Dialyzed supernatant			+	'Homogenizing'medium	0.09	69% of that of C-2
D-2	"			+	'Phosphate' medium	0.12	92% of that of C-2
VT	Undialyzed supernatant	piece of Visking tubing immersed in the enzyme solution				0.10	77% of that of C-2

From the above Table it is apparent that the 'phosphate' medium was the more suitable dialysis medium, from the point of view of stabilization of the activity of the enzyme. The 'phosphate' medium, therefore, was used in the subsequent dialysis experiments with the '*in vivo*' and the '*in vitro*' systems.

It is evident also that a greater degree of loss of amidotransferase activity took place on shaking in contact with the pieces of Visking membrane. One cannot be certain whether the loss of activity was due to absorption of the enzyme on the membrane or to interfacial denaturation. However, it may account for the additional loss of activity.

(ii) Dialysis of liver supernatants from cortisone-treated animals.

Supernatants were prepared from the livers of normal and cortisone-treated animals (p.145). In this experiment, 2 ml of each preparation was dialyzed for 8 hours against the phosphate medium, as described (p.174). Amidotransferase activity was assayed with the 'ordinary' system.

The influence of dialysis of the supernatants on the hexose-6-P amidotransferase activity is indicated in Table XX . It is apparent that dialysis of the preparation from the cortisone-treated animals did not lessen the degree of the inhibition caused by cortisone administration. It would appear, therefore, that the inhibition could not be attributed to the presence of a dialyzable inhibitor substance induced by the cortisone treatment.

TABLE XX

Influence of Dialysis on the Hexose-6-phosphate Amidotransferase Activity of Liver Supernatants from Control and Cortisone-Treated Rats.

Source of Supernatant	Glucosamine-6-P formed Undialyzed	Stored for 8 hours	After Dialysis for 8 hours
	(μmoles glucosamine-6-P formed/tube/hr)		
CONTROL ANIMALS	0.19	0.16	0.13
CORTISONE-TREATED ANIMALS	0.07	0.04	0.02

(iii) Dialysis of supernatants to which hydrocortisone succinate was added.

These trials represent the <u>in vitro</u> system. The experimental preparations were normal liver supernatants, to which hydrocortisone succinate had been added to give a high concentration of 11.5×10^{-3} <u>M</u>. To the controls, a corresponding amount of water was added. Both types of preparation were dialyzed as described in the preceding section, with reference to the <u>in vivo</u> system. The results are given in Table XXI.

TABLE XXI

Influence of Dialysis on the Hexose-6-phosphate Amidotransferase Activity of Rat Liver Supernatants to which Hydrocortisone Succinate was Added.

Specimen	Glucosamine-6-P formed		After Dialysis for 8 hours
	Undialyzed	After storage for 8 hrs at 5°C	
	(µmoles glucosamine-6-P formed/tube/hr)		
NORMAL SUPERNATANT	0.18	0.12	0.11
SUPERNATANT WITH ADDED HYDROCORTISONE SUCCINATE	nil	nil	nil

Again it is apparent that dialysis caused no reversal of the inhibition produced by the addition of hydrocortisone succinate to the normal enzyme preparation.

In summary, neither the '<u>in vivo</u>' inhibition of hexose-6-P amidotransferase by cortisone, nor the '<u>in vitro</u>' inhibition on addition of hydrocortisone succinate to supernatant from normal animals, was diminished by dialysis of the supernatant. The additional loss of activity during dialysis may have been due to interfacial denaturation of the enzyme from contact with the surface of the dialyzing membrane, or possibly by adsorption on it.

(c) Enzyme kinetic studies on the '*in vivo*' and '*in vitro*' systems.

Preliminary studies with normal rat liver supernatant indicated that the reaction velocity (μmoles of glucosamine-6-P formed/hour) of the amidotransferase reaction was a linear function of the time up to 90 minutes. The 60-minute incubation period in subsequent experiments was found to give a suitable yield of glucosamine-6-P for the colorimetric assay.

Figure 29 demonstrates the relationship between reaction velocity and enzyme concentration with supernatants obtained from normal and cortisone-treated animals, and for normal supernatants to which hydrocortisone succinate was added.

Straight-line relationships hold in each case with no suggestion of a decrease with the higher enzyme concentrations.

Figure 30 illustrates the reciprocal relationships of reaction velocity and substrate concentration (396), for the amidotransferase reaction, in supernatants obtained from normal and cortisone-treated animals. The concentration of glucose-6-P was varied as indicated in Figure 30, using the 'ordinary' assay system (p.145).

Both the supernatant from the control liver and that from the cortisone-treated animals gave a linear reciprocal relationship between the velocity and the substrate concentration, except with very high substrate concentrations when inhibition of the reaction was observed. The apparent K_m for the control preparation of the amidotransferase, calculated from the Lineweaver-Burk plot, was found to be 1.25×10^{-4} M for glucose-6-P. It may be noted from the figure, that the inhibition of amidotransferase activity after cortisone administration is neither typically 'competitive'

FIGURE 29

THE RELATIONSHIP BETWEEN ENZYME CONCENTRATION

AND AMIDOTRANSFERASE REACTION VELOCITY

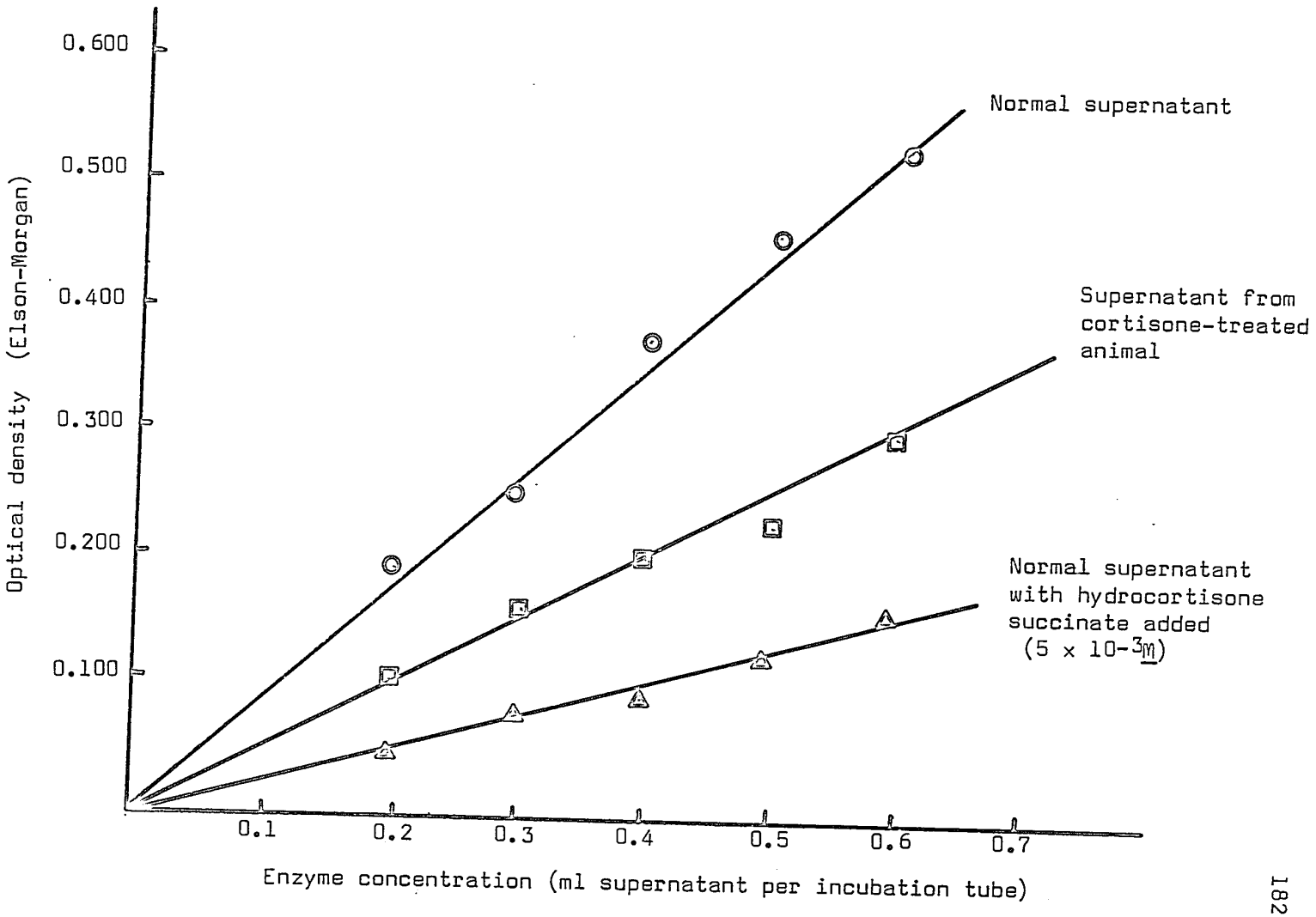
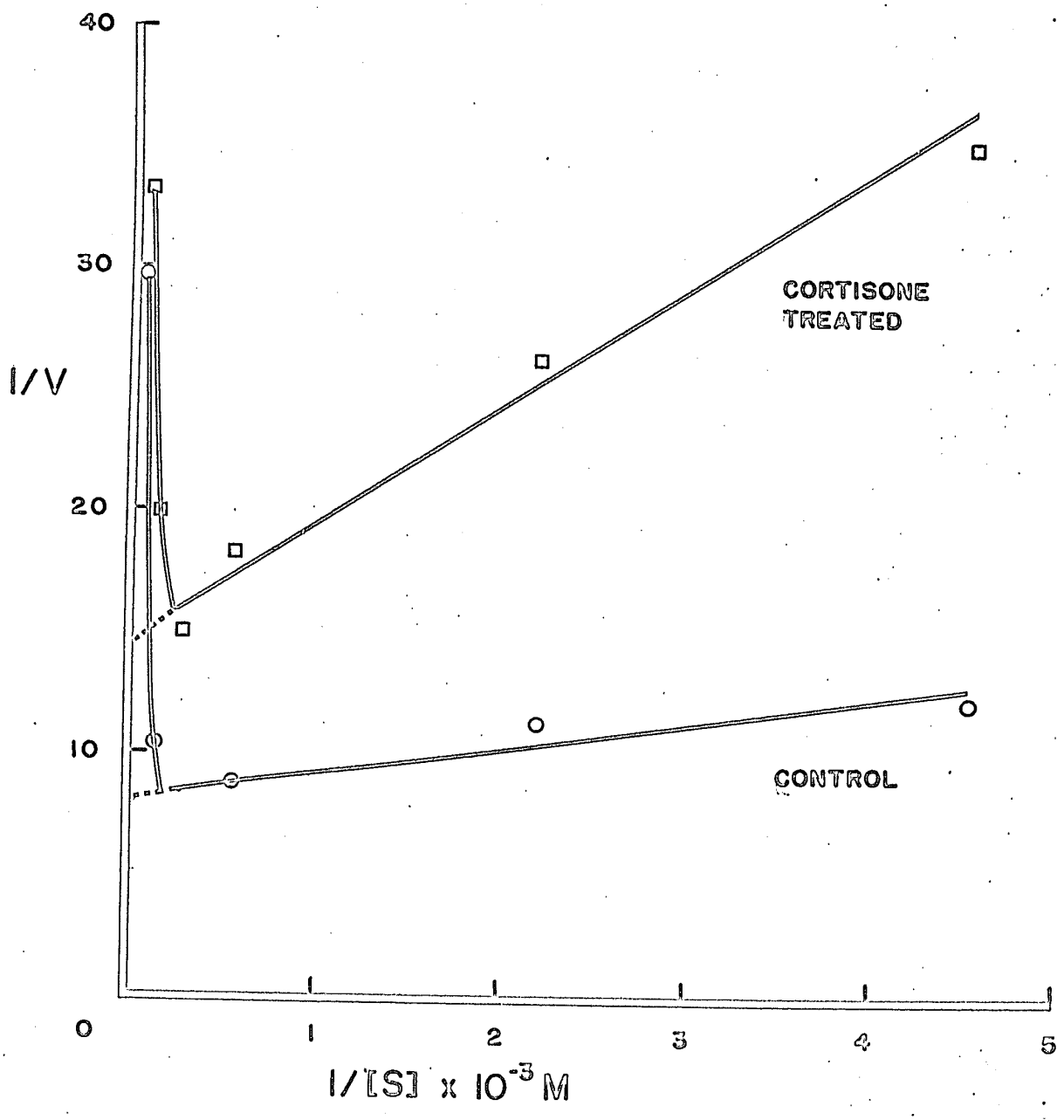


FIGURE 30

EFFECT OF SUBSTRATE CONCENTRATION ON AMIDOTRANSFERASE REACTION VELOCITY, IN LIVER SUPERNATANTS FROM NORMAL AND CORTISONE TREATED RATS



Legend

S - Substrate concentration (glucose-6-P)

V - µmoles of glucosamine-6-P produced per hour

Each point represents the mean values of 3 experiments, assayed in duplicate.

nor 'noncompetitive'. Both an increase in the apparent K_m value and a decrease in the reaction velocity were observed in the enzyme preparation from the cortisone-treated animals, as compared to the results with the controls.

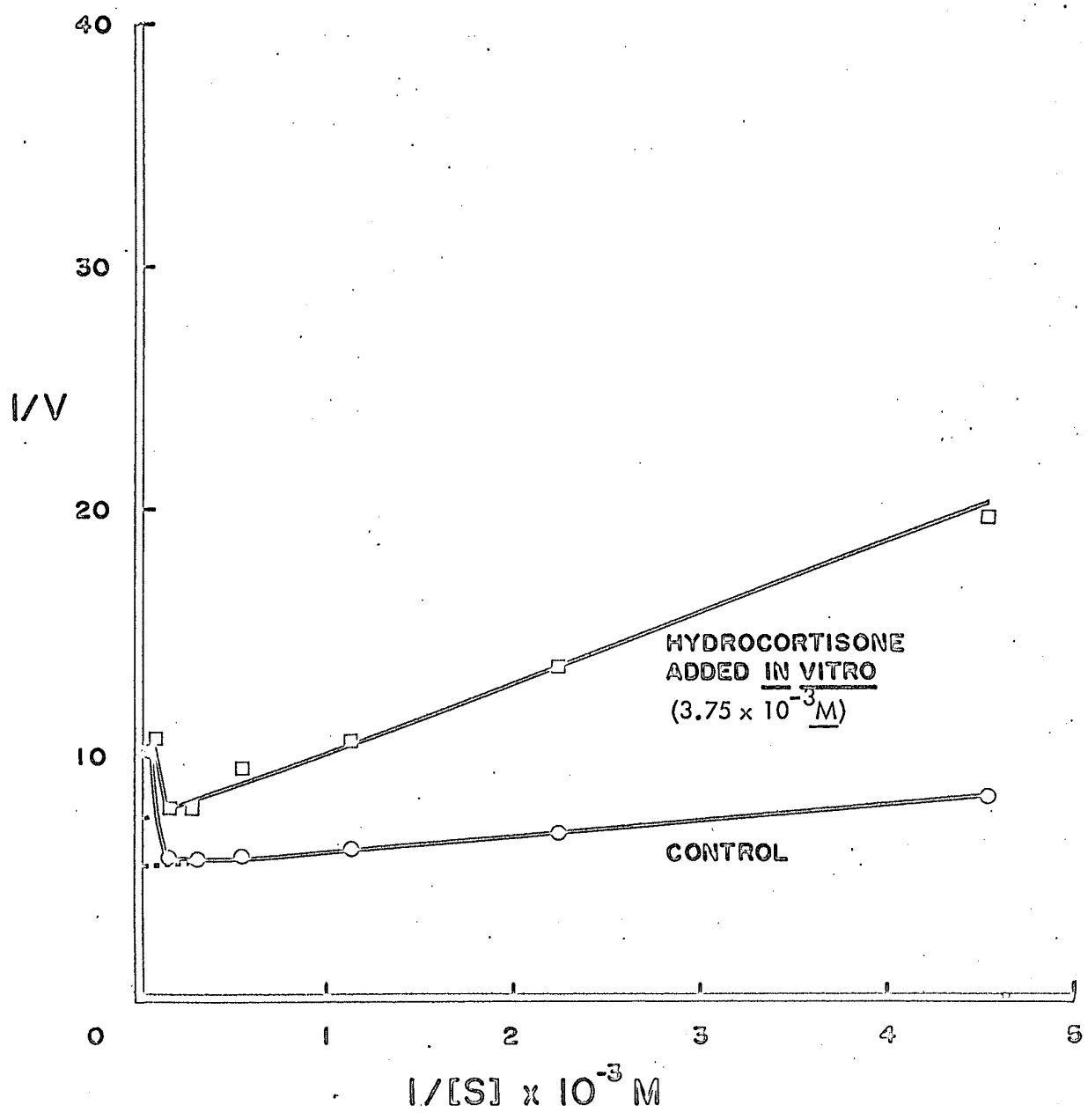
The reciprocal relationship between the reaction velocity and the substrate concentration (396) for the amidotransferase reaction in the supernatant from normal animals and the same to which hydrocortisone succinate was added as indicated in Figure 31.

Again, it is evident that the inhibition is neither of the strictly competitive nor the noncompetitive type.

The next figure (Fig.32) combines the data from Figures 30 and 31.. As expected, the two 'control' amidotransferase preparations, while having somewhat different activity, had an identical apparent K_m value. However, it is interesting to note that the amidotransferase preparation from the cortisone-treated animals and from the normal, to which hydrocortisone succinate was added, also gave closely corresponding shifts in the different K_m values. The significance of this observation is not clear and may be fortuitous.

FIGURE 31

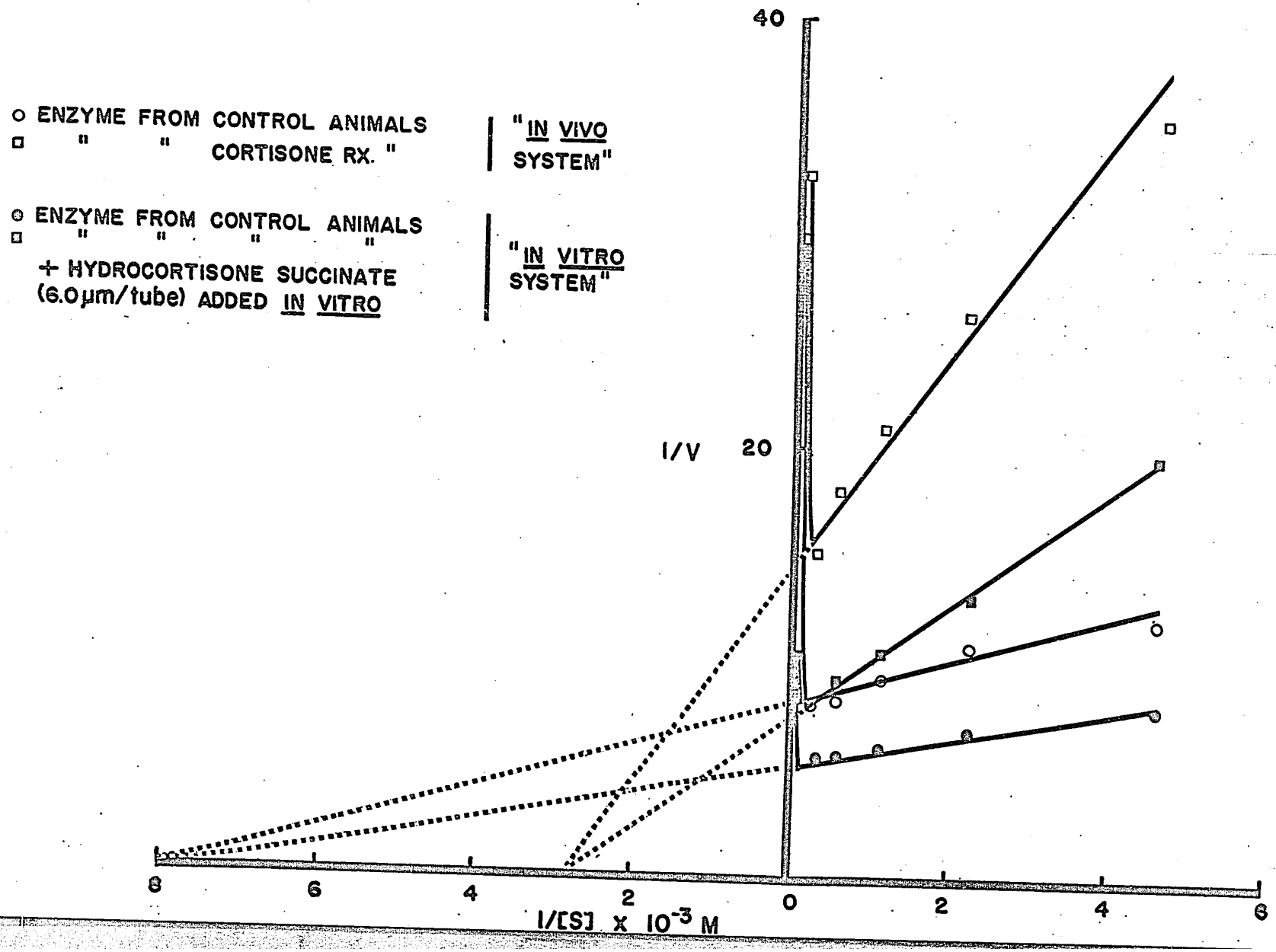
EFFECT OF SUBSTRATE CONCENTRATION ON AMIDOTRANSFERASE REACTION VELOCITY, IN LIVER SUPERNATANTS FROM NORMAL ANIMALS WITH AND WITHOUT ADDITION OF HYDROCORTISONE SODIUM SUCCINATE



Legend - as in Fig. 30.

FIGURE 32

EFFECT OF SUBSTRATE CONCENTRATION ON AMIDOTRANSFERASE REACTION VELOCITY, IN THE IN VIVO AND IN VITRO SYSTEMS.



6. THE INCORPORATION OF GLUCOSAMINE-^{14}C INTO THE PHOSPHORYLATED HEXOSAMINE INTERMEDIARIES IN THE LIVER OF NORMAL AND CORTISONE-TREATED RATS.

The question arose, as to whether cortisone may inhibit also other steps in the hexosamine intermediary pathway, in addition to the amidotransferase. The task of assaying each of the enzymes in the pathway in liver preparations from normal and cortisone-treated animals, would be protracted and at present impracticable, as many of the reactions require special substrates which are not yet commercially available. For this reason, a more indirect approach was concurred.

Since glucosamine enters the hexosamine synthetic pathway either by being phosphorylated at C-6, or first undergoing N-acetylation and then phosphorylation at the C-6 position (p.22), the use of glucosamine to start with would eliminate the amidotransferase step and thus might ascertain whether cortisone could interfere also with subsequent steps in the pathway. The procedure designed, was to administer glucosamine-^{14}C to normal and to cortisone-treated rats, as a tracer, and measure its incorporation into the phosphorylated intermediaries of the hexosamine synthetic pathway in the rat liver preparations. The ratio of the ^{14}C incorporated into a given phosphorylated intermediate, in the controls and the treated animals, at a given time, could serve as an index for comparison of the degree of tagging between one phosphorylated intermediate with another.

(1) Methods.

The analytical procedure is based on the method of McGarahan and Maley (48). Phosphorylated hexosamine intermediaries are separated

by ion-exchange chromatography on a basic Dowex-1 column in the formate form. Hexosamines and N-acetyl hexosamines are first eluted with water. Glucosamine-6-P is then eluted with 0.01N formic acid (Fraction I). The N-acetyl glucosamine-6-P is eluted with 4N formic acid (Fraction II), and the UDP-N-acetylhexosamines, with a mixture of 4N formic acid - 0.2N ammonium formate (Fraction III). McGarahan and Maley (48, 49) used both an 'interrupted' gradient elution and the 'batch' elution systems. The specific activities of the UDP-N-acetylglucosamine in the gradient elution system were apparently determined from the ultraviolet absorption of the nucleotide.

We employed the more convenient batch-elution system. The separation of the above mentioned hexosamine intermediaries in the fractions were confirmed by reference to pure standards. (Glucosamine, N-acetyl glucosamine and UPD-N-acetylglucosamine were obtained from Sigma. Glucosamine-6-P was obtained from Nutritional Biochemicals). The 20 x 1 cm Dowex-1 (formate) column initially was washed with 150 ml of water and then with 70 ml of each of the formic acid solutions. However, it was desirable in the batch-elution procedure, to estimate the amount of UDP-N-acetylglucosamine by means of the specific Elson-Morgan reaction for hexosamines, rather than with the nonspecific ultraviolet absorption. UDP-N-acetylglucosamine can be estimated after mild hydrolysis with 0.1N HCl at 100°C for 10 minutes (397). The hydrolytic treatment was followed by removal of HCl and estimation of the quantity of N-acetylhexosamine released by the Elson-Morgan procedure (23). However, the UDP-N-acetylglucosamine standard, contained in 'Fraction IV', gave a very poor colour yield by this method. It was suspected that the

poor yields were due to interference with the colour development by the presence of ammonium formate salt in 'Fraction IV'. The experiment reported in the following Table would appear to support this view.

TABLE XXII

Effect of Added Ammonium Formate on the Determination of UDP-N-acetylglucosamine by the Elson-Morgan Reaction.

UDP-N-acetyl-glucosamine added	Salt concentration	Recovery
(µmoles)	(µmoles/5.0 ml)	(%)
2.4	0	102
2.4	6 *	0
4.8	6 **	0

* ammonium formate added before hydrolysis with 0.1N HCl

** " " " after " " "

It was found that if the hydrolysis, in the presence of ammonium formate, was carried out with considerably higher concentrations of HCl (up to 2N) some colour was obtained, but the results were inconsistent.

The problem of the interference by ammonium formate in Fraction III was overcome by passage of this fraction through a polysulfonated Dowex-50 (H^+) resin column (20 x 1 cm) to remove the ammonium cation. The effluent was combined with a water wash and the solution concentrated to dryness. The residue was taken up in 0.1N HCl and hydrolyzed. The hydrolyzate was concentrated to dryness, the residue taken up in a known volume of water, and the hexosamine in the solution determined with the Elson-Morgan method. The procedure yielded better than 90% recovery of the UDP-N-acetylhexosamine standard.

Preliminary analyses with rat liver specimens indicated that only the Fraction III contained sufficient hexosamine (about 0.2 µmoles/g wet weight of tissue) to permit reliable estimation by the Elson-Morgan method. Fraction I contained too small a concentration of hexosamine intermediaries to be detectable by the Elson-Morgan reaction, even when two livers were pooled and the final material was concentrated to a volume of 2 ml. The concentrates from Fraction II gave a relatively weak colour in the Elson-Morgan reaction. Furthermore, the colour was atypical. The following Figure illustrates typical absorption spectra of the Elson-Morgan colour obtained with concentrates from Fractions II and III and with commercial standards.

It is apparent that the absorption spectrum with Fraction IV is the same as that of the commercial UDP-N-acetylglucosamine. The colour has an absorption maximum at 580 mµ. Fraction II, however, shows an additional peak at about 420 mµ. The tailing-off of this nonspecific absorption curve could affect the value of the specific absorption at 580 mµ.

For this reason the values for the hexosamine concentration in

FIGURE 33

ABSORPTION SPECTRA OF FRACTIONS 'II' AND 'III'

FROM LIVERS OF NORMAL AND CORTISONE-TREATED RATS

(Elson-Morgan Reaction)

Legend

II C - Fraction II from control animals

III C - " III " " "

II T - " II " cortisone-treated animals

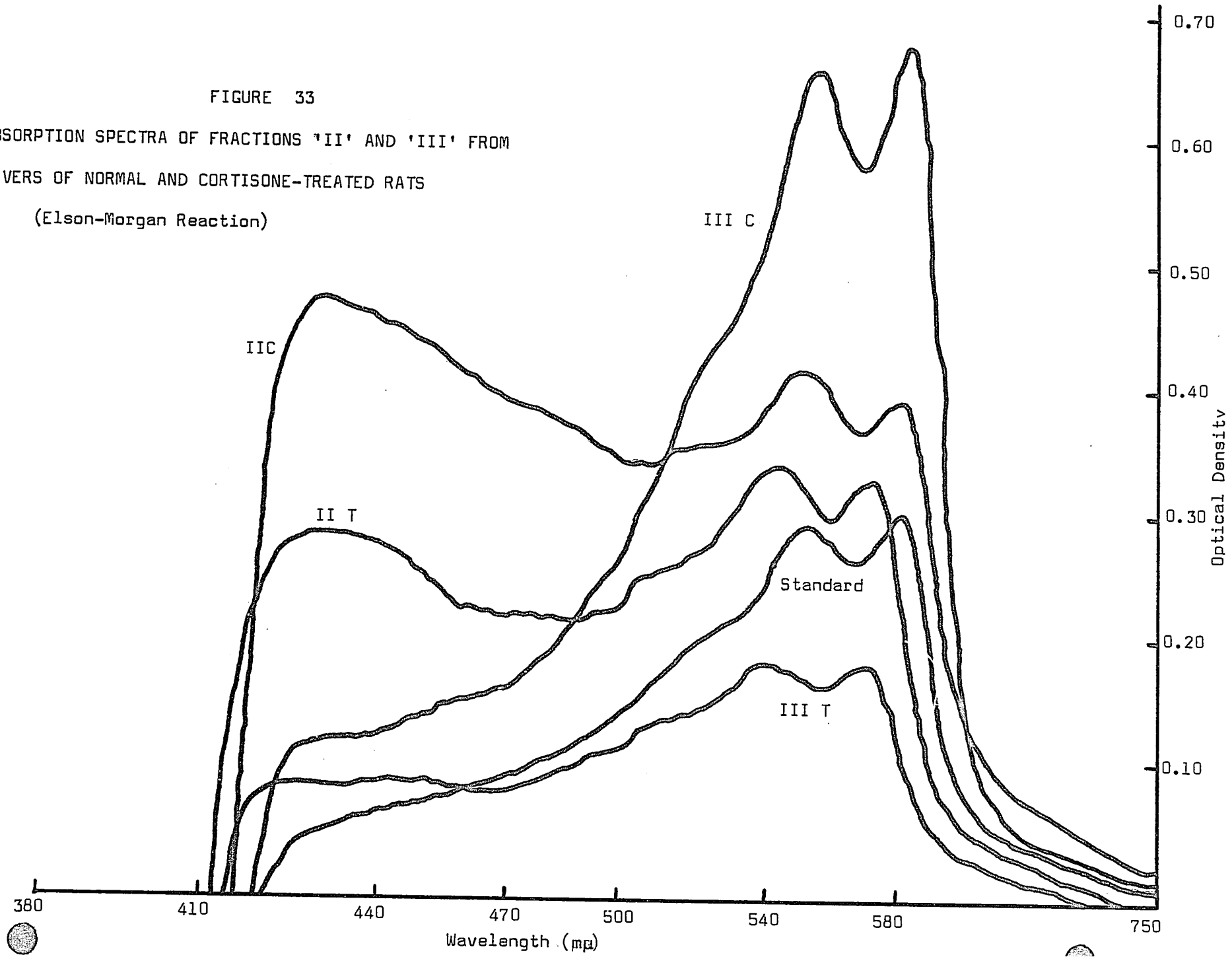
III T - " III " " " "

Standard - 0.08 µmoles of UDP-N-Acetylglucosamine

FIGURE 33

ABSORPTION SPECTRA OF FRACTIONS 'II' AND 'III' FROM LIVERS OF NORMAL AND CORTISONE-TREATED RATS

(Elson-Morgan Reaction)



Fraction II cannot be considered reliable and, therefore, are not presented.

In the experiments with the normal and the cortisone-treated rats, the latter group received a subcutaneous injection of 5 mg cortisone acetate/day for 10 days. Both of the groups received 0.02 μc of uniformly labelled D-glucosamine-U-^{14}C per gram body weight by injection into the tail vein. The animals, half an hour after the injection, were anesthetized and killed by exsanguination through a ventricular incision. The liver was removed and dropped into a preweighed beaker containing ice-cold 0.05N perchloric acid. The beaker with the perchloric acid and liver was weighed and the contents transferred to a Virtis apparatus and homogenized at high speed for 1 minute, with the flask surrounded with ice. The contents of the flask were transferred quantitatively with the acid of about 10 ml 0.05N perchloric acid to plastic centrifuge tubes and centrifuged at 10,000 x g for 20 minutes in a refrigerated 'Sorval' high-speed centrifuge. The supernatant was neutralized with 50% KOH, and the potassium perchlorate removed by centrifugation in the cold. The supernatant was applied to the Dowex-1 (formate) column, chromatographed and eluted as described. Fraction III was desalted by a passage through a Dowex-50 (H^+) column and the N-acetylhexosamine content was determined.

The radioactivity of the fractions was estimated in duplicate by applying 50 μl of the concentrated material to a filter paper (Whatman No.2) and 'counting' the specimen in a Packard '574' scintillation counter set for maximum ^{14}C-counting. The scintillating liquid was composed of toluene containing 0.03% 1,4-bis 2-(5-phenyloxazolyl) Benzene (POPOP) and 0.5% 2,5-Diphenyloxazole (PPO) obtained from Packard.

(2) Results.

Table XXIII indicates the counts recorded for each of the fractions from the Dowex-1 column. 'Series A' and 'series B' represent experiments carried out about a month apart on two groups of animals of the same age.

TABLE XXIII

Incorporation of Glucosamine-U-^{14}C into Phosphorylated Hexosamine Intermediaries in Livers of Normal and Cortisone-Treated Rats.

Experiment* No.	Animal	Radioactivity Incorporated (cpm/g liver weight) Frac.I	Frac.II	Frac.III	Ratio of Counts C/T Frac.I	Frac.II	Frac.III
SERIES A							
1	C	230	14,190	82,967	0.9	1.3	1.3
	T	235	10,548	62,126			
2	C	191	12,027	49,042	1.1	1.6	1.5
	T	180	10,320	32,952			
3	C	430	14,529	58,752	1.0	1.8	1.6
	T	441	7,617	36,621			
SERIES B							
1	C	223	17,737	120,036	1.0	1.2	1.7
	T	201	15,083	70,229			
2	C	231	23,045	93,135	1.1	1.8	1.4
	T	320	13,176	65,064			
3	C	280	10,611	64,889	0.7	1.5	1.8
	T	295	6,980	35,669			
Mean	C	264	15,356	78,136	1.0	1.5	1.5
	T	279	10,620	50,443			

* In each experiment all the steps in processing the liver and subsequent chromatography, were carried out simultaneously for the control (C) and cortisone-treated (T) animals.

It is apparent that while there is variation in the total number of counts obtained for a particular fraction in the different experiments, the ratio of counts from the control and the cortisone-treated animals followed a similar pattern in all the experiments.

It is noteworthy, that by far the greatest incorporation of glucosamine-^{14}C was found in Fraction III, which represents the UDP-N-acetylhexosamines. By contrast, Fraction I, which includes glucosamine-6-P, showed very slight incorporation of the glucosamine-^{14}C counts.

It is of particular interest that the ratios of the counts between control and cortisone-treated groups in Fraction II and Fraction III are similar, but that of Fraction I is the lower.

Table XXIV gives the concentration of UDP-N-acetylglucosamine found in Fraction III of liver extracts from normal and cortisone-treated rats. This was the only phosphorylated hexosamine intermediary whose concentration could be reliably estimated in the liver extracts.

TABLE XXIV

UDP-N-Acetylhexosamine Concentration in Livers of Normal and Cortisone-Treated Rats.

Group *	Concentration of UDP-N-acetylhexosamine	
	(μmoles/g wet weight liver)	(μmoles/100 mg DNA)
CONTROL	0.243 ± 0.05	4.3 ± 0.17
TREATED	0.234 ± 0.04	2.9 ± 0.12

* 6 animals in each group. Values are from the same experiments as in Table XXIII.

The concentration of UDP-N-acetylhexosamine in livers of cortisone-treated animals is lower than that of the controls when the results are expressed on the basis of DNA but not in terms of wet weight of liver. It would appear that individual liver cells contain less UDPN acetylhexosamine after cortisone administration than they do normally.

The significance of these findings is considered in the subsequent 'Discussion'.

DISCUSSION

At the outset of this study consideration was given to the dosage of cortisone to be administered to the rats to ascertain the effect on MPS metabolism. The large dosage (5 mg/day) was favoured for the following reasons. First, the majority of studies in the literature have been done with a similar dosage (241, 244, 315, 316, 376), thus comparison of results could be readily made. Second, since anti-inflammatory steroids are administered to patients, usually in unphysiologically large doses, it was considered that a study on the rat might throw light on connective tissue changes that may occur in the human. Third, with the rat as the experimental subject administration of cortisone (or cortisol), even in very low dosages, constitutes in a sense a 'pharmacological' rather than a physiological experiment, as the rat normally does not secrete these steroids (280). Finally, the use of large dosages produces changes sufficiently profound to allow definitive biochemical comparisons between the normal tissues and those from the cortisone-treated animal.

(1) Influence of Cortisone on the Overall Metabolism of MPS and Glycoproteins.

Our experiments with contact radioautography showed that administration of cortisone to the rat reduced greatly the incorporation of $^{35}SO_4$ into the epiphysial cartilage and skin, and to a lesser extent the incorporation into the aorta and the liver, compared to that in the

untreated controls. Since sulfate-^{35}S, administered to the normal animal, is incorporated largely into the sulfated MPS (76, 77), our results can be interpreted as indicating that the treatment with relatively large doses of cortisone produced extensive derangement of the metabolism of sulfated MPS. However, the contact radioautograms, in themselves do not tell whether the changes were the result of impaired synthesis of the MPS or of accelerated tissue catabolism, or both. This can be said also of a number of studies where sulfated MPS have been isolated from tissues (315, 316, 317).

Contact radioautography, however, does provide some information in this question since most of the mucopolysaccharides are synthesized intracellularly and secreted into the tissue matrix where the 'turnover' occurs (76, 146). The use of labelled sulfate reveals that the uptake, incorporation, retention and secretion is a process that extends over several hours (151).

In our study, therefore, we decided to measure the ^{35}S-incorporation 2 hours after the administration of the radioisotope, at which interval the intracellular synthesis is most active, but little secretion of the MPS occurs. In this way we hoped to minimize the complications from catabolism on the ^{35}S-labelled MPS. That our timing was successful is obvious from the results in Figures 5 A, 5 B and 6 A, 6 B, where there is little evidence of secretion of ^{35}S-sulfated MPS during the 2-hour period by the tissue cells of the normal or the cortisone-treated animals. The greatly diminished extent of ^{35}S incorporation in the cartilage, skin, the aorta and liver, of the cortisone-treated animals, is evidence of interference by the cortisone with the synthesis of sulfated MPS, notwith-

standing any additional influence on the catabolism of the MPS after it was secreted.

The mechanism by which synthesis of sulfated MPS is influenced by corticosteroids is not understood. Two explanations have been offered: first, that only the sulfation process is inhibited; second, that the synthesis of the entire MPS molecule is inhibited and, therefore, that a decreased incorporation of ^{35}S into MPS simply reflects this. With respect to the first-mentioned view, Balasubramian et al. (331) observed that the activity of the 3'-phosphoadenosine-5'phosphosulfate synthesizing system was decreased in rat granulomata after administration of very high doses (200 mg/kg body weight/day) of cortisol. However, the authors questioned the specificity of their finding. Moreover, inhibition of this enzyme system has not been observed with cartilage, although the ^{35}S-incorporation was markedly inhibited by cortisone or cortisol (330). The second view is supported by the work of Schiller and Dorfman (315,316) who found that cortisol (5 mg/day) administered to rats caused a decrease in the incorporation of both ^{35}S-sulfate and ^{14}C-acetate into chondroitin sulfate to the same extent. These results, together with our observations on the histochemistry and the hexosamine and hexuronic acid content of tissues (see also below) suggest that the metabolism of the entire MPS molecule is impaired and not the sulfation alone.

The histochemical studies with toluidine blue, indicated that the composition of the MPS was altered in the animals treated with cortisone. Anionic substances such as MPS bind toluidine blue, but metachromasia is considered to arise primarily from the interaction of the dye with the alternating units of hexosamines and hexuronic acids, and possibly also

with the sulfate groups (398). However, the changes observed after cortisone administration were not the same in all the tissues. For example, in the skin there was marked reduction of dye uptake with complete loss of metachromasia, while in the epiphysial cartilage the dye uptake was not diminished but the typical metachromatic reaction was abolished (Figs. 8 A and 8 B). These histochemical changes are difficult to interpret in biochemical terms. However, it is of interest that while both the hexosamine and the hexuronic acid concentration of the defatted skin of the cortisone-treated animals was decreased compared to that of the controls, there was little change in the concentration of these substances in the epiphysial regions.

The morphological changes, after cortisone administration, were particularly pronounced in the case of the epiphysis and skin (H and E sections, Figs. 7 A, B, and 10 A, B). In the former, the ossification process was abnormal, and in the latter, the loose subcutaneous tissue became much more dense. The concomitant increase in the hydroxyproline content of these tissues (Table XV) appears to be in agreement with these findings.

Periodic acid-Schiff (PAS) staining of tissues of normal and cortisone-treated rats indicated changes that were less clearcut than those observed with radioautography and toluidine blue staining. The work of Leblond (399) has indicated that the predominant PAS-positive substances in the tissues are collagen and reticular fibrils, presumably because of the carbohydrate moieties associated with these structures.

It is difficult to evaluate changes in the staining intensity in the tissues of the cortisone-treated animals in the presence of pronounced morphological changes (Figs. 7 A, B, 10 A, B). This is especially true in the case of the epiphysial cartilage which was greatly reduced

in width in the cortisone-treated animals. It is noteworthy, however, that fibre-like structures in the skin of these animals reacted with the PAS stain more intensely than in the controls, in contrast to the relatively light staining with toluidine blue.

The concentration of hexuronic acid reflects the concentration of the MPS in the tissue while the total hexosamine concentration reflects both the content of MPS and glycoproteins. The hexuronic acid concentration, on the basis of dry weight of the defatted tissue, was decreased in the skin and the aorta but not in the epiphysial region of the cortisone-treated animals (Tables XI-XIII, XIV). This suggests that a corresponding decrease occurred in the MPS concentrations of the former two tissues but not in the epiphyses. A similar pattern of change occurred in the total hexosamine content of these tissues in the cortisone-treated animals and also in the total hexosamine content in the liver (Table VIII).

It is of interest to speculate whether changes occurred also in the glycoprotein content of the tissues after cortisone administration. An approximate indication may be obtained by subtracting the content of hexuronic acid in the tissue (on a molar basis) from that of the hexosamines. It is noteworthy, however, that such a calculation would be valid only as a relative estimate of the hexosamine content of 'glycoproteins' in tissues of the control and cortisone-treated animals. This is because we did not study the optimal conditions of hydrolysis for release of hexosamines and hexuronic acids for each tissue, but used the 24-hour resin hydrolysis as an expedient basis for comparison. The results of the estimations of the hexosamine content of the glycoprotein

of tissues from normal and cortisone-treated animals can be presented most conveniently in the form of a Table.

TABLE XXV

Concentration of Hexosamines in Tissue 'Glycoprotein' of Normal and Cortisone-Treated Animals

Group	Tissue		
	Aorta	Skin *	Epiphysis
	(μmoles hexosamine/g dry tissue)		
CONTROL	11.4	3.0	7.4
CORTISONE-TREATED	9.9	2.2	-

* based on dry weight of defatted tissue.

These calculations suggest that the 'glycoprotein' content of tissues from the animals treated with cortisone was decreased compared to that of the controls. With the liver hydrolyzates a reliable estimate of the hexuronic acid content could not be obtained (p. 141). Nevertheless, the decrease in the hexosamine content in the liver of the cortisone-treated animals (expressed either on the basis of dry weight or concentration of DNA) suggests that a decrease occurred in the concentration of the MPS and/or glycoprotein.

It was established by means of ion-exchange chromatography, and with the ninhydrin or the Elson-Morgan detection system, that the predominant hexosamine in the liver of the normal and the cortisone-treated rat is glucosamine. The significant decrease observed in the total amount of hexosamines thus presumably represents primarily a decrease in the content of glucosamine. In this connection it is of interest that Spiro(12,400) found only glucosamine in the trichloroacetic acid precipitate from rat liver. He found no evidence of the presence of galactosamine or mannosamine after ninhydrin degradation to the pentose derivatives or on conversion to the N-acetyl form. In our work with the hydrolyzates from whole dry liver the presence of small amounts of galactosamine was unequivocally established on three ion-exchange chromatography systems, one of which was specific for the detection of hexosamines. It may be that much of the galactosamine in the liver is in a TCA-soluble form (e.g., UDP-N-acetylgalactosamine) and thus was not detected in Spiro's work.

A decrease in the hexosamine and hexuronic acid content of the tissues from the cortisone-treated animals as compared to that of the controls might have been anticipated from the marked reduction of the ^{35}S - uptake of the corresponding tissues from the treated animals. However, if one attempts to correlate the ^{35}S-uptake with the observed changes in hexosamine and hexuronic acid content it becomes evident that the uptake of label is not always proportional in the concentration of MPS present. For example, the epiphysis of the cortisone-treated rats showed a marked reduction in ^{35}S-uptake in the region of the epiphysial plate, but there was little difference in the hexosamine and hexuronic acid content in

epiphysis between the control and the treated group. Similarly, with the skin, while there was a pronounced decrease in the ^{35}S incorporation after cortisone treatment the diminution in the hexosamine and hexuronic acid content is demonstrable, when the results are calculated only on the basis of dry weight of the defatted tissue.

The finding of a pronounced decrease of ^{35}S incorporation in the skin and cartilage of the cortisone-treated animals, as compared to the controls, is not contradictory to the smaller changes observed in hexosamine and hexuronic acid content of these tissues. As already indicated, the amount of ^{35}S present in the radioautograms reflects mainly the incorporation of ^{35}S sulfate into sulfated MPS during a relatively short time interval (2 hours) and may be considered to represent mainly synthesis. On the other hand, the analytical estimation represents the content of MPS existing in the tissues and may be thought to reflect the total MPS pool.

Little is understood about the relationships of MPS pools in tissues, but it would appear from the work of Kaplan and Fisher (317), Schiller et al. (316), and Rice (313) that the concentration of various MPS, even within the same tissue, is affected differently by the 'anti-inflammatory' steroids. If one makes the oversimplified assumption that individual MPS within one tissue belong in the same metabolic pool, then the size of that pool is controlled by both the synthesis and catabolism of the MPS. As already mentioned, there is evidence (315, 316, 317) that administration of glucocorticoids to animals causes a decrease in the catabolism as well as the synthesis of the MPS. The mechanism by which the former is controlled is obscure. It is of interest to recall the

stabilizing effect of glucocorticoids on lysozomes (385, 386) which are reported to contain a number of enzymes that participate in MPS catabolism (p. 82). If lysozomal hydrolases, such as β-glucuronidase, β-N-acetylglucosaminidase, arylsulfatases - A and B, β-galatosidase, play a role in the catabolism of MPS in normal animal tissues, the presence of glucocorticoids may decrease catabolic activity by stabilizing the lysosomes against rupture and release of the enzymes. However,this postulated protective action of the corticoids has not yet been established for lysozomes in normal, 'noninjured' tissues (383).

(2) Site of the Inhibitory Action of Cortisone on the Biosynthesis of MPS.

Having demonstrated that cortisone administration to the rat resulted in inhibition of the synthesis of MPS, we proceded to determine the possible sites of inhibition in the synthetic pathway of MPS, under identical conditions of steroid treatment.

The observed decrease in glucosamine-6-P synthesis by the liver supernatants from the cortisone-treated animals directed our attention first to the amidotransferase-catalyzed reaction, since this represents the first step in the principal hexosamine biosynthetic pathway (19,21). This inhibition was studied, therefore, in some detail.

We found that the activity of the amidotransferase decreased only after several days of cortisone administration to the animals. The same delay in inhibition was apparent whether the activity of the enzyme was assayed in supernatants or the whole homogenates, but the values with

the homogenates were considerably lower. The delayed pattern of decrease in activity is different from what has been commonly observed with a number of 'induced' enzymes in liver, after administration of glucocorticoids, where a sharp increase in activity often occurs within a few hours after glucocorticoid administration (p.77). Thus, it would appear unlikely that the decrease in activity of amidotransferase in the treated animals is attributable to the induction of enzymes involved in gluconeogenesis, such as the hexose-6-P phosphatases (361), or the transaminases (335). Nevertheless, it is important to rule out the possibility of an effect of hexose-6-P phosphatase on the amidotransferase system. If the phosphatase were active it could influence the assay values for the amidotransferase activity by hydrolysis of the substrates glucose-6-P or fructose-6-P. This possibility, however, was excluded in the case of the assays done with supernatants, by demonstration of the absence of the phosphatase. The whole homogenates, on the other hand, may be expected to contain hexose-6-P phosphatase. This circumstance may account, at least in part, for the comparatively low activity observed in the whole homogenates by Pogell and Gryder (21), and Bollet and Schuster (326). Although hexose-6-P phosphatase does not affect the validity of amidotransferase assay with liver supernatant, it is possible that in the intact liver the phosphatase could influence the amount of substrate available to the amidotransferase even though these two enzymes appear to be in different subcellular fractions (Table XVII). However, the argument may be raised that in such an event an increased hexose-6-P phosphatase activity in the cortisone-treated animal may accentuate the inhibition on glucosamine-6-P synthesis

by removing available substrate (glucose-6-P or fructose-6-P) for the amidotransferase reaction. However, since the effective glucose-6-phosphate pool may be influenced by other factors such as enhanced gluconeogenesis as a result of glucocorticoid administration (338, 340, 342), it is difficult to evaluate the effectiveness of the phosphatase in determining the availability of glucose-6-P.

The validity of the amidotransferase assay values in the supernatants was indicated also by identification of the product, glucosamine-6-P on ion-exchange chromatography for both the control and the cortisone-treated animals. Identification of the glucosamine-6-P peaks both by the Elson-Morgan reaction and measurement of the radioactivity unequivocally established that less glucosamine-6-P was formed by the liver supernatants from cortisone-treated animals, than the same quantity of supernatant from the controls. The fact that no glucosamine was identified either by radioactivity measurement or the Elson-Morgan reaction on ion-exchange chromatography of the incubation mixtures indicates that dephosphorylation of the glucosamine-6-P did not occur and thus can be excluded as an interfering factor. Nor is it likely that glucosamine-6-P formation, under our conditions, would be affected by the presence of phosphofructokinase as the incubation was carried out in the absence of ATP and in the presence of EDTA, which binds magnesium. The possibility of glucosamine-6-P deaminase activity in the cortisone-treated animal cannot be excluded, but this enzyme has been reported to be present only in very low concentration in the liver (401) .

It is probable, therefore, that the observed decrease in the formation of glucosamine-6-P by the liver supernatants from the cortisone-

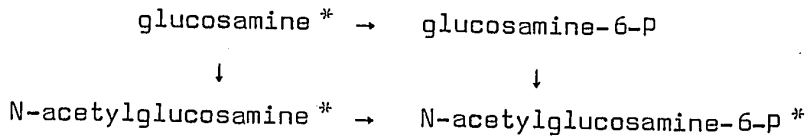
treated animals was due to a decrease of the activity of the amidotransferase per se, and not to other reactions that may have metabolized the substrate or product. Nevertheless, it is difficult to be certain whether the decreased activity of the amidotransferase would, in fact, lead to a decrease in the amount of glucosamine-6-P formed in the livers of the cortisone-treated animals. As shown (p.190), glucosamine-6-P is present in too small a concentration to be amenable to direct measurement. However, our results indicate that the concentration of hexosamine was diminished in the livers of the cortisone-treated animals. The identification of the hexosamines in the liver hydrolyzates as being composed mainly of glucosamine is in agreement with the view that suppression of its formation had occurred under the influence of cortisone.

Our studies on the incorporation of glucosamine-^{14}C into the phosphorylated intermediaries (p.196) also fit in with the view that the predominant block in the hexosamine synthetic pathway is at the amidotransferase step. The ratio of isotope incorporated (in the control: to the cortisone-treated) into N-acetylglucosamine-6-P (Fraction III) and that in the UDP-N-acetylglucosamine (Fraction IV) is very similar. There is no evidence, therefore, that any additional blockage occurred in the hexosamine synthetic pathway of the cortisone-treated animal, between the above-mentioned two intermediareis (see also Fig.2, 'Introduction'. p.22).

The reason for the greater number of counts/g wet weight of liver in FractionsII and III in the control as compared to that in the cortisone-treated animals is not clear. However, as the glucosamine-^{14}C was

administered to the animals on a body weight basis, the controls received a considerably higher total dosage since they weighed, on the average, twice as much as the cortisone-treated animals.

The relatively very low number of counts present in Fraction I is in agreement with the observations of McGarahan and Malley (48). According to these authors, glucosamine predominantly enters the pathway by being acetylated first and then phosphorylated, rather than the reverse, i.e.,



Evidence has been presented (48) that the glucose in the liver (which is present at a relatively high concentration) competes with glucosamine as substrate for a hexokinase. However, this is not the case between N-acetylglucosamine and glucose. Our finding of low radioactivity in Fraction I which includes glucosamine-6-P, indicates that this situation also applies in the case of the cortisone-treated animal. It must be pointed out, however, that although glucosamine-6-P standards are eluted with 'Fraction I', neuraminic acid and N-acetylglucosamine-1-P may also be included in this fraction (48, 49). Furthermore, this fraction, which contains only a few counts, is eluted immediately after very highly labelled glucosamine and N-acetylglucosamine which are included in the initial water wash of the column. For these reasons the author feels that the reliability of the radioactivity measurements in Fraction I (as representing labelled glucosamine-6-P) is in doubt and further conclusions are unwarranted.

UDP-N-acetyl hexosamines are considered to be the direct precursors of MPS and glycoproteins (p.26). We found the concentration of UPD-N-acetyl hexosamine in the liver of cortisone-treated animals to be decreased when the results were expressed on the basis of DNA but not on the basis of wet weight of tissue. Little is known about how the levels of the hexosamine nucleotides are controlled or how they influence the rate of MPS or glycoprotein synthesis. Kornfeld et al. (25) have postulated that a feedback control exists which tends to maintain the UDP-N-acetyl hexosamine pool constant, by inhibiting the amidotransferase reaction when the amino sugar nucleotides accumulate. Nevertheless, this view may be an oversimplification as these workers (25) also found that the concentration of the sugar nucleotides (in terms of wet weight of liver) does not change after puromycin administration, even though their data with glucosamine-^{14}C indicated that UDP-N-acetylhexosamines are not metabolized and would be expected to accumulate. Our results suggest that during cortisone treatment the amidotransferase reaction is inhibited and we could find no evidence for the presence of another block in the hexosamine synthetic pathway. The argument could be advanced that if the amidotransferase step is rate-limiting in the synthetic pathway, then the levels of UDP-N-acetylhexosamine in the livers of the cortisone-treated rats would decrease compared to levels in the control animals, if other related reactions remained constant. We found this to be the case when the UDP-N-acetylhexosamine content of the liver was expressed on a per cell basis. Nevertheless, the pool size of UDP-N-acetylhexosamine in the livers of the cortisone-treated animal could be influenced also by a variety of other factors.

The effect of cortisone on protein metabolism is of particular importance, especially in view of the fact that parts of the protein in glycoproteins may be thought of as 'acceptors' of the hexosamines of the amino sugar nucleotides (63-65). A somewhat similar situation may apply in the 'protein-polysaccharide' complexes of MPS (141, 142).

(3) Relationship Between the '*In Vivo*' and '*In Vitro*' Inhibition of the Amidotransferase Reaction by Glucocorticoids.

The observed inhibition of the amidotransferase activity,'*in vitro*', (i.e., inhibition by the addition of hydrocortisone succinate to normal rat liver supernatants), was of particular interest, since a study of this system might yield clues as to the mechanism of action of glucocorticoids '*in vivo*'. However, our findings have indicated that the *in vivo* inhibition of the amidotransferase (i.e., inhibition by the daily administration of cortisone to rats) might not have an identical mechanism of action as the *in vitro* effect, for the following reasons.

First, while the *in vivo* inhibition of amidotransferase activity occurs only after several days of cortisone administration to rats, the *in vitro* effect was obtained immediately on addition of hydrocortisone succinate to normal supernatant. (Figs. 27, 28).

Second, if the delay in the decrease in activity of the amidotransferase after subcutaneous cortisone administration is due to delayed absorption of the steroid, then this delay might be overcome by giving large doses of a soluble derivative of the steroid intravenously. Since it has been shown (402) that hydrocortisone administered intravenously reaches maximum concentration in the hepatocytes in 45 min-

utes, one would anticipate, that if the immediate *in vitro* effect has a similar mode of action as the *in vivo* effect, then the decrease in amidotransferase activity should occur at or about that time. However, our results clearly demonstrated that no effect on the activity of the amidotransferase is observed at least up to 8 hours after the intravenous administration of very large doses of hydrocortisone succinate, thus suggesting that the mechanism of action of the *in vivo* and the *in vitro* effects are dissimilar.

Third, the suppression of the amidotransferase activity *in vitro* was observed only with high concentrations of the steroid (i.e., in the order of 10^{-3}M), while the levels attained by subcutaneous administration would be much lower (403). Furthermore, incubation of rat plasma with an excess of cortisone followed by addition of the incubated plasma to normal rat liver supernatant, did not produce inhibition of the amidotransferase (p.168).

There are, on the other hand, some features of similarity between the behaviour of the supernatant from the *in vivo* and the *in vitro* systems. The inhibition produced in both systems appears to be irreversible, in that the inhibitory action was not diminished by dialysis of the preparations(Tables XX,XXI).or by high concentrations of substrate (G-6-P) (Figs.30, 31, 32).

The most striking similarity between the two supernatant preparations was their kinetic behaviour in terms of velocity in relation to the substrate concentration. The Lineweaver-Burk plots for the two systems are similar and their apparent K_m values are practically the same, thus suggesting that the affinity of the enzyme for the inhibitor

also is similar.

If it could be demonstrated that the *in vitro* effect has a similar mechanism of action to the *in vivo* effect, it may be argued that at least one way by which glucocorticoids can affect the activities of enzymes is by direct interaction with them. The evidence thus far presented does not allow this conclusion to be drawn with certainty. However, further experiments on purification of the amidotransferase from the two systems and a study of the physical-chemical properties of the inhibited enzyme as compared to the normal enzyme may produce some further clues as to how steroids affect the activity of enzymes.

SUMMARY AND CONCLUSIONS

1. Administration of cortisone, in relatively large doses, to rats, decreased the incorporation of injected ^{35}S-sulfate into the epiphyses, skin and to a lesser extent, aorta and liver, as compared to the degree of incorporation in normal animals. With the aid of coated radioautograms it was further demonstrated that 2 hours after the injection of the radioisotope most of the label was still within the cells in both groups of animals. However, considerably less label had been incorporated intracellularly, or excreted extracellularly, in the livers of the cortisone-treated animals. Since injected radiosulfate is known to be incorporated mainly as ^{35}S-sulfate in sulfated mucopolysaccharides (MPS), we interpreted our results as indicating that the synthesis of sulfated MPS had been inhibited by the cortisone. An additional effect on the catabolism of the MPS after they had been secreted cannot be excluded.

Corroborative histochemical evidence was obtained of pronounced alterations in the MPS of the tissues, especially in the skin and cartilage.

2. Analysis of tissues for hexosamine and hexuronic acid content (by a resin hydrolysis procedure that allows survival of the hexuronic acid) indicated that the aorta and liver of the cortisone-treated animals contained less hexosamine (on a dry weight basis)

than these tissues of the controls. Similarly, the hexosamine concentration of the defatted skins was decreased after cortisone treatment, but no change was observed in the case of the epiphysis. The change in the hexuronic acid concentration paralleled that of the hexosamines in the skin, aorta and the epiphysis, but it was not possible to obtain a reliable estimate of the hexuronic acid concentration in the liver. The results indicated that the concentration of MPS, and probably of glycoproteins, was decreased in the skin, aorta and liver, with smaller changes in the epiphysis. Ion-exchange chromatography of the liver hydrolyzates, with both the ninhydrin and the Elson-Morgan detection systems, indicated that glucosamine and a small amount of galactosamine were present in the liver, and that cortisone administration had decreased the concentration of glucosamine in the liver of the treated animals.

3. The activity of fructose-6-P-L-glutamine amidotransferase was decreased in the liver after the third day of cortisone administration ('in vivo system') and it persisted as long as the treatment was continued. The validity of the amidotransferase assay was demonstrated for the supernatants from the liver homogenates, but not for the whole homogenates. Assays for the amidotransferase, with ^{14}C-labelled glucose-6-P and cold glutamine as the substrates and with the aid of ion-exchange chromatography of the products, indicated that glucosamine-6-P was the only hexosamine intermediate produced during incubation of the liver supernatant from the normal and the cortisone-treated animals. Thus, the decrease in activity of the

amidotransferase might be one way by which cortisone exerts its inhibitory action on MPS and glycoprotein synthesis, at least in the liver.

4. A decrease in activity of the amidotransferase was observed also when the soluble derivative, hydrocortisone sodium succinate, was added in relatively high concentration to supernatant from the liver homogenate from normal animals ('in vitro system').

5. Studies to establish whether the decrease in activity in the 'in vivo system' was caused by the same mechanism as with the 'in vitro system' afforded evidence that the mechanisms may be different:

While the response in vitro was immediate upon addition of the steroid to the normal supernatant, the response in vivo was observed only after several days of cortisone administration. Furthermore, when the hydrocortisone was administered intravenously no decrease in the amidotransferase activity was observed at the time when peak concentration of the steroid was expected to be reached in the liver cells. On the other hand, the amidotransferase reaction velocity, in the two systems, varied in a similar manner when measured with various concentrations of the substrate (glucose-6-P). A shift in the apparent K_m values as well as in the maximum velocity was evident in both cases. The absence of a dialyzable inhibitor of the amidotransferase was demonstrated with both the systems, and the observed decrease in the transferase activity in both systems was apparently irreversible. The decrease in activity is tentatively attributed to inhibition. However, the possibility that a suppression of enzyme

systems *in vivo* has occurred as well cannot be ruled out.

6. Experiments on the administration of glucosamine-^{14}C to normal and cortisone-treated animals demonstrated incorporation of the label predominantly into the N-acetyl glucosamine-6-P and the UDP-N-acetylhexosamine in the liver. Relatively little incorporation of the label was found in the fractions which include glucosamine-6-P. Thus there is evidence that in the cortisone - treated animal, as well as the normal, free glucosamine enters the biosynthetic pathway predominantly by way of the N-acetyl glucosamine-6-P. The ratios of radioactivity (between the control and the cortisone-treated animals) found in the various phosphorylated intermediaries do not suggest the presence of a block in the biosynthetic pathway for hexosamines other than that at the amidotransferase step.

The author considers, therefore, that at least one way by which cortisone exerts its inhibitory action on MPS and glycoprotein synthesis is by inhibiting the first step of the hexosamine intermediary biosynthetic pathway.

CLAIMS TO ORIGINAL RESEARCH

The author considers points 2 - 6, and part of the work with coated radioautograms (point 1) mentioned in the foregoing summary to be original contributions.

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LIST OF ABBREVIATIONS

G-6-P	-	Glucose-6-phosphate
F-6-P	-	Fructose-6-phosphate
ATP	-	Adenosine triphosphate
UTP	-	Uridine triphosphate
EDTA	-	Ethylene diaminetetracetic acid Tetrasodium salt
AcCoA	-	Acetyl coenzyme A
Amidotransferase	-	D-fructose-6-P-L-glutamine amidotransferase

Appendix Table i.

Effect of Daily Injections of Cortisone Acetate on the 'Glucosamine-6-P' Synthesizing Capacity of Rat Liver Homogenates.

No. of Exp'ts.**	No. of Daily Injections		'Hexosamine Products' Formed*		
			Cortisone-Treated	Controls	Ratio Treated/Control
			(µmoled/ml/hr per 100 mg liver)		(%)
2	0		0.058	0.062	94
			0.054	0.076	72
		Mean	0.056	0.069	83
1	1		0.078	0.076	100
2	2		0.072	0.058	125
			0.044	0.032	137
		Mean	0.058	0.045	131
3	3		0.001	0.040	2
			0.077	0.109	70
			0.008	0.036	22
		Mean	0.028 ± 0.07	0.062 ± 0.23	31
4	5		0.094	0.130	72
			0.004	0.062	6
			0.004	0.044	9
			0.010	0.070	14
		Mean	0.028 ± 0.02	0.077 ± 0.06	25
5	7		0.012	0.052	23
			0.094	0.112	84
			0.004	0.104	38
			0.022	0.068	32
			0.030	0.058	51
		Mean	0.032 ± 0.04	0.079 ± 0.09	46
2	9		0.004	0.038	22
			0.016	0.070	23
		Mean	0.010	0.054	23
1	26		0.026	0.048	54

* Each value represents the mean of two simultaneous incubation experiments.

** Each experiment represents the simultaneous preparation and assay of homogenates from liver of control and cortisone-treated animals.

Appendix Table ii.

Effect of Daily Injections of Cortisone Acetate on the Glucosamine-6-P Synthesizing Capacity by Rat Liver Supernatants.

No. of Exp'ts**	No. of Daily Injections		'Gm-6-P' Formed *		Ratio Treated/Control
			Cortisone-Treated	Controls	
			(μmoles/ml/hr per 100 mg liver)		(%)
3	0		0.172	0.188	91
			0.192	0.172	111
			0.181	0.168	107
		Mean	0.181	0.177	102
1	1	Mean	0.145	0.149	97
2	2		0.149	0.149	100
			0.143	0.161	89
		Mean	0.146	0.155	95
3	3		0.142	0.162	89
			0.132	0.189	71
			0.110	0.124	51
		Mean	0.128± 0.12	0.165± 0.15	70
5	5		0.092	0.137	61
			0.130	0.220	59
			0.094	0.232	40
			0.087	0.206	42
			0.128	0.210	61
		Mean	0.106± 0.05	0.202± 0.09	53
3	7		0.039	0.164	23
			0.038	0.157	21
			0.048	0.177	27
		Mean	0.041± .015	0.166± 0.11	24
3	9		0.054	0.178	31
			0.078	0.175	51
			0.072	0.168	48
		Mean	0.068± 0.05	0.173± 0.14	43
1	10	Mean	0.078	0.216	34
2	12		0.057	0.212	37
			0.094	0.224	38
		Mean	0.076	0.218	38
1	26		0.072	0.189	40

* Each value represents the mean of two simultaneous incubation experiments
** As in Table i .

Appendix Table iii.

Influence of Storage on the Capacity of Liver Supernatant Fractions to Synthesize Glucosamine-6-P *

No. of Exp'ts **	No. of Daily Injections		'Hexosamine Products' Formed: Cortisone-Treated	'Hexosamine Products' Formed: Controls	Ratio Treated/Controls
			(µmoles hexosamine/ml/hr per 100 mg liver)		(%)
2	0		0.086	0.082	105
			0.084	0.082	102
		Mean	0.085	0.082	103
2	3		0.017	0.082	19
			0.042	0.093	45
		Mean	0.030	0.088	34
2	5		0.025	0.083	28
			0.028	0.083	32
		Mean	0.027	0.083	30
1	9		0.043	0.092	46

* Liver supernatants from normal and cortisone-treated rats, stored for 5 hours at 4°C.

** As in Table i .