STUDIES ON BIOLOGICALLY IMPORTANT POLYMERS AND RELATED COMPOUNDS.

THESIS

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in the

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by

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PREFACE.

I wish to express my grateful thanks to Professor J.M. Robertson, under whose supervision this work was carried out. I also express my gratitude to Dr. W.R. Rees for his constant advice and encouragement.

A preliminary report of the work described in Part I, Section I, has appeared in the following publication:-

L.M. Fogarty and W.R. Rees, Nature, 193,1180,(1962).

and a short paper discussing some of the work described in Part II, Section II, was read at the following meeting:-

Fourth International Seaweed Symposium,Biarritz,1961.

I wish to record my thanks to Professor P. O'Colla, University College Galway, Ireland, for the gift of sulphated polysaccharides from the sea-:weeds Furcellaria fastigitata and Chondrus crispus.

W.M.F.

Abbreviations used.

adenosine 2'(3') monophosphate	A2'(3')MP
adenosine-5'phosphate	A5'P, AMP.
adenosine 2'(3')5'diphosphate	A2'(3'):5DP.
adenosine 2':5'diphosphate	A2':5'DP.
adenosine 3':5'diphosphate	A3':5'DP,PAP.
adenosine 5'-sulphatophosphate	APS.
adenosine 3'-phosphate-5'-sulphatophosphate	PAPS.
adenosine 5'-diphosphate	ADP.
adenosine triphosphate	ATP.
uridine diphosphate glucose	UDPG.
uridine diphosphate acetyl-:glucosamine	UDPAG.
uridine diphosphate glucuronic acid	UDPGA.
inorganic pyrophosphate	PP.
p-nitrophenylsulphate	p-NPS.
p-nitrophenol	p-NP.
ethylenediaminetetraacetate	EDTA.
2-amino-2 hydroxymethyl-propan - 1:2-diol with HCl	Tris (buffer)
4-amino-4'-chlorodiphenyl	CAD.
2-cyanoethylphosphate	CEP.
dicyclohexylcarbodiimide	DCC.
dicyclohexylurea	DCU.
millimole	m.mole,mM.
micromole	µM.

millimicromole	mμM.
milligram	mg.
microgram	μg.
general method	G.M.

NOTE: All temperatures are expressed in degrees Centigrade.

The words sulphation, sulphurylation and sulphatation are synonymous.

CONTENTS.

GENERAL INTRODUCTION.

I. PRESENT KNOWLEDGE CONCERNING ADENOSINE 3'-PHOSPHATE 5'-SULPHATOPHOSPHATE (PAPS) AND ITS ROLE IN SULPHATE METABOLISM.

Sulphate is bound mostly in ester linkage in a fairly large variety of compounds present in living organisms. Most important among these compounds are the sulphated mucopolysaccharides. The occurrence of sulphate esters has been known for many years, but only recently has much interest been shown in their metabolism. Sulphate esters may be classified as follows: See also Fig. 1.

1. Aryl sulphates.

These commonly occur in urine, both under normal physiological conditions and after the adminis-:tration of phenols Fig. 1(a) and phenol precursors (1). Elsewhere few have been found. Tyrosine-O-sulphate occurs in fibrinopeptide (2) while the sulphate esters of various pigments have been found in bile (3).

2. Steroid sulphates.

This group includes three different types of

compound:-

(a) compounds of the type of esterone sulphate Fig. 1(b) which is an aryl sulphate.

(b) compounds typified by androsterone sulphate Fig. 1(c) in which the parent hydroxyl is that of a secondary cyclic alcohol (4).

(c) compounds such as ranol sulphate (5). These are true alkyl sulphates being derivatives of primary alcoholic groups in the side chain of the steroid molecule, Fig. 1(d).

3. Carbohydrate sulphates.

This group contains many types of compounds, including the sulphates of the mucopolysaccharides of connective tissue (6), the sulphated polysaccharides of marine algae (7) and the sulpholipids of nervous tissue (8).

4. Alkyl sulphates.

As well as the bile salts this group includes choline-O-sulphate, Fig. 1(e), a metabolite

of several moulds (9) and the sulphates of primary aliphatic alcohols. (10).

5. Mustard oil glycosides.

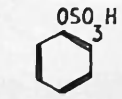
This group includes the sulphate esters of compounds which contain the grouping $=N-O.SO_3H$. e.g. sinigrin (11), Fig. 1(f).

6. Sulphamates.

Two main types occur in biological materials: (a) the sulphamate derivatives of glucosamine as found in heparin (12) and (b) the aryl sulphamates Fig. 1(g), found in urine after the administration of certain arylamines (13).

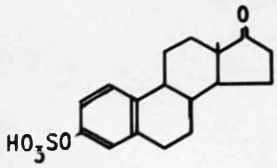
While knowledge concerning the chemistry of sulphate esters dates back to the beginning of the century, current concepts of the metabolism of these substances have resulted from developments during the past decade. However, in 1861, Boedeker, performed the first known metabolic experiment in this field when he administered a cartilage jelly from autopsy material

SOME NATURALLY OCCURING
SULPHATE COMPOUNDS



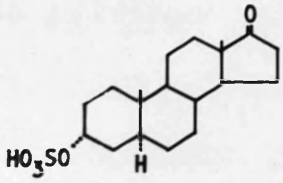
phenyl sulphate

(a)



esterone sulphate

(b)



androsterone sulphate

(c)

$C_{27-28}H_{43-45}(OH)_4 \cdot OSO_3H$

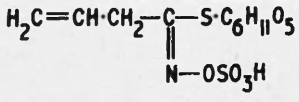
renol sulphate

(d)

$^{-}O_3SO \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}(CH_3)_3$

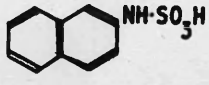
choline - O - sulphate

(e)



sinigrin

(f)



2—naphthylsulphamic acid

(g)

FIG. I.

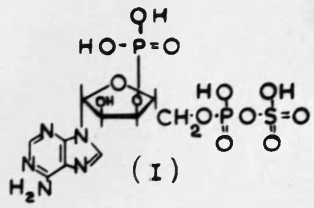
to volunteers and observed an increased excretion of sulphate in the urine (14).

The pioneer studies of De Meio and his co-workers (15-17) laid the foundations of our present knowledge concerning the metabolic role of adenosine 3'-phosphate 5'-sulphatophosphate (PAPS). He showed that enzymes in rat liver homogenates could synthesize aryl sulphates from phenols and inorganic sulphate in the presence of adenosine triphosphate (ATP). An extension of this work by Bernstein and McGilvery (18, 19) demonstrated that enzymes from rat liver induced reaction between sulphate ions and ATP to form an "active intermediate" which in turn in the presence of phenol gave rise to phenylsulphate. Kinetic studies by Segal (20) led to the same conclusion. He showed that the rate curves of the phenol-sulphate conjugating system of rat liver conformed to the kinetics of a two-step reaction. The rate constants of the separate reactions (i.e. (a) activation and (b) transfer) were determined and shown to be proportional to enzyme concentration.

De Meio (21) separated the system into two fractions, the sulphate activating portion which synthesizes "active sulphate" and the sulphate trans-:ferring enzyme which transfers sulphate from "active sulphate" to a phenol. This active intermediate was shown to be an adenylic acid derivative (22,23) and was finally identified by Robbins and Lipmann (24,25) as adenosine 3'-phosphate 5'-sulphatophosphate (PAPS). Fig. 2.

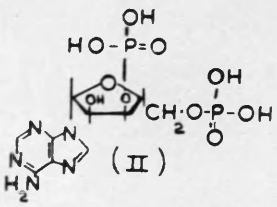
Thus, these workers isolated the "active sulphate" by ion-exchange chromatography (Dowex - 1) and showed it to contain adenine, ribose, phosphate and sulphate in the molar ratios of 1:1:2:1. Acid hydrolysis of the compound indicated a phosphate on the 2' or 3' position of the ribose moiety, thus excluding the presence of a pyrophosphate group. Phosphate attachment to the 3' position was confirmed:-
(a) by a negative periodate reaction and (b) by use of the specific rye grass 3'-nucleotidase which liberated phosphate from the compound. The sulphate group was released completely by treatment with 0.1 N HCl at 37^{o}

STRUCTURAL FORMULAE OF PAPS, PAP & APS.



(I)

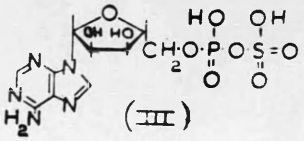
"Active Sulphate" (PAPS)



(II)

A 3':5'DP.

(PAP)



(III)

APS

Figure 2

for 30 minutes. Its point of attachment was indicated by titrimetric data which revealed the production of an equivalent of secondary phosphate. It was therefore deduced that the 5' position of the ribose was substituted by a sulphatophosphate group. Final confirmation of the proposed structure was eventually obtained by chemical synthesis. (58).

Further investigation showed that enzymatic synthesis of PAPS required two enzymes (26,53). The first, ATP - sulphurylase catalyzes the displacement of pyrophosphate (PP) from ATP with formation of adenosine 5'-sulphatophosphate (APS) Fig. 2. The second reaction catalyzed by APS-kinase is the phosphorylation of the 3'-hydroxyl group of APS to form PAPS.

$$(1) \quad ATP + SO_4^{2-} \xrightleftharpoons{\text{ATP-sulphurylase.}} APS + PP.$$

$$(2) \quad APS + ATP \xrightleftharpoons{\text{APS-kinase.}} PAPS + ADP.$$

The ATP-sulphurylase of yeast is a stable acidic protein which has been purified extensively (53-55) and the following remarks are largely confined to this system. The outstanding characteristic of the

sulphurylase reaction is the extremely unfavourable equilibrium for APS formation (54). Since the equilibrium is so unfavourable the forward reaction proceeds to a reasonable extent only when both products, PP and APS, are removed. Pyrophosphate (PP) is removed by hydrolysis and APS by its conversion to PAPS by APS-kinase.

ATP-sulphurylase is completely specific for ATP but will use a number of inorganic anions in place of sulphate (55). With all of these anions, of which molybdate is the most active, an unstable anhydride which hydrolyzes spontaneously is presumably formed between AMP and the anion. The overall reaction then observed is a splitting of ATP to AMP and PP. APS-kinase of yeast and other tissues has not been studied as extensively, although the yeast enzyme has been purified. The fact that ATP-sulphurylase has been found in liver homoganates indicates that enzymatic mechanisms of PAPS formation are likely to be similar in liver and yeast.

The complete enzyme system that synthesizes

PAPS has been found in a variety of plant, animal and microbial sources. Liver (19), Neurospora sitophila (23), yeast (26) chick embryo (27), mast cell tumour (28) hen oviduct (29) and the marine snail Busycon (30) include some of the sources that have been investigated.

It has been noted, however, by several workers, that the sulphate activating system is readily demonstrated in tissues (e.g. yeast) which apparently do not contain sulphuryltransferases, * which are enzymes that transfer sulphate from PAPS to acceptor molecules. This would seem to suggest that PAPS may have functions other than that of a sulphate donor. Some indications that this might be so, have indeed, been found. For instance, the formation of PAPS appears to be a necessary

* While some authors refer to an enzyme transferring sulphate as a "sulphokinase", the alternative and less ambiguous term "sulphuryltransferase" has been used in this account. O.Hoffmann-Ostenhof, Adv. in Enzymology, 14, 219, (1953) reserves the term transferase for "those enzymes which transfer a part of a donor molecule except hydrogen or electrons, to an acdeptor molecule, and neither the donor nor the acceptor may be water".

step in the reduction of sulphate to sulphite by micro organisms (31,32). Again, the reported occurrence of a succinoadenosine 5'-sulphatophosphate in salmon liver (33,34) suggests as yet unsuspected functions for sulphatophosphates. It seems possible that the role of PAPS and related sulphatophosphates in metabolic processes may be more extensive than is apparent at present.

Thus, two separate biological roles are envisaged at the moment both dependent upon the activ-:ation of sulphate to PAPS. In one, the sulphate may be reduced as indicated in the above paragraph, or alternatively, and this is the matter of greater concern in this present investigation, it may be transferred enzymatically.

As mentioned, transfer of sulphate from PAPS is catalyzed by a group of enzymes called sulphuryltransferases.* The general reaction leads to the formation of a sulphate ester and PAP, as follows:

$$\text{(3)}\quad \text{PAPS} + \text{X} \xrightarrow{\text{x-sulphuryltransferase}} \text{X-sulphate} + \text{PAP}$$

A number of sulphuryltransferases is now known (35) and

* See footnote page 10.

among those which have been studied is phenol sulphuryltransferase which transfers sulphate from PAPS to a phenol to form an aryl sulphate. This liver enzyme has been obtained by Nose and Lipmann in a fairly pure state (36).

Phenol sulphuryltransferase can also be obtained admixed with other transferases by suppressing the sulphate activating system in liver preparations by removal of sulphate ions. This enzyme will also catalyze the transfer of sulphate from p-nitrophenyl-:sulphate (p-NPS) to a suitable phenol with formation of p-nitrophenol (p-NP) and arylsulphate:

(4) p-NPS + PAP $\rightleftharpoons$ p-NP + PAPS

(5) PAPS + R.OH $\rightleftharpoons$ PAP + $R.O.SO_3H$.

This reaction requires the presence of adenosine 3':5'-diphosphate (PAP) as a co-factor and can in fact be utilized as an assay system for PAP (37): the concentrat-:ion required is very low, saturation being reached at 2×10^{-6} M PAP.

Specificity with regard to sulphate acceptor has not been exhaustively studied but apparently several

phenols can act as such (37). A number of discrepancies appear in the reported properties of this system: the pH optimum has been given as ranging between pH 6.0 and 8.5 (37-39) apparently depending on the composition of the buffer. A requirement for magnesium ions has been claimed by Segal (40) but denied by Lipmann (37); a requirement for -SH groups has been noted by Gregory and Lipmann (37) but not by Brunngraber (38). The reasons for such discrepancies are not clear but may lie in the different experimental techniques. However, the existence of a number of different sulphuryltransferases should not be excluded.

Among other reactions which have been observed in vivo and in vitro are the sulphurylation of tyrosine derivatives (39) arylamines (41), steroids (42,43), charonin (44) - a sulphated polyglucose, cerebrosides (45), bilirubin (46), choline (47), and mucopolysaccharides (29,48,49). Vestermark and Bostrom (57) have observed that a number of aliphatic alcohols and polyhydroxy compounds can be conjugated as sulphates by extracts of rat liver.

The work summarized above shows that the major route of sulphate ester synthesis is by a sulphuryltransferase - catalyzed transfer of sulphate from PAPS. A closely related route, is the sulphate transfer from an aryl sulphate to a phenol by a reaction involving the intermediary formation of PAPS (37), as mentioned earlier. See p. 12 eq. 4-5. It differs in that ATP and APS are not involved. More recently Wortman (56) has demonstrated phenol and mucopolysaccharide sulphuryltransferase activities in beef cornea epithelial extracts and has coupled these enzymes to serve as an assay procedure for the study of the latter enzyme. There are, however, some indications that other transfer routes may exist. Thus, it has been shown (44,50) that extracts of the digestive gland of the mollusc Charonia lampas will incorporate sulphate from p-nitrophenylsulphate (p-NPS) into glucose -6-sulphate or charonin sulphate (a sulphated polyglucose found in the digestive gland of C.lampas). Because of the inhibition of this transfer by phosphate or fluoride it was suggested that an aryl sulphatase was involved since both of these ions are general sulphatase inhibitors. Purified liver aryl

sulphatase from this organism could also transfer sulphate from S^{35} labelled p-NPS to crude but not to purified preparations of charonin sulphate (50). Whether the effect was a net synthesis of sulphate ester linkages in the polysaccharide or merely a sulphate exchange reaction, is not clear from the available data. Egami (51) has since claimed that both aryl and glucosulphatases are required, the former transferring sulphate from p-NPS to an unidentified co-factor in crude preparations of charonin sulphate and the latter subsequently transferring the sulphate from the sulphated co-factor to the carbohydrate.

Roy (52) has obtained another indication of a route not directly involving PAPS. He showed that 17-oxo-steroids greatly increased the rate of aryl sulphamate synthesis by rat liver homogenates although not by guinea pig liver preparations. Results obtained were consistent with the hypothesis that 17-enol sulphates were being formed by PAPS and that the sulphate was subsequently transferred from them to the arylamine by a reaction not involving arylamine sulphuryltransferase.

It is difficult to assess the general importance of the above type of reaction and in the case of the charonin sulphate it is the first suggestion that sulphatases may act as transferases in conjunction with sulphate donors.

II. PRESENT KNOWLEDGE CONCERNING THE ISOLATION AND STRUCTURE OF SOME MUCOPOLYSACCHARIDES.

A. Isolation and Purification of Mucopolysaccharides.

Recent years have witnessed a widespread and evergrowing interest in the group of compounds called mucopolysaccharides. An all embracing definition is difficult; to regard them as polymers of high molecular weight containing hexosamine is reasonably satisfactory. They exist free or associated with inorganic cations or with proteins and may be divided into two main groups: (1) Neutral mucopolysaccharides, e.g. chitin which contains only N-acetyl-glucosamine.

(2) Acid mucopolysaccharides, which in addition to hexosamine contain a uronic acid e.g. glucuronic acid as in hyaluronic acid and in other instances, sulphate, as in the chondroitin sulphates and heparin. See Table I.

In order to determine the nature and the amount of mucopolysaccharides in a tissue specimen two main principles may be used:

(1) direct acid hydrolysis of the tissue and analysis of the resulting mixture of monosaccharides or

(2) extraction and fractionation of the polysaccharides and determination of their physical and chemical properties.

The first method does not give any detailed information regarding the polysaccharide pattern in the tissue. Several polysaccharides may exist with all or some of their building units in common.

The ideal method for the preparation and fractionation of the polysaccharides should allow all the components to be extracted quantitatively from any tissue and to be quantitatively separated into well

defined fractions, each consisting of a chemically and physically individual polysaccharide. To date such a method has not been evolved and most of our knowledge of the polysaccharide pattern derives from the combination of the results obtained by different authors using different techniques.

At the beginning of this century extraction with alkali was the general method used for the isolation of polysaccharides from tissues and this method was used later on in most of the work done by the leading workers in this field. The greatest disadvantage in the alkaline extraction technique is the possibility of degradation of the polysaccharide molecules. Moreover the possibility exists that not all the polysaccharides are extracted.

Blix and Snellman (60) showed that the physical properties of chondroitin sulphate prepared by the alkaline extraction method are different from those given by milder methods.

To-day most investigators agree that the use of proteolytic enzymes for the breakdown of tissue

TABLE I.

CONSTITUENTS OF SOME MUCOPOLYSACCHARIDES.

NAME.	CONSTITUENTS.			
	HEXOSAMINE	URONIC ACID	GALACTOSE	SULPHATE.
Hyaluronic Acid	Acetylglucos -amine	glucuronic acid	-	-
Chondroitin	Acetylgalactos -amine	glucuronic acid	-	-
Chondroitin Sulphate A	"	"	-	+
Chondroitin Sulphate B	"	idurnoic acid	-	+
Chondroitin Sulphate C	"	glucuronic acid	-	+
Heparin	Glucosamine	glucuronic acid	-	+
Heparitin Sulphate	Acetylglucos -amine	glucuronic acid	-	+
Keratosulphate	"	-	galactose	+

proteins (61,62) in order to release the mucopoly-:saccharides gives a better recovery of material. Moreover these methods do not appear to degrade the polysaccharides provided they are not attacked by the enzymes used in the procedure.

The tissue should be finely ground or minced and the fats extracted in order to facilitate the penetration of the proteolytic enzyme into the tissue. A preliminary heat coagulation is of importance in the case of tryptic digestion, since the denatured proteins are more easily attacked by the enzyme.

When this treatment is complete the solution contains amino acids, peptides, salts and nucleic acids or their breakdown products and in most cases a few per cent of polysaccharide. In order to recover the polysaccharides in a reasonably pure form the mixture has to be subjected to some further purific-:ation procedure. Protein precipitating agents have been used but they have a tendency to precipitate polysaccharide together with impurities. A method

frequently used for the removal of proteins is the so-called Sevag technique (63). This method, originally described by Morner (64) consists of denaturing protein by shaking the aqueous solution with a mixture of chloroform and amyl alcohol. Schiller et al. (65) have used trichloroacetic acid to precipitate proteins in this context.

Probably the simplest way to dispose of most of the impurities is to precipitate the poly-:saccharide either by alcohol or by quaternary ammonium compounds. Alcohol precipitation sometimes yields a product contaminated with protein and/or protein breakdown products, depending on the organ from which the polysaccharides have been isolated. Extraction of the alcohol precipitated material with phenol to remove protein has been used. This technique has two distinct advantages. First, the removal of the materials produced by proteolysis is fairly good and secondly losses at this stage can easily be demonstrated by adding alcohol to the phenolic solution and analyzing the precipitate. One disadvant-:age is that 90% phenol is an unpleasant solvent to

work with, especially on a large scale.

Dialysis to remove impurities is not always to be recommended, since, depending on the quality of the dialysis membranes, some losses of polysaccharide may occur. Further purification is frequently required. Protein adsorbents such as Lloyd's reagent and Fuller's earth (67) have been used. These adsorbents, however, have a tendency to adsorb carbo-:hydrate together with protein.

Scott (68) has described a method for deproteinizing mucopolysaccharide solutions which is based on the use of quaternary ammonium compounds for the fractionation of acidic polysaccharides. In this method the solution is treated with a quarternary ammonium detergent e.g. cetyl-trimethyl-ammonium bromide at an alkaline pH. Both polysaccharides and proteins are thus precipitated. The formation of complexes between polysaccharides and proteins is prevented both by the alkaline reaction and also as the result of complex formation between the quarternary

ammonium compound and the protein. At a faintly acid pH the complexes between the proteins and the quarternary ammonium compound are soluble whereas the polysaccharide quarternary ammonium complexes are still insoluble. These precipitates can be brought into solution by treatment with strong salt solutions which also dissociates the complexes. Hence the polysaccharide may be subsequently recovered by precipitation with alcohol.

The most commonly used method for the fractionation of polysaccharide mixtures has been the fractional precipitation with organic solvents from aqueous solutions containing different metal ions. A fractionation scheme based on the addition of increas-:ing amounts of ethanol to the polysaccharide solution in the presence of calcium ions has been described by Meyer et al (61). Fractionation with alcohol has, however, certain disadvantages. It is difficult to apply on a small scale, moreover the fractions are frequently cross-contaminated.

Zone electrophoresis has been applied to the fractionation of polysaccharides (69). (71). More recently electrophoretic separations have been carried out on cellulose columns; these are claimed to give better results (60).

Paper chromatographic methods (72) have been examined but polysaccharides do not give discrete spots on paper. As mentioned earlier, Scott (68,73) found that quarternary ammonium compounds gave insoluble precipitates with acidic polysaccharides. Some of these complexes are more readily induced to dissociate in salt solution than others; increased salt concentration will in turn induce the remainder to dissociate. This phenomenon formed the basis of a paper chromatographic separation devised by Marsden and Kent (96) in which the paper was irrigated by an aqueous solution continuously increasing in salt concentration.

The modified cellulose ion-exchange materials introduced by Sober and Peterson (74) for fractionation

of proteins have been used by Ringertz and Reichard (75,76) for separation of acid polysaccharides. The fractionation is carried out on ECTEOLA-cellulose (77) at acid pH and separations obtained either by step-wise, or gradient elution chromatography. This adsorbent, ECTEOLA-cellulose contains basic groups derived from triethanolamine which are bound to the cellulose through reaction with epichlorohydrin.

B. The Structure of some Mucopolysaccharides.

Much work still remains to be done to elucidate the complete structure of many sulphated polysaccharides. In some cases considerable progress has been made. Many proposed structures are tentative and still await a rigorous proof. This situation arises largely because of the charged nature of the polysaccharides. Difficulties, as already mentioned, are involved in isolation and purification. For experimental purposes it is prudent to manipulate these materials as their salts. A major handicap in structural investigation lies in the difficulty of

acetylation and methylation. The presence of inorganic ions attached to the sulphate groups renders the resulting salt difficult to disperse in solvents such as pyridine, which is an excellent solvent for acetylation. Similarly, methylation becomes difficult because the sulphate group hinders the introduction of methyl groups. If it were possible to remove the sulphate groups without affecting the glycosidic link-:ages an examination of the products of hydrolysis of the methylated desulphated polysaccharide and a comparison with those of the original methylated substance would fix the positions of the sulphate groups. Unfortunately these groups are resistant to alkaline hydrolysis under ordinary conditions and acid hydrolysis in aqueous solution splits the polymeric linkages.

Heparin and the chondroitin sulphates are the most widely studied members of the sulphated mucopolysaccharides. Three different chondroitin sulphates have been distinguished and these have been designated chondroitin sulphate A, B and C respectively.

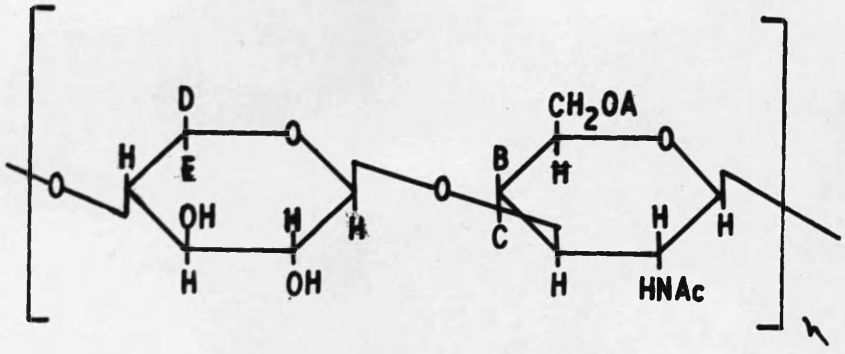
Their proportions vary in different tissues. The distinct identity of chondroitin sulphate C remained in doubt until it was shown to be the only polysaccharide isolated from a chondrosarcoma while from another only chondroitin sulphate A was isolated.

Because of their similarity and the confusion that exists in the literature it is appropriate that chondroitin sulphates A and C should be discussed together. Early workers regarded chondroitin sulphate as a single entity but many of their findings can be reinterpreted in the light of later results. Chondroitin sulphate was first isolated in 1861 by Fischer and Boedeker (59) and Krukenberg also reported its presence in cartilage (131). Hebting demonstrated (78) that hydrolysis of chondroitin sulphate with oxalic acid gave a sulphate-free and acetyl-free disaccharide, named chondrosine, which he could convert into a crystalline ethyl ester hydrochloride. D-glucuronic acid and D-galactosamine were established as the monosaccharide constituents (79) and Davidson and Meyer (80) demonstrated that chondrosine was a β-1,3

linked glucuronide (Fig. 3). Reduction of chondros-:ine gave a glucopyranosylamino galactitol from which glucose was obtained on incubation with B-glucosidase but not with α-glucosidase. Oxidative deamination, followed by periodate oxidation showed that the glucuronidic bond was on C_3 of galactosamine.

It was not until 1951 that Meyer and Roppert reported that two types of chondroitin sulphate, viz. A and C were present in hyaline cartilage. The properties of chondroitin sulphates A and C were summarized (81) as follows:
chondroitin sulphate A had $(\alpha)_D$ -28 to -32°, required 30-40% ethanol to precipitate its calcium salt, was hydrolysed by testicular hyaluronidase and contained equimolar amounts of D-galactosamine and D-glucuronic acid. Fifty per cent of the sulphate was liberated after hydrolysis for one hour at 100° with N HCl. Chondroitin sulphate C had $(\alpha)_D$ -16 to -22°, required 40-50% ethanol to precipitate its calcium salt, was hydrolysed by testicular hyaluronidase and contained equimolar amounts of D-galactosamine and D-glucuronic

STRUCTURE OF THE REPEATING UNIT
OF SOME MUCOPOLYSACCHARIDES



mucopolysaccharide	A	B	C	D	E
chondroitin sulphate A	H	OSO_3H	H	COOH	H
chondroitin sulphate B	H	OSO_3H	H	H	COOH
chondroitin sulphate C	SO_3H	OH	H	COOH	H
hyaluronic acid	H	H	OH	COOH	H

FIG. 3.

acid. Fifty per cent of the sulphate was again liberated after hydrolysis for one hour at 100° with N HCl.

Both A and C were devoid of any blood anticoagulant activity. One explanation advanced for the different optical rotations and solubility properties was that C had a lower molecular weight than A. Samples of crystalline chondrosine having identical infrared spectra have been obtained from both A and C so the difference does not lie in the glucuronosyl - galactosamine glycosidic linkage.

Matthews (82) reported that chondroitin sulphates A and C could be differentiated by their infrared spectra in the region 700 to 1000 $cm.^{-1}$, where C had unique bands at 1000 $cm.^{-1}$, 820 $cm.^{-1}$, and 775 $cm.^{-1}$, while A had bands at 928 $cm.^{-1}$, 852 $cm.^{-1}$ and 725 $cm.^{-1}$. Following an earlier assignment by Orr (83), Matthews suggested that in A the sulphate group occupied an axial position while in C the sulphate occupied an equatorial position.

This prompted the suggestion that in A the sulphate group was attached to C_4 of the hexosamine moiety while in C it was attached to the C_6 of the hexosamine. Hoffman et al. (84) using testicular hyaluronidase and B-glucuronidase produced further evidence regarding the position of the sulphate linkage when they showed that the glucuronic acid was sulphate-free. Hence it was concluded that the sulphate groups in both A and C must be on the galactosamine residue and that the difference in the two molecules must be due to its point of substitution. Chondroitin sulphate B, to be discussed later, is known by methylation studies to have its sulphate group attached to C_4 of its galact-:osamine residues and since its infrared spectrum is comparable with that of chondroitin sulphate A, it was argued that A had C_4-galactosamine linked sulphate and C had a C_6-galactosamine linked sulphate.

Meyer and Chaffee (85) in a study of the polysaccharides present in pig skin obtained a component containing hexosamine, acetyl, uronic acid and sulphate in equimolar ratios. This polymer

differed significantly from chondroitin sulphates A and C, was recognised as a different polysaccharide and is now referred to as chondroitin sulphate B. Unlike the other two, it is not attacked by testicular hyaluronidase, its negative optical rotation $(\alpha)_D$-55 to 63° is much greater than A or C; it requires 18 to 25% ethanol to precipitate its calcium salt and has an appreciable anticoagulent activity. It is sometimes called β-heparin.

Hydrolysis of chondroitin sulphate B (86) with Dowex -50 (H^+) resin led to the identification of the uronic acid by paper chromatography as iduronic acid - the C_5 epimer of D-glucuronic acid. A crystalline disaccharide which on further hydrolysis gave galactosamine and iduronic acid was also obtained. The presence of iduronic acid was confirmed and finally proved when, after hydrolysis of the desulphated and borohydride reduced polysaccharide, idose was identified. The configuration of the hexosaminidic linkage remains to be proved though it is thought to be the same as in A and C, i.e. repeating sequences with alternating

β 1,4 acetylhexosaminidic and β 1,3- uronidic acid units. Methylation studies (87) have established that in chondroitin sulphate B the sulphate groups are attached to C_4 of the amino sugar.

Heparin is a blood anticoagulant present in connective tissue where it appears to be localised in the mast cells. It is the most highly sulphated polysaccharide known to exist and has been found use-:ful in surgery and in the treatment of thrombosis. Many of the early preparations of heparin were impure and this situation led to considerable differences in reported results. Jorpes and Bergstrom (88) charact-:erized the hexosamine residue as D-glucosamine; this finding was confirmed by Korn (89). Heparin was first suspected to contain uronic acid residues by Howell (90) and this supposition was in part confirmed by Jorpes (91). Final confirmation as to the nature of the uronic acid constituent was obtained by Foster et al. in 1961 (92). Successive removal of N-sul-:phate groups and selective N-acetylation, followed by hydrolysis yielded D-glucuronic acid.

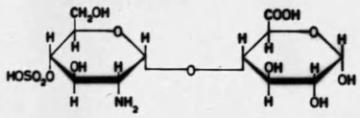
There was considerable controversy among the earlier workers as to whether or not an N-acetyl group was present in heparin. Varying acetyl contents were reported until Meyer and Schwartz (93) demonstrated that a heparin preparation could be resolved into two components by electrophoresis. The fast moving component which contained all the anticoagulant activity contained no acetyl groups, whereas the immobile component which was biologically inactive contained 19% acetyl.

Following exclusion of N-acetyl groups, it was suggested (94) that a sulphamic acid type linkage was present and this was demonstrated when the nitrogen of heparin was set free as aminonitrogen after hydrolysis in 0.04N HCl at 100° for three hours. Under these conditions exactly equivalent amounts of sulphate and aminonitrogen were liberated. A substituted amidosulphuric acid, $-NHSO_2OH$ group was therefore postulated.

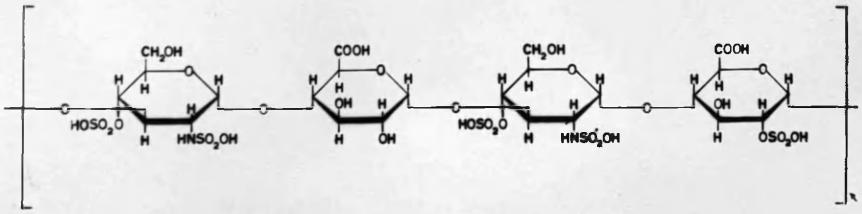
A pure preparation of the free acid of

heparin (95) was shown to consume four equivalents of alkali. Three of the acid groups were titratable below pH 3, corresponding to three sulphuric acid groups. The fourth titrated between pH 3 and 7 as would be expected of a uronic acid residue. Since only one of these sulphate groups could be attached to nitrogen, the remainder must be attached as O-sulph-:ates. Wolfrom et al. (132) oxidized sodium heparinate with sodium metaperiodate. Titration showed that there was initial rapid consumption of one mole of periodate per tetrasaccharide unit but no formic acid or formaldehyde was liberated. Furthermore, N-acetyl desulphated heparin consumed one mole of periodate per disaccharide unit, again without formation of formal-:dehyde or formic acid and with destruction of the hexuronic acid portion only. Partial acid hydrolysis of heparin in 0.5N H_2SO_4 at 100° for 18 hours gave a disaccharide designated heparosin sulphuric acid. This contained one hexosamine residue, one hexuronic acid residue and one ester sulphate group. The sequence in the disaccharide is thought to be glucosamine - glucuronic acid (Fig. 4a), the glucuronic acid being

Structural Formulae of Heparosin Sulphuric Acid (a) and Repeating Unit of Heparin. (b).



(a)



(b)

Fig. 4

the reducing moiety. This disaccharide consumed three moles of periodate with formation of one mole of formic acid but no formaldehyde. Both sugar units were attacked. Selective acetylation of the amino group of the glucosamine residue resulted in a disaccharide which consumed only two moles of periodate per mole. Again, one mole of formic acid but no formaldehyde was produced. In this instance, however, only the hexuronic acid moiety was attacked. Favouring pyranoid rings, the above data suggest structure (a) Fig. 4 for heparosin sulphuric acid and structure (b) for heparin. The strongly positive rotation of heparin indicates α-glycosidic linkages.

III. PRESENT KNOWLEDGE CONCERNING THE ENZYMATIC SYNTHESIS OF ACID POLYSACCHARIDES.

The introduction of new techniques in conjunction with the development of methods for the small scale isolation of polysaccharides and their components has increased enormously the possibilities

of carrying out metabolic studies.

The first studies in this field involved the well known phosphorylase reaction (97) which in conjunction with "branching factor" was invoked at the time to explain the synthesis of glycogen. Shortly afterwards, a somewhat similar complement of enzymes was found in plants (98, 99). For example, potato phosphorylase in the presence of primer molecules, e.g. maltodextrins, catalyzes transfer of glucose residues from α-glucose -1- phosphate to form linear α-1:4- linked polymers resembling natural amylose. Subsequently, however, Q-enzyme, by a trans-glycosidic mechanism converts these linear chains into the branched structure of amylopectin (100,101).

It may be presumed that unsuccessful attempts to find similar enzyme systems for the bio-:synthesis of other polysaccharides were made. Thus it might be envisaged that xylose -1-phosphate under the influence of an analogous phosphorylase and in the presence of a suitable primer might give rise to

xylan synthesis.

A decade ago the concepts of the enzymatic synthesis and degradation of glycogen and starch were thought to be almost complete and that further progress would consist only in adding detail to the already well defined outlines. Subsequently, however, the wide-spread discoveries of sugar-containing nucleotides (102); knowledge of the interrelations among these various nucleotide derivatives and their involvement in carbohydrate interconversions created a new interest. Thus, to give but a few examples, soon after Leloir's report on the discovery of uridine diphosphate glucose (UDPG), enzymes were reported which utilized this compound as the glucose donor in trehalose, sucrose, glycoside and even cellulose synthesis. The situation has been reviewed by Strominger (136). Furthermore, the possibility has been raised (103-105) that UDPG is involved both in glycogen and starch synthesis. Thus, the role of phosphorylase is again at issue and it has been suggested that its primary role is that of a degradative enzyme.

The study of mucopolysaccharide biosynthesis has derived great benifit from the application of results gained in other fields of carbohydrate metab-:olism. The numerous examples of the participation of various nucleoside diphosphate derivatives such as UDPG in saccharide synthesis makes it possible that these substances may be the specific precursors of the mucopolysaccharides as well. It was suggested by Leloir (106) that uridine diphosphate acetylglucosamine (UDPAG) could possibly participate in the formation of chitin. Likewise, Storey and Dutton (107) suggested that uridine diphosphate glucuronic acid (UDPGA) was a precursor of uronic acid- containing mucopolysacch-:arides. (Fig. 5). Some of these hypotheses are now in the process of being experimentally substantiated.

Thus, some instances of mucopolysaccharide synthesis have already been reported e.g. chitin (109) and hyaluronic acid (110, 111). The formation of these substances has been shown to follow a pathway in which uridine nucleotide sugar derivatives represent the final stage before polymerization to a macromolecule.

POSSIBLE METABOLIC STEPS IN THE BIOSYNTHESIS OF CHONDROITIN SULPHATE AND HYALURONIC ACID.

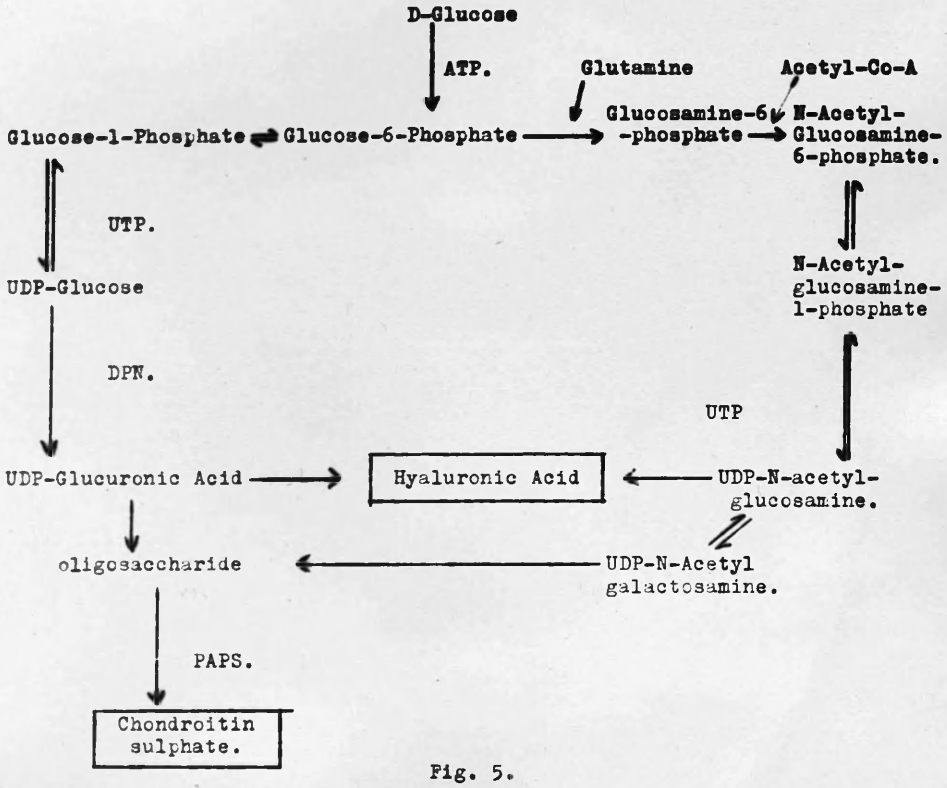


Fig. 5.

(Adapted from P.W. Kent, "Some Biochemical aspects of sulphated Mucosubstances", Biochemical Soc., Symposium No. 20. The Biochemistry of Mucopolysaccharides of Connective Tissue. Edited by F. Clark and J.K. Grant, Cambridge University Press (1961)).

A particulate enzyme from Neurospora crassa has been found to catalyze the synthesis of insoluble chitin directly from UDPAG (109.) The reaction proceeds by glycosyl transfer from the nucleotide linked sugar to preformed chitodextrin chains. Proof that the product of the reaction is chitin rests upon:

(a) the demonstration that N-acetyl-glucosamine is incorporated as a unit from UDPAG.

(b) the finding that after partial acid hydrolysis, all of the isotope in synthesized C^{14}- chitin can be recovered as C^{14}-N-acetylglucosamine after chitinase digestion and

(c) the formation of a series of C^{14}-oligosaccharides after partial acid hydrolysis of the synthesized C^{14}-chitin.

The net synthesis of chitin has been shown in experim-:ents in which the increase in hexosamine in the insoluble material after incubation has been measured chemically.

An enzyme that catalyzes the synthesis of hyaluronic acid from uridine diphosphate glucuronic

acid and uridine diphosphate N-acetylglucosamine has been obtained from a strain of group A streptococcus (111). The synthesis, which requires Mg^{++}, Mn^{++} or Co^{++}, was demonstrated with tritium labelled precur-:sors and the product was found, by specific degrad-:ations to be labelled in the appropriate corresponding portion. Net synthesis of hyaluronic acid was demonstrated and the results obtained indicated a 10-fold increase in non-dialyzable uronic acid and hexosamine during the incubation. It was concluded that 90% of the hyaluronic acid isolated was synthesized in vitro. Attempts to demonstrate a primer effect of either hyaluronic acid or testicular hyaluronidase treated material were uniformally negative.

The biosynthesis of the sulphated mucopoly-:saccharides presents a special problem: the introduction of the sulphate group into the molecule requires the activation of the sulphate group to adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) and the subsequent transfer of the sulphate moiety to

a carbohydrate acceptor. The incorporation of the sulphate radical and the stage in the biosynthesis of the macromolecule at which this step occurs, poses a unique problem. Some progress is being reported in this respect, as will be discussed later.

It is natural that the introduction of isotopic methods is proving to be fruitful in the elucidation of mucopolysaccharide metabolism. The techniques have been used mainly for two purposes. First the origin of the different moieties of the mucopolysaccharides have been investiaged in bacteria and mammals using precursors such as S^{35}- sulphate and variously labelled C^{14}- glucose. Secondly the turnover rates of the different mucopolysaccharides have been studied. Administration of radioactive sulphate has been shown in cartilage to result in its incorporation into chondroitin sulphate (112, 113). In studies on hyaluronic acid (114-116) synthesized by streptococci, C-1 labelled glucose appeared as glucosamine and glucuronic acid in the polymer, and in each case the radioactivity was retained by the C-1 carbon atom of

these sugars, implying that the sugar skeleton was incorporated without scission. Similar results pertain to the formation of hyaluronic acid in animal tissues (117).

Thus, tracer methods may give valuable hints about possible reaction sequences in biosynthetic path-:ways and this type of work should be regarded as preliminary to a closer investigation of the enzymic reactions involved.

The original observation of D'Abramo and Lipmann (118) on the incorporation of S^{35} from S^{35}-sulphate and S^{35}-labelled PAPS into chondroitin sulphate in an extract of embryonic chick cartilage has been followed by similar studies on the mucopoly-:saccharides of hen oviduct (119,122) by Suzuki and Strominger. It was demonstrated (120) that an enzyme from the isthmus of hen oviduct could catalyze the transfer of S^{35}-sulphate from PAPS to added mucopoly-:saccharide acceptors. The reaction had an absolute dependence on added acceptor. A number of mucopoly-

:saccharides including chondroitin sulphates A, B or C and a heparitin-type sulphated heptasaccharide were active in the system. (Heparitin sulphate is the name given to a polysaccharide resembling heparin but of low sulphate and low anticoagulant activity. Crude heparitin sulphate obtained as a side product during the commercial preparation of heparin has been separated into several fractions. A family of substances appear to be present containing variable amounts of sulphate and acetyl groups (172). Brown (173) has suggested a heptasaccharide structure for a heparitin type sulphated material isolated from the liver of a patient with Hurler's syndrome). Chemical and enzymic analysis of the radioactive products formed with chondroitin sulphate A and the heparitin-like heptasaccharide as acceptors have shown that a different radioactive product is formed in each case and that these radioactive products have similar properties to those of the acceptor employed. It is of interest that the isthmus of hen oviduct, which was used in these investigations also contains uridine diphosphate-N-acetylgalactosamine sulphate, but participation of

this substance in the transfer of sulphate to mucopoly-:saccharides has not been observed.

Transfer of sulphate from S^{35}- PAPS to N-acetylgalactosamine, N-acetylgalactosamine sulphate and oligosaccharides of varying degree of sulphation has been observed (121) using the enzyme prepared from the isthmus of hen oviduct. The preparation can catalyze either the introduction of a first sulphate residue into N-acetylgalactosamine as the monosaccharide or contained in oligosaccharides or can catalyze the introduction of a second sulphate group into N-acetyl-:galactosamine already bearing a sulphate residue. Enzymatic sulphation of N-acetylgalactosamine apparently leads primarily to the 4-sulphate (123), the position the sulphate group occupies in chondroitin sulphates A and B.

A series of even and odd numbered oligosacch-:arides prepared from chondroitin and chondroitin sulphate A were shown to act as sulphate acceptors. It is interesting to note that the velocity of the transfer

reaction increased with increasing chain length of the acceptor. Notable exceptions to this, however, were the relatively high rates of sulphation of the tri - and pentasaccharides from chondroitin. Of the compounds examined, bovine corneal chondroitin was sulphated at the fastest rate. Radioactive chon-:droitin and chondroitin sulphate were prepared (122) by transfer of sulphate from S^{35}- PAPS, catalyzed by the sulphuryltransferase of hen oviduct. Oligosacch-:arides were then isolated by digestion of these products with testicular hyaluronidase and identified. The results were comparable to those obtained with monosaccharides and oligosaccharides as acceptors. It has been suggested that two types of sulphation occur, viz. sulphation of an unsubstituted N-acetylgalactos-:amine residue in the mucopolysaccharide acceptor and sulphation of an N-acetylgalactosamine sulphate residue in the acceptor with formation of N-acetylgalactosamine disulphate. It is worthy of emphasis that the products obtained were analyzed only by methods applicable to radioactive compounds and confirmation of these elegant results by measurements of net sulphate gain is

desirable.

In view of the results obtained above it is interesting to note that sheep intestinal mucosa preparations which will synthesize mucopolysaccharides (134) will not transfer sulphate to any of the simple carbohydrates assayed with this system (135). The sugars used included D-glucose, D-glucosamine, D-galactosamine, N-acetylglucosamine, UDPG and UDPAG.

Sulphuryltransferases with specificity towards various mucopolysaccharides have been detected. Thus, Davidson and Riley (124) have obtained an enzyme from rabbit skin which transfers sulphate from S^{35}-PAPS more readily to chondroitin sulphate B than to several other similar mucopolysaccharides e.g. chon-:droitin sulphates A and C. It is noteworthy that these workers found that uridine triphosphate but not the triphosphates of adenosine, cytosine or guanosine stimulated the sulphation of chondroitin sulphate B.

Extracts prepared from embryonic calf

cartilage have been shown to catalyze sulphate transfer from S^{35}- PAPS to both desulpho - chondroitin sulphate and to partially sulphated chondroitin sulphate (125).

Wortman (126) has demonstrated both phenol and mucopolysaccharide sulphuryltransferase activities in beef cornea epithelial extract. These enzymes have been coupled to serve as an assay procedure for the study of the latter enzyme. The following reaction sequence represents the basis of the assay procedure used -

(a) Phenol sulphuryltransferase -

$$p\text{-}NPS^{35} + PAP \longrightarrow p\text{-}NP + PAPS^{35}$$

(b) Mucopolysaccharide sulphuryltransferase -

$$PAPS^{35} + \text{corneal mucopolysaccharides} \longrightarrow PAP + \text{sulphated corneal mucopolysaccharides.}$$

The extract was shown to synthesize a sulphate -S^{35}- containing substance with the same electrophoretic and chromatographic mobility as authentic chondroitin sulphate. Data presented indicates the dependency on corneal mucopolysaccharides for the appearance of p-NP from p-NPS. Under certain experimental conditions a

dependency on PAP has been shown.

Eiber and Danishefsky (127) have demonstrated that $Na_2S^{35}O_4$ injected intraperitoneally gives a maximum incorporation of S^{35} in dog liver heparin after 28 hours. Jorpes et al. earlier showed (128) that exogenous sulphate is taken up in the mast cells. Working on mouse mast cell tumour slices Korn (129) demonstrated the incorporation of C^{14}-glucose and S^{35}-labelled sulphate into a mucopolysaccharide which was indistinguishable from heparin. Subsequently this author (133) presented data which indicates that the soluble fraction of mast cells contains the necessary enzymes to synthesize PAPS from ATP and SO_4^{2-} and then transfer the sulphate group to heparin. However, evidence to suggest the net transfer of sulphate to the polymer remains to be presented. Ringertz (130) has concluded from experiments with transplantable mouse tumours that the nucleotide adenosine 5'-sulphato-:phosphate (APS) is an intermediate in the transfer of sulphate to heparin. Heparin, it was shown was sulphated more rapidly than chondroitin sulphate A,

while hyaluronic acid was completely inactive as a sulphate acceptor.

The trend demonstrated in this summary appears to indicate that

1) PAPS is the sulphate donor in sulphate transfer reactions to mucopolysaccharides,

2) that transfer of sulphate to the polymerized material appears to be favoured,

3) that specificty of sulphuryltransferases towards certain mucopolysaccharides has been detected in some instances and

4) while tracer methods have been used in all of the work reported, quantitative determination of sulphate transferred would confirm and enhance the results already obtained.

IV. SCOPE OF THE PRESENT WORK.

For convenience of presentation the account given of the investigations described in this thesis

has been divided into two parts. Part I deals with the preparation of the substrates required for the enzymic work discussed in Part II.

Part I.

The present investigation was initiated with the aim of studying the role of the nucleotide adenosine 3'-phosphate-5'-sulphatophosphate (PAPS) in the sulphation of polysaccharides. In order to investigate the enzymatic transfer of sulphate from PAPS, a method for the chemical synthesis of this compound was worked out. The preparation, purificat-:ion and identification of PAPS is described. An account is given of a method for the desulphation of heparin based on that of Kantor and Shubert (137) for the removal of the sulphate groups from chondroitin sulphate.

Part II.

In this section a series of in vitro experiments demonstrating sulphate transfer,

is described. The aim of these experiments was to study the role of PAPS in the sulphation of poly-:saccharides and also to obtain some information about the nature of the sulphate acceptor molecules involved.

One of the chief obstacles in sulphate transfer experiments is the difficulty of isolating the polysaccharide material from incubation digests in a quantitative manner and in a pure form. A method is described for removal of (low molecular weight) substances which interfere with subsequent sulphate determinations. This technique involves gel filtration using Sephadex G-25.

Liver preparations have been shown to transfer sulphate from heparin to p-nitrophenol (p-NP). Evidence is presented to indicate that adenosine 3':5' diphosphate (PAP) is a co-factor in this transfer reaction.

P A R T I

The preparation of enzyme substrates.

S E C T I O N I.

The preparation and purification of adenosine 3'-phosphate 5'-sulphatophosphate.

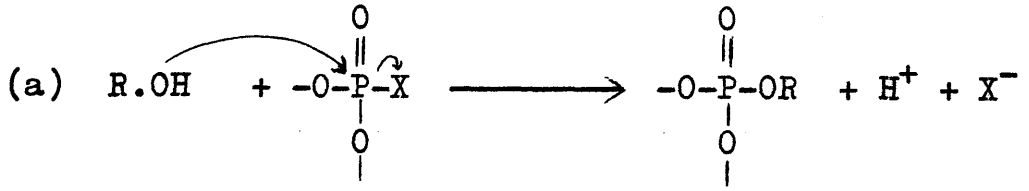
1. Introduction.

Nucleotide co-enzymes have assumed steadily increasing importance in our understanding of biochem-:ical reactions during recent years. This group now comprises not only nucleoside pyro- and triphosphates concerned in transphosphorylation; the oxidation reduction co-factors di- and triphosphopyridine nucleotide and flavine adenine dinucleotide but also many nucleotides involved in the transfer of amino acids, sugars etc.

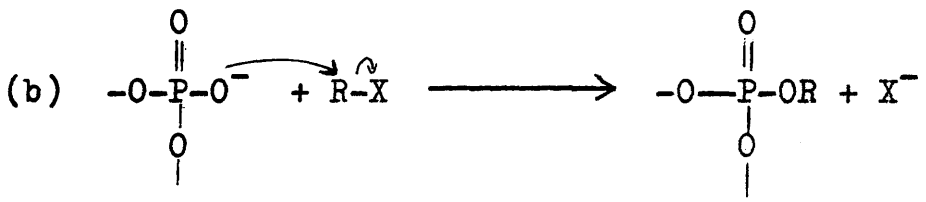
Methods for the chemical synthesis of nucleotide co-enzymes were first developed by A.R. Todd and his colleagues as an extension of their work on new routes for the phosphorylation of alcohols and phenols and on general methods for the synthesis of pyrophos-:phates.

Chemical phosphorylation may be considered

in analogy with the esterification of carboxylic acids, as (a) the electrophilic attack of an activated phos-:phoryl group on a hydroxyl group:



or (b) as a nucleophilic attack of a phosphate anion on the carbon of an alkyl halide or related compound:



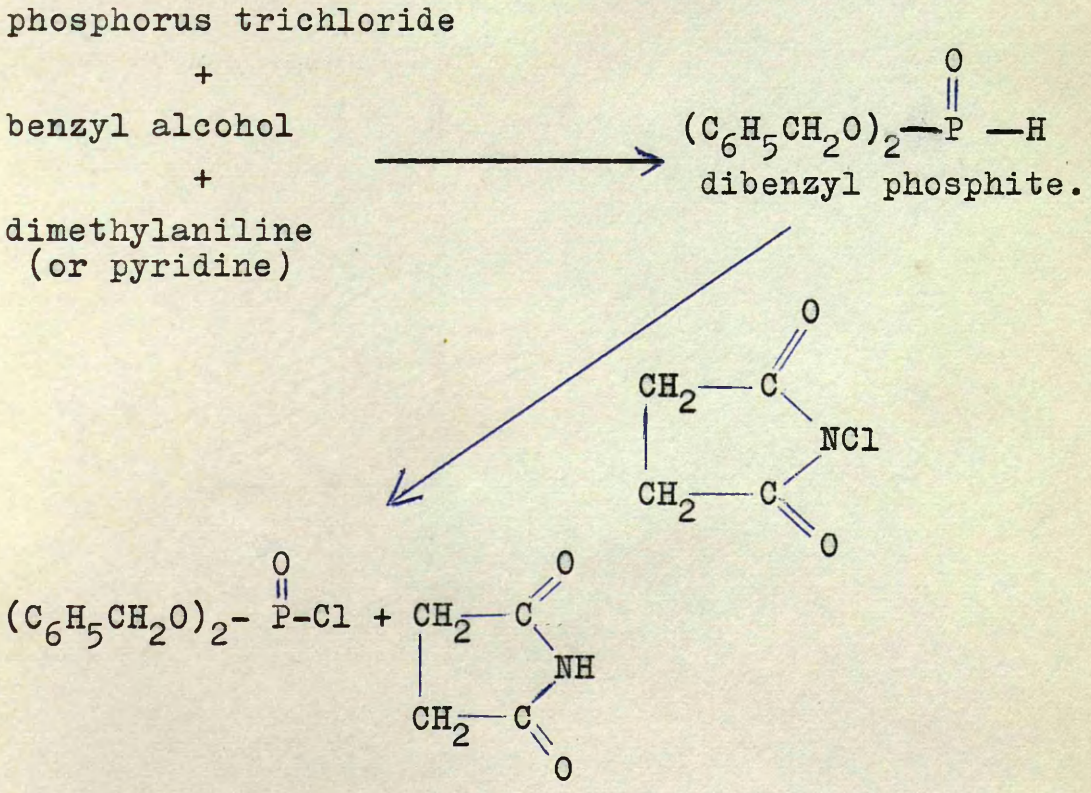
Most often chemical phosphorylation has been realized by the general process shown in equation (a) above; the phosphorylating agents have been the activated derivatives of phosphoric acid itself or its mono or diesters.

The most important feature of chemical phosphorylation which should be emphasized, is the polyfunctional nature of phosphoric acid and hence the chief problem in activating a phosphoric acid molecule to a suffic-:iently powerful phosphorylating agent is to overcome the negative charges on the oxygen atoms. Thus the

simple anhydride of phosphoric acid, pyrophosphoric acid, is completely devoid of phosphorylating capacity under mild conditions. Similarly, dialkyl pyrophos-:phates lack this function but tetraalkyl pyrophosphates possess the character of phosphorylating agents. One of the major problems in the development of efficient phosphorylating agents has therefore been to confer on the phosphorus atom of the phosphoric acid molecule, which is usually surrounded by electronegative oxygen atoms, an electropositive character.

Many reagents (138, 139) have been employed for the synthesis of phosphate esters but none has proved to be completely general in its application. The limitations of existing reagents are particularly evident in the synthesis of nucleotides where such considerations as acid lability of purine glycosidic bonds, alkaline lability of the 6-amino group of cytosine (140) and the catalytic reduction of pyrimid-:ine rings (141) must be taken into account. Multi-:functional reagents such as phosphorous oxychloride (142-144) and polyphosphoric acid (145) are of limited

value because of the complex mixture of products they produce and their use is practical only in those cases where the reaction products can withstand hydrolysis. One of the more successful reagents so far employed in the nucleotide field is dibenzyl phosphorochloridate (146). It may be prepared as follows:



dibenzyl phosphorochloridate.

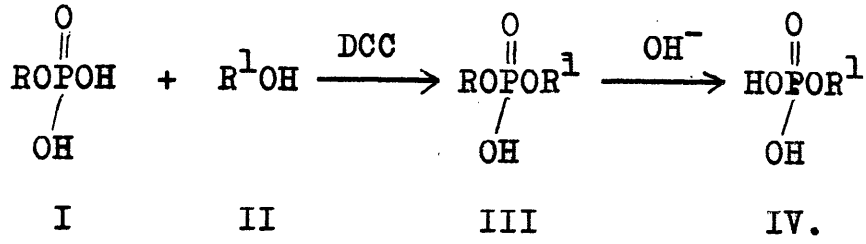
It phosphorylates most primary alcoholic

functions and the benzyl groups can be removed from the intermediate phosphotriesters by catalytic hydro-:genolysis using a palladium catalyst. This reagent however, has two very distinct disadvantages. First it is unstable and very sensitive to traces of water. Secondly, during the prolonged reaction time required to phosphorylate secondary alcoholic functions the reaction solvent i.e. pyridine, causes debenzylation (147) of the intermediates thus giving lower yields of the desired products. Furthermore, dibenzyl phosphorochloridate is not an extremely powerful reagent as shown by its inability to phosphorylate guanosine nucleosides (143). Tetra-p-nitrophenyl-:pyrophosphate (144) was developed to fill this latter need, but in this case the p-nitrophenyl protecting groups must be removed from the intermediate phosphod-iester by drastic alkaline hydrolysis or by a specific enzymic procedure. Likewise O-benzylphosphorous - O, O-diphenylphosphoric anhydride (148) was developed to provide a more powerful phosphorylating agent but because of the difficulties encountered (149) in removing benzyl groups from cytosine-containing nucleotides by hydrogenolysis, even with palladium

catalysts, alternate reagents were sought.

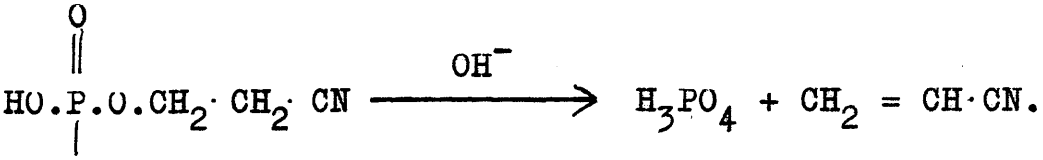
In view of the above limitations it is apparent that a phosphorylating agent for use in the nucleotide field should satisfy the following requirements; it should be very powerful and it should be a monofunctional reagent from which the protecting groups can be removed by very mild and specific methods. In addition it is desirable to have a simple procedure for both the preparation of the reagent and its use. Since nucleotides are unaffected by mild alkali, a phosphorylating agent with protecting groups sensitive to mild alkali would appear to be more generally useful.

In a recent communication (150) a new approach to the synthesis of phosphate esters was reported. This method was based on earlier observations (151) that phosphodiesters III can be readily prepared from a monoalkylphosphate I and an alcohol II by allowing them to react in anhydrous pyridine with dicyclohexylcarbodiimide (DCC).



I II III IV.

This latter reagent acts by abstracting the elements of water from the reactants thus forming the posphod-:iester III. Mechanisms of this reaction have been discussed by Khorana (178). In this phosphorylation procedure an alkyl phosphate I is chosen, such that after coupling it to the alcohol II, as shown in the reaction scheme above, its alkyl group can be select-:ively removed. The net result of the reaction is then the conversion of the alcohol II to its phosphate ester IV. The alkyl phosphate used was 2-cyanoethyl-:phosphate V since it was shown (152) to break down



V.

under very mild alkaline treatment with liberation of orthophosphate, presumably by the above reaction.

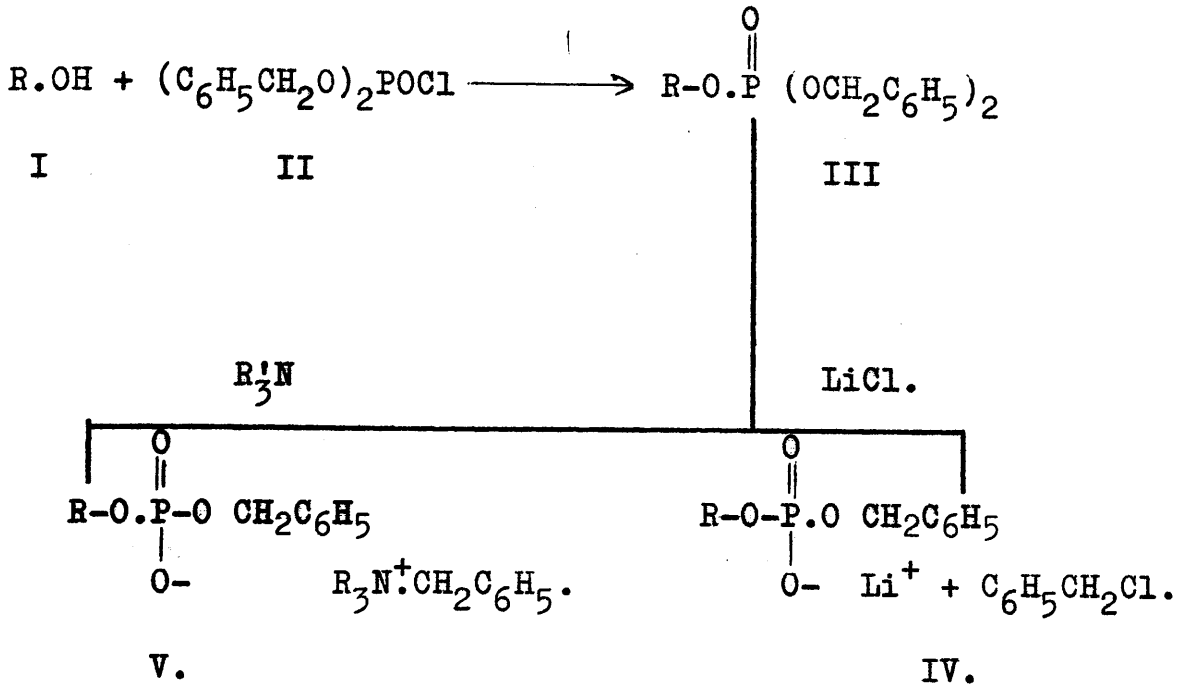
In general, phosphorylating agents are very sensitive to traces of water; however, 2-cyanoethyl-:phosphate (CEP) offers the advantage of being completely stable and is converted to the active phosphorylating species only when brought into react-:ion with DCC (153). Water will decompose this active intermediate and care should be taken to remove as much as possible from the reaction mixture. However, in practice absolutely anhydrous conditions are not required because an excess of DCC is usually added and last traces of water are removed by the hydration of DCC to form dicyclohexylurea.

2. Literature Accounts of the Preparation of Adenosine 3'-phosphate 5'-sulphatophosphate using dibenzylphosphorochloridate.

Chemical synthesis of adenosine 3'-phosphate 5'-sulphatophosphate (PAPS), starting with adenosine, requires firstly the preparation of adenosine 3':5' diphosphate and secondly the sulphation of this product to form PAPS. The phosphorylation of the unprotected nucleoside using an excess of phosphoryl-

:ating agent would be expected to give the 2'(3'):5'-diphosphates. The first point of attack on the nucleoside would be the 5'-hydroxyl function, followed by attack on either the 2'-or 3'-hydroxyl functions. Attack on either of these positions may cause steric hindrance to any subsequent attack on the adjacent hydroxyl group.

The isomeric diphosphates of adenosine (i.e. A2':5'DP and A 3':5'DP) are basic structural units in some nucleotide co-enzymes. Thus, A2':5'DP comprises part of the structure of triphosphopyridine nucleotide, whereas the isomeric A 3':5'DP is a component of co-enzyme A and PAPS. A mixture contain-:ing the two isomers has been synthesized by Todd and his co-workers (154); they succeeded in phosphorylating adenosine using an excess of dibenzyl phosphorochlorid-:ate. The intermediate was selectively monodebenzyl-:ated, a process of removing one benzyl group from each dibenzyl phosphate residue. For example if an



alcohol of the general formula I is allowed to react with dibenzyl phosphorochloridate II it affords the ester III. Selective debenzylation can be accomplished by "quaternization" - a process depending on the transfer of a benzyl residue from oxygen to nitrogen with form-:ation of a quaternary salt. A strong base such as N-methylmorpholine (161) has been found satisfactory. Debenzylation can also be brought about by a base hydrochloride (162) e.g. lithium chloride. An equilibrium is set up between the triester and LiCl on the one hand, and the lithium salt of the diester and benzyl chloride on the other. Precipitation of this lithium salt from solution leads to quantitative

reaction. In both methods of debenzylation the monobenzyl ester is produced as an anion and therefore a second debenzylation which would produce a doubly charged anion is not favoured.

The remaining benzyl groups were subsequently removed by hydrogenolysis and the isomeric diphosphates then isolated by ion-exchange chromatography. Attempts by these workers to resolve the isomers however were without success.

Baddiley et al. (155) prepared the mixed diphosphates by a similar method and succeeded in separating the isomers on Dowex-1 ion-exchange resin using a gradient elution with calcium chloride and hydrochloric acid. Subsequently these workers (156) prepared PAPS by reacting A 3':5'DP with pyridine sulphurtrioxide in aqueous sodium bicarbonate.

3. Initial attempts to prepare adenosine 2'(3'):5'-diphosphate using dibenzyl phosphorochloridate.

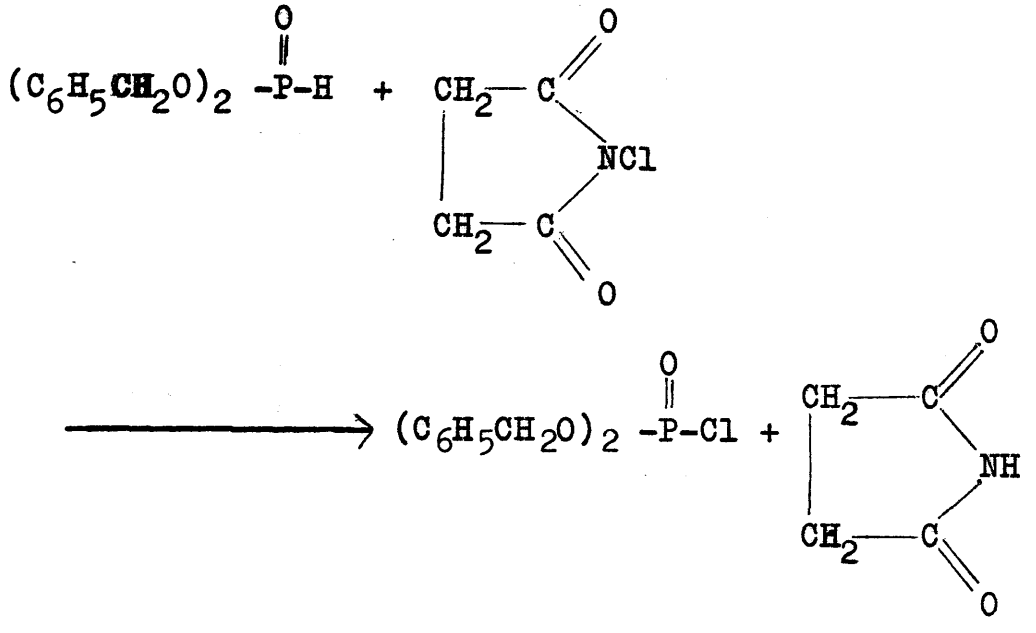
(a) Preparation of dibenzyl phosphite.

The preparation of dibenzyl phosphite (146) in a crude state is relatively easy but its purification by high vacuum distillation is difficult and even hazardous. The reagent dibenzyl phosphor-:ochloridate (146) itself is unstable and must be prepared from dibenzyl phosphite immediately before use. Although the reagent has been used with success in the synthesis of a number of nucleotides, it should be noted that it is not very powerful and is often unsatisfactory - for example, for the phosphorylation of the secondary hydroxyl groups in deoxyribonucleo-:sides (159) and the hydroxyl group in serine (160).

A solution of phosphorus trichloride (Expt. I) in dry benzene was cooled to 0^{o} and a mixture of dibenzyl alcohol and diethylaniline previously cooled to zero added dropwise with continu-:ous stirring. The addition was carried out at such a rate that the reaction temperature remained below 5^{o}. The mixture was then stirred for a further $1\frac{1}{2}$ hours. After the addition of water the phases were allowed to separate and the organic phase was further washed

successively with water, ammonia and finally water before drying with sodium sulphate overnight. The solution was filtered and the filtrate concentrated under reduced pressure on a boiling water-bath until all the benzyl chloride was removed. The residue set to a white solid mass after standing for several days at -14°. This product was further purified as follows: gaseous ammonia was bubbled into the crude material and the acidic impurities which separated out were filtered and N-methylmorpholine was added to the filtrate which was distilled immediately in an atmosphere of nitrogen at a pressure of 10^{-3}mm; oil bath temperature 200-210° which corresponded to a temperature of 175-185° inside the distilling flask. The first few ml. were rejected and the remainder collected until most of the material had distilled or until the material in the distilling flask showed signs of decomposition (a sudden darkening in colour) - in which case the system was immediately flooded with nitrogen, the oil-bath removed and the vacuum system turned off. Dibenzyl phosphite set as a white solid. M.P. 18-19°.

(b) Preparation of dibenzyl phosphorochloridate.



Dibenzyl phosphite was added (Expt. 2) to dry benzene. After the addition of N-chlorosuccin-:imide the mixture was shaken until all the chlorin-:ating agent had dissolved and then allowed to stand at room temperature for 1½ - 2 hours. Succinimide was filtered off and then benzene removed under reduced pressure and the resulting oil used directly.

(c) Attempts to phosphorylate adenosine.

Adenosine was dissolved in boiling anhydrous pyridine (Expt. 3) and the solution was cooled to -30°

before it was added to dibenzyl phosphorochloridate. The mixture was kept at -30° for 30 minutes and thereafter at 0-2° for 18 hours.

2-ethoxyethanol was added and the faint precipitate of pyridine-hydrochloride which had separated was collected after 30 minutes and the filtrate evaporated in vacuo to yield a gum. After a further addition of 2-ethoxyethanol and subsequent evaporation the syrup was dissolved in 2-ethoxyethanol (100 ml.) containing fused lithium chloride and heated at 100° for 3 hours. This process of anionic deben-:zylation should selectively remove one benzyl group from each phosphate thus permitting hydrogenolysis to proceed more smoothly (see later).

Addition of ether to the cooled solution (0°) precipitated a gum which solidified when shaken with ethanolic acetone to give a light coloured powder (4 grs.) I in low yield. The ethanolic-acetone supernatant was concentrated in vacuo to give a syrup, which was redissolved in 2-ethoxyethanol

containing lithium chloride and again heated at 100°/3 hours. The solution was then worked up as before to give an off-white powder (3½ grs.) II. The ethanolic-acetone supernatant was again concentrated and worked up as before after treating with lithium chloride and gave a further 1½ grs. of material III. The three precipitates were combined to give product A (9 grs.)

The ethanolic-acetone supernatant from the above still yielded a gum (B) on concentrating in vacuo. A small portion of this was dissolved in water and submitted to electrophoresis. It indicated an ultra violet absorbing material which moved very slightly towards the cathode as might be expected of a fully benzylated derivative of a nucleotide. Attempts to mono-debenzylate this material using 1) potassium acetate (Expt. 4) and 2) N-methylmorpholine (Expt. 5) were without success. An attempt was made to hydrogenate the fully benzylated material (Expt.6) but the mixture failed to absorb any hydrogen. Fully benzylated materials have not been hydrogenated with

any success except in the case of adenosine 5'-dibenzyl phosphate.

The combined precipitates (A above) were treated with N-NaOH (200 ml.) to dispose of any cyclic phosphates and to allow the subsequent hydro-:genation to proceed smoothly. After 40 hours the solution was neutralized to pH 7, extracted with ether and the aqueous phase concentrated to 150 ml. The nucleotide solution was converted to the lithium salt by passing it through a column of Dowex-50 (x4) (lithium form) resin which was washed with water (500 ml.) and the eluate and washings concentrated in vacuo. The residual syrup was converted to a solid by shaking with ethanolic acetone and the resulting solid collected by centrifugation and after washing with further ethanolic-acetone, dried to give a white powder (4.1 grs.). This material was dissolved in water (150 ml.) containing acetic acid (2.0 ml.) and palladium chloride (100 mgs.) was added. The material was then hydrogenated (Fig. 6).

HYDROGENATION OF ADENOSINE-2'(3')MONOBENZYL PHOSPHATE - 5'- MONOBENZYL PHOSPHATE

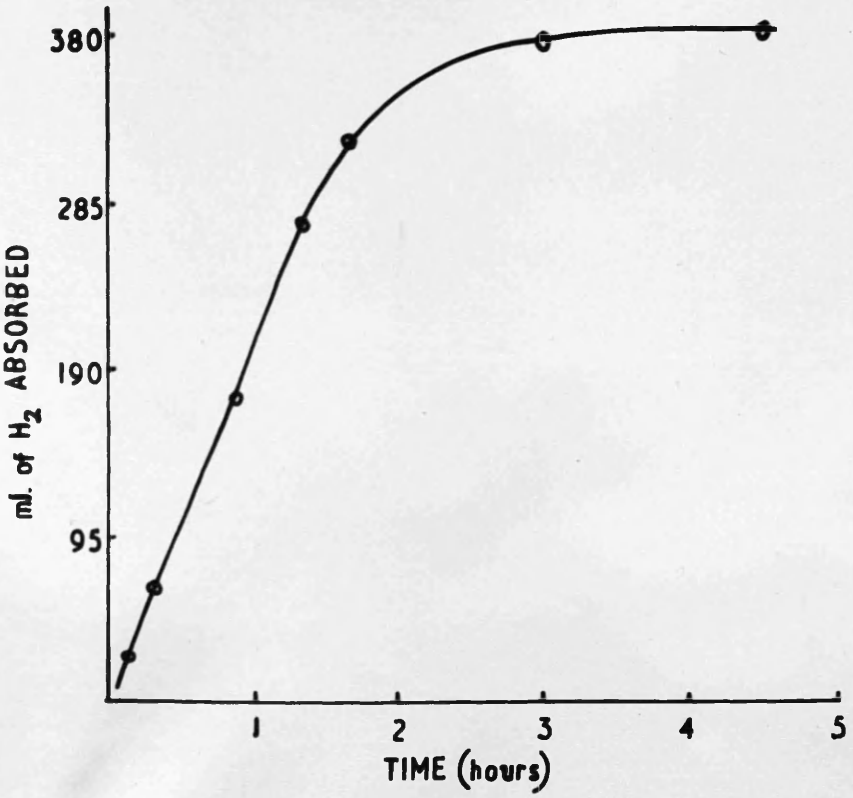


FIG 6

After removal of catalyst the solution (containing 0.285 g. of nucleotides calculated as adenosine 2'(3'):5'diphosphate, based on optical density at 260 mμ) was adjusted to pH8 with ammonia, diluted to 500 ml. with water and applied to a column of Dowex-2 (x8) (chlorideform) resin. The column was washed with water and thereafter monophosphates (0.1 gr.) were eluted with 0.003 N HCl (25 ml. fractions being collected with an automatic fraction collector). The optical density of each fraction was read at 260 mμ on a Unicam spectrophotometer. Diphosphates (0.08g.) were then eluted with 0.03N HCl.

Several attempts were made to repeat this experiment but the process of monodebenzylation never worked with any success. It was therefore decided to investigate 2-cyanoethylphosphate as a possible alternative to dibenzyl phosphorochloridate as an agent for the preparation of the mixed diphosphates of adenosine.

4. Preparation of adenosine 2'(3'):5' diphosphate using 2-cyanoethylphosphate.

As mentioned in the Introduction to this section (page 61), 2-cyanoethylphosphate possessed many of the characteristics of the ideal phosphoryl-:ating agent e.g. ease of preparation, stability, ease of removal of protecting groups etc. It has been used to prepare monophosphates (150). No account, however, of its use to prepare adenosine 3':5' diphosphate had been reported and this possibility was therefore investigated.

(a) Preparation of 2-cyanoethylphosphate (149).

Freshly distilled phosphorus oxychloride was mixed with anhydrous ether and to this solution was added slowly a mixture of anhydrous pyridine and hydracrylonitrile at -15° (Expt. 7). After the addition was complete the mixture was stirred for a further hour and then poured with stirring into a mixture of water, pyridine and ground ice. A solution of barium acetate was added and after

standing for two hours the barium phosphate precip-:itate was filtered on a Buchner funnel. To precipitate the product two volumes of 95% ethanol were added slowly with stirring to the clear filtrate. The barium salt of 2-cyanoethylphosphate was collected after one hour and washed with 50% ethanol and then with 95% ethanol and air dried to give 35grs. of product.

A standard solution containing 1 milli-mole/ml. for use in phosphorylation reactions was prepared in the following way; the dried barium salt was dissolved in water with the aid of Dowex-50 (H^+ form) resin. The material was then poured on to a column of Dowex-50 (H^+form) resin which was washed with water to pH 7. Pyridine was added to the effluent and the solution concentrated in vacuo to about 20 ml. This solution was transferred to a graduated (50 ml.) flask and diluted to the mark with pyridine. The reagent prepared in this way was stable for 2-3 months.

(b) Initial attempt to phosphorylate adenosine with 2-cyanoethylphosphate (CEP).

Adenosine (1m.mole) was dissolved in boiling anhydrous pyridine and then cooled to room temperature (Expt. 8). CEP (3 m.moles) was added and the mixture concentrated in vacuo on a water bath (temperature 25°) to an oil. A further amount of anhydrous pyridine was added to the oil and the mixture was again concentrated. This procedure was repeated a total of three times to ensure removal of last traces of water. Finally the oil was dissolved in anhydrous pyridine (15 ml.) and dicyclohexylcar-:bodiimide (DCC) was added. The mixture was shaken thoroughly and allowed to stand at room temperature. The progress of the reaction was followed by removal of aliquots of the reaction mixture after set inter-:vals. These were heated with ammonia to remove the cyanoethyl blocking group and then submitted to electrophoresis; the increase in the amount of ultra-:violet absorbing material which moved towards the anode indicating that the nucleoside was being phos-:phorylated.

After two days most of the adenosine appeared to have been used up. Water was added to the reaction mixture and after 30 minutes the dicyclohexylurea (DCU) was filtered on a Buchner funnel. One volume of ammonia (0.88) was added to the filtrate which was then heated at 60° for 1 hour. This treatment removes the protecting cyanoethyl groups. The solution was concentrated in vacuo on a water-bath at 35° to dryness. The white solid was dissolved in water (25 ml.), any remaining DCU was filtered off and the precipitate washed with a further 25 ml. of water. The filtrate and washings were combined and the solution (containing 259 mgs. of nucleotide calculated as adenosine (2') 3':5 diphos-:phate, based on optical density at 260 mμ) was concentrated to 10 ml. and then freeze dried to give a white powder which was examined by paper electro-:phoresis and paper chromatography. The evidence indicated a monophosphate as the major product and a minor component which was probably a diphosphate.

An explanation was sought for the high yield of monophosphate obtained. It was decided, in the first instance, to investigate the alkaline lability of the 3' and 5' monophosphates of adenosine under conditions similar to those which prevailed in the above experiment, during the removal of the cyanoethyl groups from the nucleotide intermediates. It was thought that the 3'-phosphate (which is, under acid conditions, more labile than the 5' isomer) might be the subject of some hydrolysis, thereby accounting for the high yield of monophosphate obtained and identified as adenosine 5'-phosphate.

(c) Investigation of the alkaline lability of adenosine 3'-phosphate and adenosine 5'-phosphate.

The respective nucleotides (L. Light & Co. Ltd.) were dissolved (Expt. 9) in water (1.0ml.) and ammonia (0.88, 1.0 ml.) added to each and the solutions heated at 60° for 1 hour in a water bath. Samples were examined by paper electrophoresis and paper chromatography and for release of inorganic

phosphate. In both cases there was no evidence to indicate any degradation or release of inorganic phosphate.

(d) The effect of increasing the ratio of phosphorylating agent to adenosine.

It is apparent that the low yield of adenosine 2'(3'):5' diphosphate may be attributed to the difficulty of phosphorylating the secondary alcoholic groups (i.e. the 2' or 3'-positions of the nucleoside). One method which suggested itself as a means of increasing the yield of diphosphates was to increase the ratio of CEP to adenosine (Expt.10).

Adenosine (4 m.moles) was dissolved in boiling anhydrous pyridine (60 ml.), cooled to room temperature (Expt. 10) and CEP (16 m.moles.) added and the mixture concentrated in vacuo, followed by further addition and concentrating again, as described earlier. Finally pyridine (60 ml.) was added to the resultant oil, followed by DCC (64 m.moles). The reaction mixture was allowed to stand at room

CHROMATOGRAPHIC SEPARATION OF ADENOSINE PHOSPHATES ON DOWEX–2 (×8) ION EXCHANGE RESIN

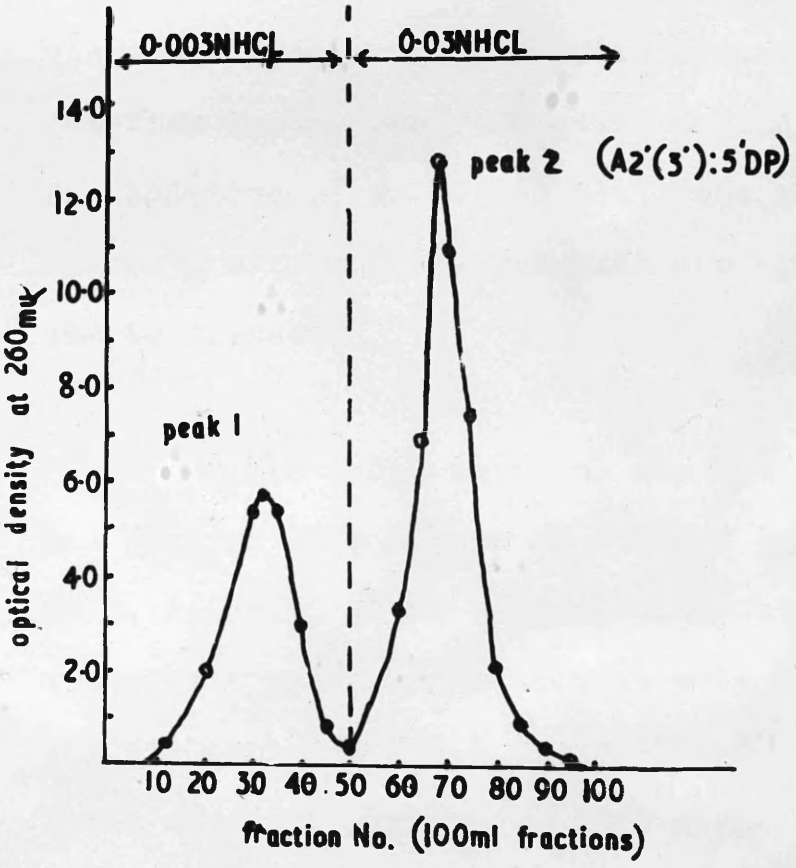


FIG. 7.

temperature. Aliquots (0.1 ml.) were withdrawn after 4, 9, 21 and 24 hours, treated with ammonia (0.88) at 60° for 1 hour and examined by paper electrophoresis. After 24 hours the major product indicated that it might be a diphosphate, although there was still some unreacted adenosine. The reaction was stopped (26 hours having elapsed) by the addition of water (60 ml.); the solution filtered after 30 minutes, treated with ammonia and concentrat-:ed to dryness.

The solid material was dissolved in water and applied to a column of Dowex-2 (x8), chloride form, resin. After washing with water the mono-:phosphate material was eluted with 0.003 N HCl and the diphosphates (Peak II) with 0.03 N HCl. (Fig. 7) Yield of diphosphates, at this stage was 30% based on adenosine.

(e) The effect of temperature on the yield of diphosphates.

With a view to further increasing the

yield of adenosine 2'(3'):5 diphosphate it was decided to repeat the preceding experiment and on this occasion to carry out the phosphorylation reaction at 37° instead of at room temperature (Expt. 11). It was thought that such an increase in temperature might substantially increase the yield.

Adenosine (4 m.moles) was phosphorylated at 37° (other details as Expt. 10) and after 26 hours was worked up as previously described. The nucleotide material was applied to a column of Dowex-2 (x8) ion exchange resin and phosphate esters eluted by stepwise gradient using hydrochloric acid (Fig. 8). Yield of diphosphates 44% (approx).

5. Methods of Isolating the Nucleotides and identification of the products.

(a) Calcium salt method.

Initially (Expt. 10) nucleotide material

CHROMATOGRAPHIC SEPARATION OF ADENOSINE PHOSPHATES ON DOWEX-2(x8) ION EXCHANGE RESIN

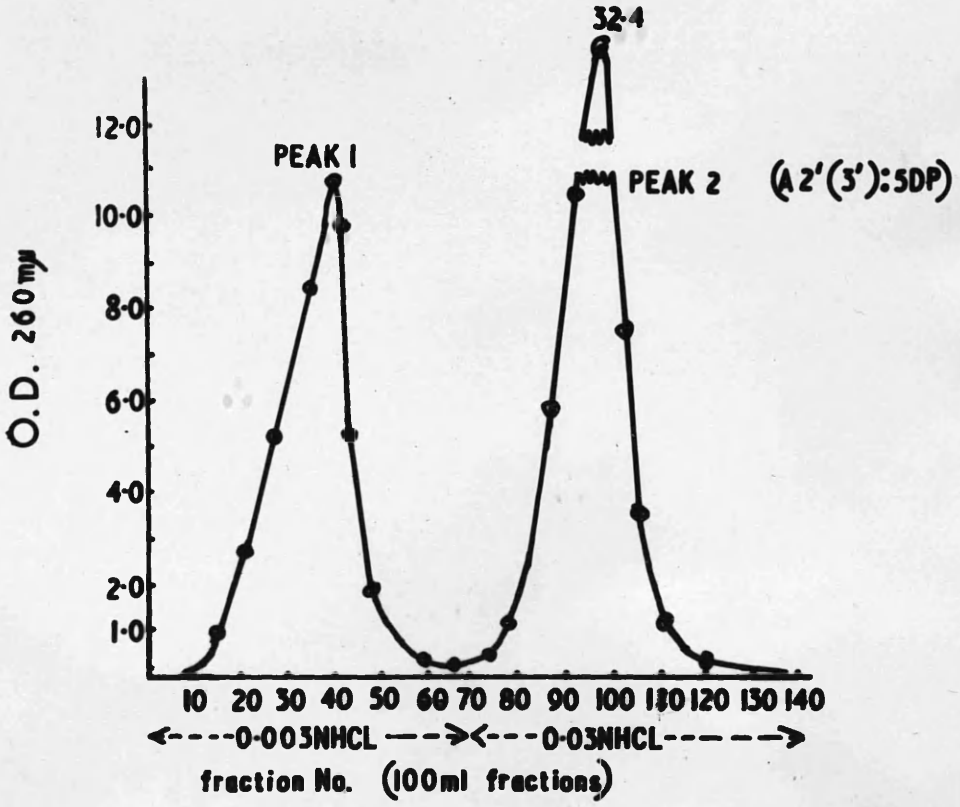


FIG 8

was isolated as the calcium salt. While this method is simple and straightforward it has the disadvantage that large volumes of liquid must be concentrated and in addition it was found that the product was impure.

The appropriate fractions (Expt.10, Peak II, Fractions 52-85) were bulked and neutralized to pH 7.0 with a suspension of calcium hydroxide and concentrated to a small volume on a rotary evapor-:ator and then freeze dried. The white solid was washed with ethanolic-ether (50 ml. portions) until the supernatant was free of chloride ions and finally with 3 x 50 ml. portions of ether. The solid was collected by centrifugation and air dried to give a white powder (1.7 grs.). Spectrophoto-:metric analysis (based on optical density at 260mμ) showed that the nucleotide content of the solid was equivalent to 0.739 gr. (43%). Further purification was effected through adsorption of the nucleotide material on charcoal (Expt. 13) at acid pH. The column was washed with water and then with sodium

bicarbonate and finally the nucleotide material was eluted with 50% aqueous ethanol containing 2% ammonia. The eluate containing the nucleotide material was concentrated to dryness in vacuo on a rotary evaporator. The solid was washed with ethanolic-ether and air-dried. The purity of the nucleotide was shown (by ultraviolet absorption at 260 mμ) to be 97%.

(b) Lithium salt method.

In subsequent experiments (e.g. Expt.11) an alternate method was used to isolate the nucleo-:tide material. It was first concentrated by ion-exchange. Thus, fractions 79-120, approximately 4 litres, (Peak 2. Expt. 11), were bulked and adjusted to pH 8 with ammonia. The volume was diluted three times with water (to ensure retention of the nucleotide by the resin) and the solution applied to a column of Dowex-2 (x8), chloride form, resin (2 x 3.2 cms.). The column was washed with water (1 L.) and then eluted with 0.06N HCl acid,

ELUTION OF A(2')3':5'DP FROM DOWEX-2 (x8) ION EXCHANGE RESIN

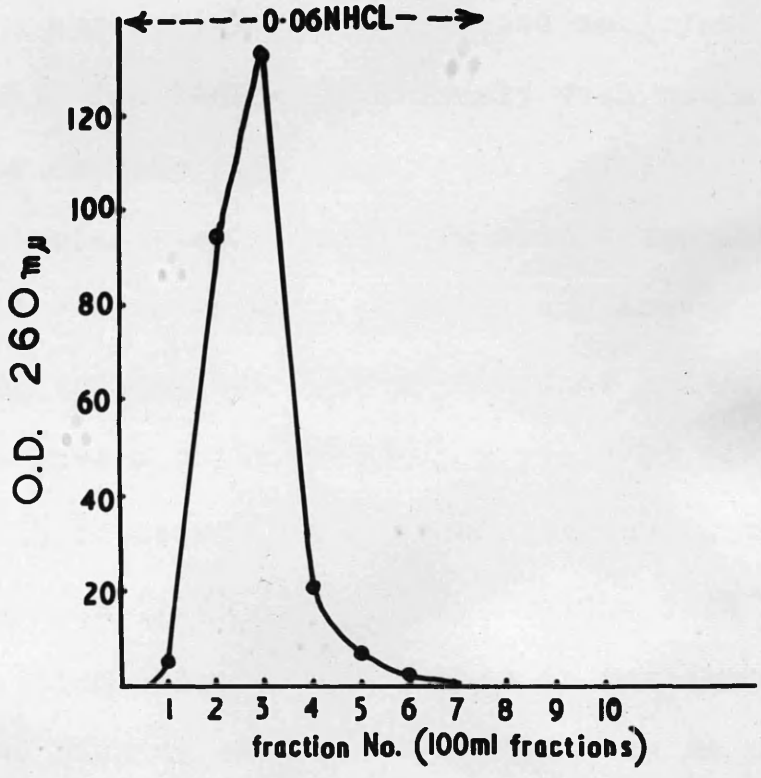


FIG 9

which displaced the nucleotide. Fractions (100ml.) were collected and each fraction read on a Unicam spectrophotometer at 260 mμ. (Fig 9). The appropriate fractions (approx. 600 ml.) were then bulked, adjusted to pH 6.5 with lithium hydroxide and the solution concentrated to dryness. The solid was stirred vigorously with methanol (25 ml.) and acetone (125 ml.) added. The nucleotide material was collected by centrifugation and repeat-:edly treated with methanol and acetone until the supernatant was free of chloride ions and air dried to give a white solid in a yield of 44% (0.84 gr.). This procedure is based on the method of Smith and Khorana (179) and is based on the fact that whilst lithium chloride is soluble in methanolic-acetone the lithium salt of the nucleotide is not.

(c) Analysis and Identification of Adenosine 2'(3'):5'-diphosphate (Expt. 15).

The ultraviolet spectrum of A 2'(3'):5'DP compares with that of an adenine containing compound (Fig. 10), λmax. 258 mμ and λmin. 227 mμ. Phosphate

ABSORPTION SPECTRUM OF A2(3'):5DP

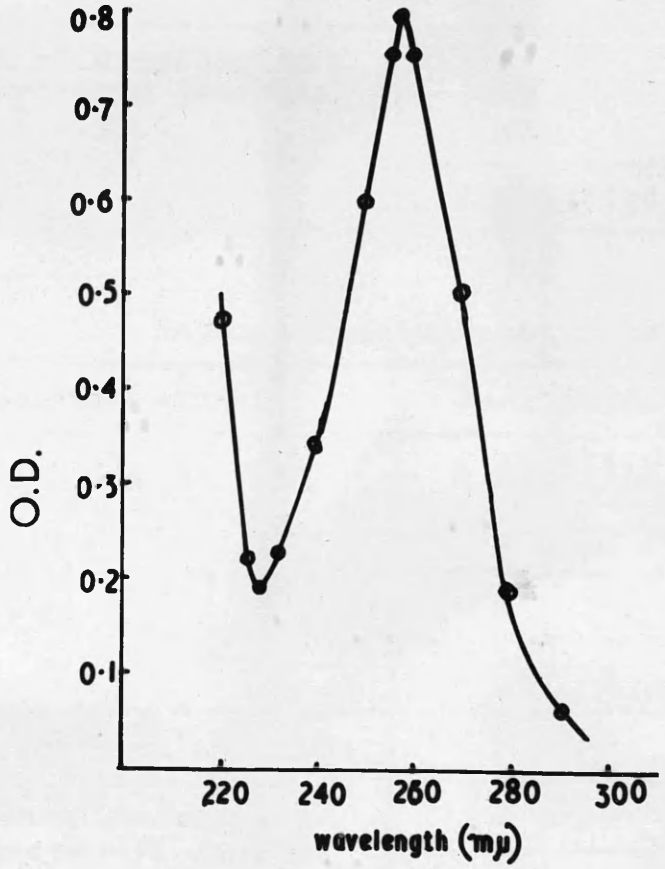


FIG 10

TABLE 2.

PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS OF A (2')3':5'DP.

Paper Electrophoresis (G.M. 2).

Buffer	Nucleotide	Rf.
0.05M ammonium formate pH 3.5 500V/1 hour.	A 5'P	5.5
	ADP	7.4
	A (2')3':5DP	8.0
M/50 dipotassium hydrogen phosphate pH 7.5 750V/1¼ hours.	A 5 P	10.0
	ADP	11.5
	A 2'(3'):5'DP	12.8

Paper chromatography (G.M. 2).

Solvent system	Nucleotide	Rf.
n-proply alcohol -ammonia-water, (6:3:1 v/v). Ascending technique	A 2'(3'):P	0.32
	A 5'P	0.28
	A 2'5'DP	0.10
	A 3'5'DP	0.10
saturated ammonium sulphate - 0.1M ammonium acetate-isopropyl alcohol (79:19:2 v/v). Ascending technique	A 2'P	0.30
	A 3'P	0.22
	A 5'P	0.35
	A 2':5'DP	0.49
	A 3':5'DP	0.41

analysis (G.M. 1) showed that the ratio of adenine to phosphorous was 1:1.97. One phosphate is hydrolyzed in IN HCl at 100° after 30 minutes, as is the case with adenosine 2' or 3'-phosphate (164). The second phosphate is rather acid stable corres-:ponding to adenosine 5'-phosphate. The position of the more easily (30 min.) hydrolyzed phosphate was further identified by its behaviour towards hydrolysis with a specific phosphatase (163) from germinating rye-grass (Expt.15). This enzyme is known to hydrolyse a phosphate group at the 3' position in adenosine and its phosphates, but is without action on phosphate groups attached to other positions. Incubation of this extract with A 2' (3'):5' DP released 49% of the easily hydrolyzable phosphate or 24.5% of the total phosphate, thus indicating that the two isomers exist in approxim-:ately equal amounts.

The mixture was examined (Table 2) by paper chromatography and paper electrophoresis (G.M. 2). Resolution of the isomers was effected

CHROMATOGRAPHIC SEPARATION OF A2':5'DP & A3':5'DP ON DOWEX-1 (x2) RESIN

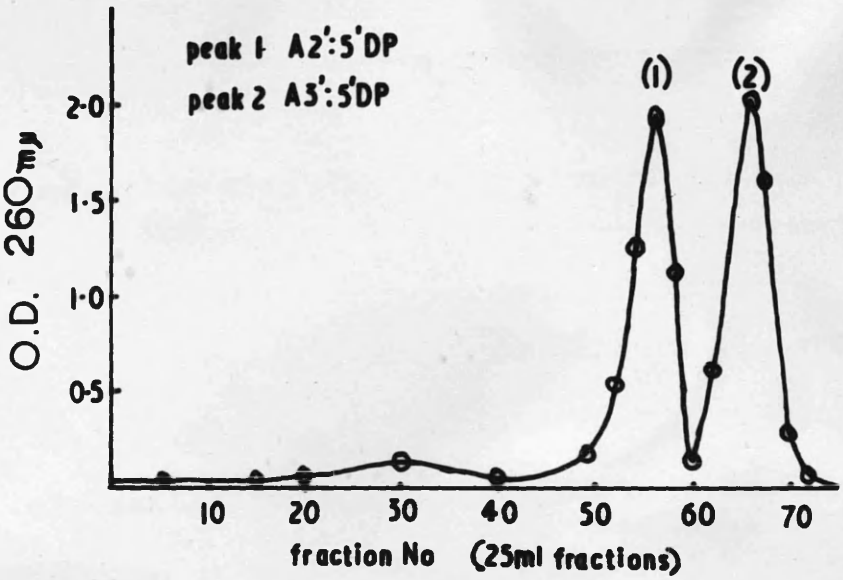


FIG II

by paper chromatography in the solvent system ammonium sulphate/ammonium acetate/isopropanol. This is the only system known to separate the isomers on paper (Fig.13).

6. Separation of adenosine 2':5'diphosphate and adenosine 3':5' diphosphate by ion-exchange chromatography and preparation of PAPS.

(a) Ion-exchange separation of A 2':5'DP and A 3':5' DP.

Baddiley and co-workers (155) succeeded in separating the two isomers by ion-exchange chromatography employing gradient elution with calcium chloride and hydrochloric acid and their method has been used here with excellent results.

A 2' (3'):5'DP was adsorbed (Expt.16) on Dowex - 1 (x2), chloride form, resin. The column (1 x 65 cms.) was then washed with water (200 ml.). Elution was carried out with calcium chloride and

hydrochloric acid; the concentration range was from 0.03M Ca Cl_2 and 0.0045 N HCl to 0.07M Ca Cl_2 and 0.007 N HCl. A 2':5'DP was eluted ahead of A 3':5'DP (Fig.11). This was in agreement with the order of elution of monophosphates of adenosine where the 2'-precedes the 3'-phosphate. The diphosphates were isolated from the approp-:riate fractions by adsorption on charcoal as described. Examination of the products by paper chromatography (Fig.13) showed a clean resolution and the two isomers were characterized by their activity as substrates for the specific 3'-phos-:phatase from rye grass (A 2':5'DP is inactive).

(b) Preparation of Adenosine 3'-Phosphate 5'-sulphatophosphate (PAPS).

The pyridine-sulphur trioxide complex (G.M. 3) has been shown (156) to react with adenosine 3':5' diphosphate (PAP) in sodium hydrogen carbonate solution to give PAPS and a sulphur free nucleotide which was identified as adenosine 2':3' cyclic phosphate 5'-phosphate.

The formation of this cyclic phosphate is readily explained on the assumption that an initial reaction product was adenosine 5'-phosphate 3'-sulphatophosphate. Such a mixed anhydride would be unstable, decomposing spontaneously to inorganic sulphate and a 2':3' cyclic phosphate.

The experimental conditions for this synthesis were chosen in order to limit the possible introduction of more than one sulphate group into the nucleotide.

The pyridine-sulphur trioxide complex (88 mgs.) was added (Expt. 17) with stirring to a solution of the lithium salt of A 3':5'DP (23 mgs.) and sodium bicarbonate (132 mgs.) in water (1.3ml.). The reaction was carried out in a water bath at 45°. After 40 minutes at this temperature the solution was cooled, diluted with ice-water (50ml.) adjusted to pH 6.0 with 1N formic acid and passed through a column (2 x 8 cms.) of Norit A charcoal-celite 535 (4:3 by weight). All operations

carried out at 0 - 4°. The column was washed with water (60 ml.) Nucleotides were eluted from the column with 50% aqueous ethanol which contain-:ed 2% ammonia. The eluate was passed through a column of Dowex - 1 (x 2), chloride form, resin (2 x 8 cms.) which was washed with water (200 ml.) Gradient elution was carried out using 1.95N lithium chloride (666 ml.) as eluent in the reser-:voir and water (1 L.) in the mixing flask. Fractions (25 ml.) were collected and the optical density of each fraction read at 260 mμ. Two peaks were observed (Fig. 12), the first corresponding to A 3':5'DP, probably containing a trace of adenosine 2':3'-(cyclic) phosphate - 5' phosphate while the latter corresponded to adenos-:ine 3'-phosphate 5'-sulphatophosate. The fractions containing the desired nucleotides were isolated by adsorption on charcoal as described above, followed by concentration of the ethanolic-ammonia eluate under reduced pressure at 32-34°. The concentrated eluate (5 ml.) was passed through a column (1 x 1.5 cms.) of Dowex - 50 (x8) resin

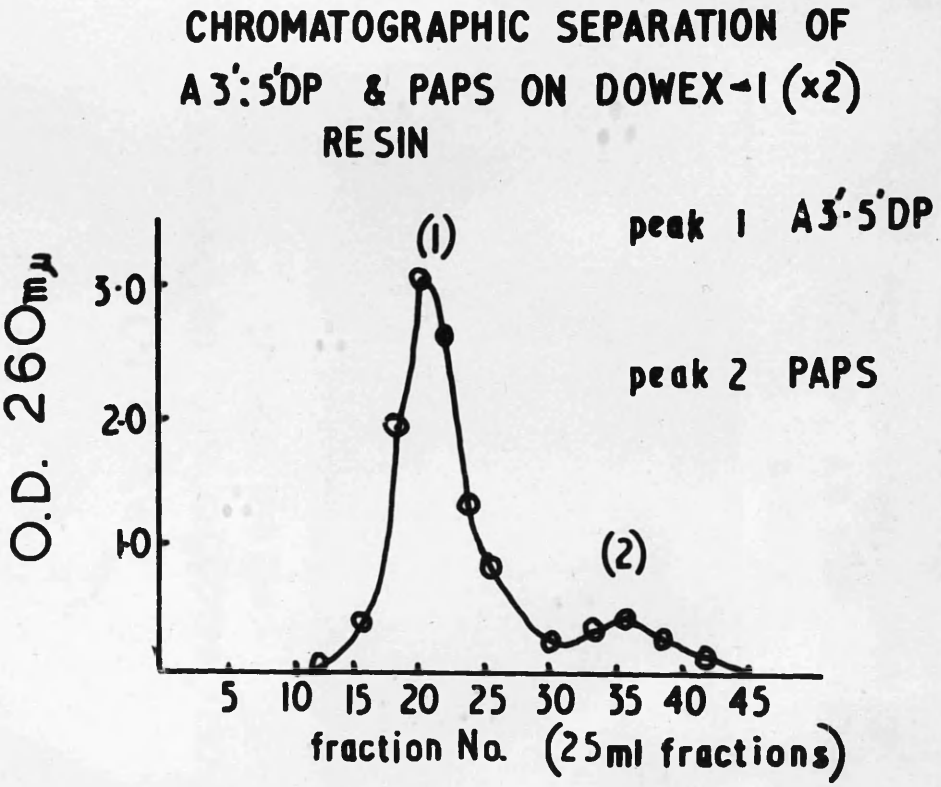
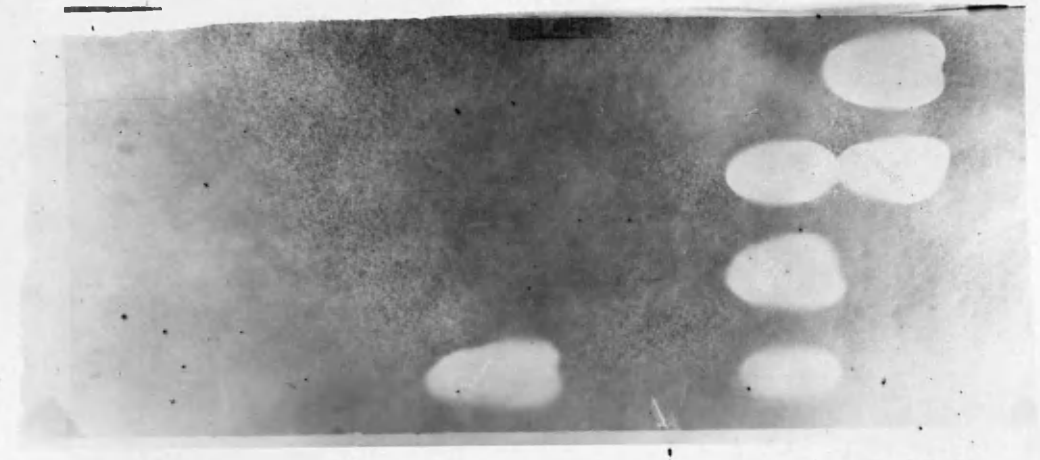

CHROMATOGRAPHIC SEPARATION OF
A 3':5'DP & PAPS ON DOWEX-1 (x2)
RESIN
O.D. 260mμ
3·0
2·0
1·0
(1)
(2)
peak 1 A3'·5'DP
peak 2 PAPS
5 10 15 20 25 30 35 40 45
fraction No. (25ml fractions)

FIG 12

PAPER CHROMATOGRAPHY OF
A2':5'DP, A3':5'DP, A2'(3'):5'DP
& PAPS.



SOLVENT SYSTEM:-
SATD.$(NH_4)_2SO_4$ / 0·1M. CH_3COONH_4 / ISO-PrOH
(79:19:2, V/V)

FIG. 13

(lithium form) and the column washed with a further 5 ml. of water. The eluate and washings were combined and freeze-dried to yield a light brown coloured solid (2.4 mgs.). Examination of this product by paper chromatography indicated it was almost homogenous; a trace of adenosine 3':5'-diphosphate was present (Fig.13). Final confirm-:ation of the compound as PAPS was shown by its ability to act as the sulphate donor in the enzym-:ically catalyzed transfer of sulphate to p-nitro-:phenol to form p-nitrophenylsulphate (Expt. 25).

SECTION II.

Preparation of other Substrates.

I. Introduction.

In the present study a method for the cleavage of sulphate from polysaccharide sulphates was sought so that the resulting partial or totally desulphated polymers might be investigated as possible acceptor substrates in enzymically cat-:alyzed transfer reactions using PAPS.

The application of classical techniques in the structural studies of polysaccharide sulphates has not been successful. For example, the presence of a high percentage of sulphate groups in the heparin molecule is undoubtedly one of the complicating factors. Thus, a satisfactory method of desulphation would also be of value in furthering structural studies of such a compound. Some progress toward this end was made with the introduction of a chemical method of desulphation by Wolfrom and Montgomery (165). The technique involves the use of concentrated sulphuric acid

and acetic anhydride. It is probable that the active desulphating agent is the acetylium ion, CH_3-CO^+, which is known to be produced when strong acids are added to acetic anhydride (166). However, the use of sulphuric acid tends to create experimental difficulties and may cause some undesirable transformations including degradation of the polysaccharide molecule.

More recently Kantor and Schubert (167) have described a milder method for the desulphation of chondroitin sulphate. This method utilizes methanolic-HCl and if the repeating unit of chondroitin sulphate be represented by KOOC - R - OSO_3K the reaction appears to be a transesterification -

$$KOOC - R-OSO_3K + 2\,CH_3OH + HCl \longrightarrow CH_3OOC - R - OH + KO_3S.OCH_3 + KCl + H_2O$$

Thus, treatment of dry potassium chondroitin sulphate with dilute HCl in methanol at room temperature yielded a non-dialyzable product

with no ester sulphate and with methylated carboxyl groups, which on hydrolysis with alkali yields a material which has been identified as desulphated chondroitin sulphate.

2. Desulphation of chondroitin sulphate.

Potassium chondroitin sulphate was prepared as follows: chondroitin sulphate (3.0grs. L.Light & Co.) was dissolved in water (Expt. 18) and applied to a column of Dowex - 50 (H^+ form) resin (3.4 x 10 cms.). The column was washed with water to pH 7.0 and the eluate and washings combined and adjusted to pH 7.0 with KOH and concentrated to (approx.) 40 ml. on a rotary evap-:orator. Addition of ethanol (2 volumes) yielded a white precipitate which was air dried to give 2.1 grs. of product.

Acid methanol (G.M. 4) was prepared by adding acetyl chloride (5 ml.) to dry methanol (1 L.) and allowing the solution to age at least

a day before use, in order to complete the methanolysis of acetyl chloride. Titration with 0.1N NaOH showed the normality of the HCl to be 0.063 N.

Finely powdered, well dried potassium chondroitin sulphate (1.25 gr.), SO_4^{2-} content -15.3% (G.M. 5) was shaken with acid methanol (210 ml.) for 24 hours at room temperature (Expt. 19). Both starting material and product are insoluble in methanol. The mixture was centri-:fuged and the residue was similarly shaken for two further 24 hour periods after addition of fresh methanolic -HCl in each instance. After 72 hours the insoluble residue was dissolved in water (20 ml.) and dialyzed against cold running water for 24 hours; the product was then precipitated by adding ethanol (200 ml.), separated by centrifug-:ation, washed with ethanol and then ether and dried in vacuo. Yield 0.712 g.

Treatment of this methyl ester of desul-

:phated chondroitin sulphate (500 mgs.) with aqueous 0.1N NaOH for 48 hours removed the methyl groups. The solution was acidified and dialyzed against distilled water, adjusted to pH 7.0 with NaOH and the sodium salt of desulphated chondroit-:in sulphate precipitated by the addition of five volumes of ethanol. The solid was collected by centrifugation, washed with ethanol and then ether and dried in vacuo to give a white powder. Yield 0.445 g. (SO_4^{2-} content - 1.5%)

3. Desulphation of heparin.

(a) Desulphation of heparin using methanolic-HCl.

Heparin (L.Light & Co.) was purified (168) as the barium salt and the converted to its potassium salt (Expt.20).

It was decided to investigate the use of methanolic-HCl as a means of removing sulphate from the heparin molecule, in view of the success of

DESULPHATION OF HEPARIN BY TREATMENT WITH METHANOLIC–HCl

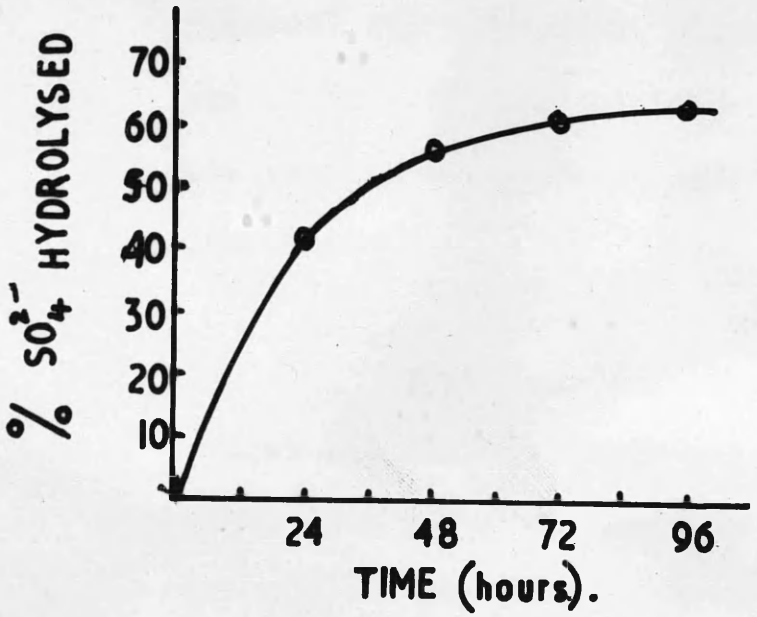


FIG 14

this method when used with chondroitin sulphate. Finely powdered, well dried potassium heparinate was shaken for 24 hours at room temperature (Expt. 21) with methanolic-HCl (160 ml.). The mixture was centrifuged and the residue was similarly shaken for three further 24 hour periods with fresh methanolic -HCl added each day. A small portion of the insoluble polysaccharide was removed, for subsequent sulphate analysis, at the end of each 24 hour period. Fig. 14 illustrates the rate of cleavage of sulphate from the poly-:saccharide.

After four days the mixture was centri-:fuged and the insoluble material washed several times with methanol and then dissolved in water (25 ml.). The polysaccharide material was precip-:itated by the addition of dry ethanol (120 ml.). The precipitate was collected by centrifugation and washed with ethanol, ether and finally dried over P_2O_5 to give the methyl ester of desulphated heparin (560 mgs.). The polysaccharide methyl

ester (550 mgs.) was saponified by allowing it to stand for 48 hours in 0.099N NaOH (12 ml.). The solution was acidified and dialyzed for 24 hours against repeated changes of distilled water and the free acid precipitated by addition of five volumes of dry ethanol. The basic sodium salt was obtained by dissolving the free acid in water, adjusting the solution to pH 7.0 with 0.1N NaOH and precipitating the product (420 mgs.) with ethanol.

It is apparent therefore that application of the method of Kantor and Schubert (167) to heparin succeeds in removing a considerable pro-:portion (63%) of the ester bound sulphate. While this technique does not furnish a totally desul-:phated polymer, it nevertheless provides a material suited to the study of transfer experim-:ents involving PAPS.

Shortly after this section of the work was completed a similar method (169) was published

by other workers, thus confirming the results obtained above.

(b) Selective N-de Sulphation of Heparin.

Heparin may be selectively treated with acid to hydrolyze the N-sulphate linkages only (186). Heparin (Expt.22) was dissolved in 0.04 N HCl and heated at 100° for 3 hours. Dialysis, followed by precipitation with ethanol yielded the product (SO_4^{2-} content 27%).

4. Isolation and Purification of Hyaluronic Acid.

Hyaluronic acid possesses the same structural units (e.g. glucuronic acid and glucos-:amine as an N-acetyl derivative) as does heparin, although it is devoid of any sulphate. However, because of the obvious similarities it seemed an ideal polysaccharide to substitute for heparin in

some enzymic experiments. Meyer et al. (81) have isolated hyaluronic acid from pig skin together with chondroitin sulphate B and we have used their method with slight modification. Firstly, it was found essential to slice the skin into small sections before passing through a power driven meatgrinder. Secondly it was necess-:ary to allow the tryptic digestion to proceed for 36 hours to almost liquify the material. The method of extraction was as follows: Fresh skin obtained chilled from the slaughter house was scraped as free of fat as possible. After cooling it was cut into small portions and passed twice through a meat grinder (Expt. 23). The ground skin was suspended in water, acidified to pH 1.5 and pepsin (B.D.H.) added. The suspension was covered with a layer of toluene and incubated at 37° for 40 hours, with occasional stirring. Most of the toluene was removed and the pH adjusted to 7.5 with NaOH. After addition of trypsin and a fresh layer of toluene the solution was incubated at 37° for 36 hours. The solution was filtered

and to the chilled filtrate calcium acetate was added to 2.5% and glacial acetic acid to 0.25N, followed by 1.25 volumes of ethanol. The mixture was kept at 0 - 4° overnight and centrifuged; the precipitate was washed and suspended in 5% sodium acetate containing 0.5N acetic acid and the solut-:ion stirred with chloroform-amyl alcohol (9:1 $^{v}/_{v}$). Ethanol was added to the aqueous layer and the precipitate collected and again dissolved in sodium acetate-acetic acid solution. Further purification was effected by reprecipitating with ethanol and passing through a column of Amberlite IR-120 (H^{+}) resin, followed by separation of the polysaccharides by fractional addition of ethanol in the presence of calcium ions. Two main components were obtained - fraction I precipitating at 21% ethanol saturation and having an I.R. spectrum comparable with chondroit-:in sulphate B. Fraction II, hyaluronic acid, was precipitated at 30% ethanol saturation and corres-:ponded to that obtained by Meyer et al. (81). The

sulphate content was 0.5% (G.M. 5).

Two other polysaccharides, one a sulphated material from the red seaweed Furcellaria fastigitata and the other a sulphated polysaccharide from Chondrus crispus were used in the investigations described in Part II, Section II. The former was shown to be a sulphated polysaccharide containing galactose, xylose, 3, 6 -anhydrogalactose and a uronic acid (204) while the latter in addition to sulphate contains D-galactose, L-galactose and 3:6-anhydro-D-galactose (205) as the main components.

P A R T II.

Some studies on the enzymatic transfer of sulphate.

S E C T I O N I.

Preparation of a sulphate transferring enzyme and some initial experiments.

I. Introduction.

The earliest interest in the biological role of sulphate esters centred around the detoxif-:ication of phenols by esterification with sulphate in vivo. The work on phenylsulphate and glucuronide excretion is summarized by Williams (1). The synthesis of phenylsulphates in liver slices and in aerobic liver homogenates was first accomplished by De Meio and co-workers (16); these workers showed that ATP was an essential requirement of the system (17). The following remarks expand somewhat on what was said in the General Introduction.

The preliminary results of Bernstein and McGilvery (19) indicated that at least two steps were required for the synthesis of phenylsulphates in vitro. The first step involves the formation of an active sulphate intermediate and the second the condensation of the intermediate with the phenol.

They reached this conclusion on the basis of kinetic data. De Meio et al. (21) separated the two activities. The first, due to the "activating system" (see below) survives heating for 15 minutes at 52^{o}, a treatment that inactivates the other (i.e. the transferring) enzyme.

1. "Activating system".

$$ATP + SO_4^{2-} \longrightarrow PAPS.$$

2. Transferring enzyme.

$$PAPS + p\text{-}NP \longrightarrow PAP + p\text{-}NPS.$$

Further investigation (26,53) indicated that enzymatic synthesis of PAPS required two enzymes: (1) ATP-sulphurylase which catalyzes dis-:placement of pyrophosphate from ATP with formation of adenosine 5'-sulphatophosphate (APS), Fig. 2, and (2) APS- kinase which catalyzes the phosphoryl-:ation of the 3'-hydroxyl group of APS to form PAPS.

1. $ATP + SO_4^{2-} \xrightleftharpoons{\text{ATP-sulphurylase}} APS + PP.$

2. $APS + ATP \xrightleftharpoons{\text{APS-kinase}} PAPS + ADP.$

ATP-sulphurylase has been purified extensively from baker's yeast by Robbins and Lipmann (53,54) and by Wilson and Bandurski (55). The purification of yeast sulphurylase is based on its acidic (anionic) properties. Wilson and Ban-:durski used protamine fractionation followed by starch gel electrophoresis. Robbins and Lipmann used a combination of salt precipitation, acid fractionation and electrophoresis to obtain an enzyme purified about 2000-fold which appeared homogenous by preliminary electrophoretic and ultra-centrifugal analysis. Because of its great elec-:trophorectic mobility the protein may be assumed to have multiple anionic charges at neutral or alkaline pH. This cannot be due to nucleotide or nucleic acid derivatives, since the purified enzyme has a typical protein ultra-violet absorption spect-:rum with a peak at 278 mμ and a 280 mμ to 260 mμ

absorption ratio of 1.7. It is worthy of note that formation of APS from ATP and SO_4^{2-} occurs to only a very slight extent in the absence of other enzymes that remove the products from the reaction mixture.

The properties of this enzyme from other sources have not been investigated in detail.

The APS-kinase reaction has not been studied in as great detail as ATP-sulphurylase. The yeast enzyme has been purified and the reaction catalyzed by it has been shown to have the expected stoichiometry.

$$APS + ATP \longrightarrow PAPS + ADP.$$

The general requirements of the enzyme are similar to those of a number of other ATP-transphosphorylases. The presence of a divalent cation is required for enzymic reaction. Mg^{++} is active over a wide range but becomes inhibitory at high concentrations if small amounts of APS are used as substrate.

The most interesting characteristic of APS-kinase is its great affinity for APS. Experimentally the highest initial rate of phosphorylation was found at the lowest concentration of APS that could be tested. From the point of view of PAPS synthesis it is appropriate that APS-kinase has a high affinity for APS, since as mentioned above, the ATP-sulphurylase reaction is unfavourable from both the equilibrium and kinetic standpoint.

A number of preliminary reports of the properties of liver phenol sulphuryltransferase have appeared (25,37). The only detailed investigation of the enzyme, however, is that reported by Nose and Lipmann (36). The enzyme was found in the fraction of rabbit liver proteins that precipitates with low concentrations of ammonium sulphate. This property of the enzyme led to its separation from steroid sulphuryltransferase activity in liver extract. The critical question of whether one or several phenol sulphuryltransferases are present in liver has still not been answered definitely. The variety of

materials that will serve as substrates for phenol sulphuryltransferase of rabbit liver (37) includes such diverse compounds as phenol, resorcinol, 3-pyridinol, hydroquinone, phloroglucinol and methyl-:salicylate. Other phenolic compounds such as salicylic acid and ∝-naphthol will not act as substrates.

2. The preparation and assay of a sulphate transferring enzyme.

Nitrophenol esters have been used extensively for the determination of hydrolyases; the phosphate having been introduced by Huggins and Smith (170) for phosphatase activity. Roy, (171) in particular, has used the sulphate ester for arylsulphatase determinat-:ion. p-Nitrophenol, which behaves as a transfer indicator because of the disappearance of the yellow coloured anion on esterification, has been used by Gregory and Lipmann (37).

p-NP ⟶ p-NPS.
yellow colourless.

Thus, the formation of p-nitrophenyl sulphate (p-NPS) by transfer of the sulphate group from PAPS to p-nitrophenol (p-NP) under the influence of phenol sulphuryltransferase, can be estimated from the decrease in absorption by the p-nitrophenol anion at 400 mμ.

The procedure adopted for the preparation of phenolsulphuryltransferase was similar to the method of Bernstein and McGilvery (18). The livers of fasted male rats (Rattus Norvegicus, albino) were removed immediately after slaughter (Expt.24), and homogenized in a volume of 0.15 M KCl containing 0.001 M. ethylenediamminetetraacetate (EDTA) pH 7.2, four times their weight. The homogenate was centri-:fuged at 20,000 xg. for 1½ hours and the protein precipitating between 1.5 M and 2.3 M ammonium sulphate concentration collected by centrifugation, dissolved in distilled water and stored at -14°.

PAPS was assayed using p-NP as an acceptor substrate (Expt. 25) and the enzyme extract prepared

above. The method used was that of Gregory and Lipmann (37). A phosphate buffer was used and potassium fluoride added to the incubation mixture. Phosphate and fluoride are general sulphatase inhib-:itors and are known to inhibit a sulphatase which degrades PAPS (120). Nitrophenolate disappearance was followed by removal of an aliquot of the incub-:ation mixture, diluting to a standard volume with N/100 NaOH and recording the intensity of the colour produced (Fig. 15) using an EEL photoelectric color-:imeter (filter 601). Thus, in the experiment quoted (Expt. 25) which is typical of many such assays carried out, 42.8% of the available sulphate as PAPS was transferred to p-NP.

The rat liver extract was also assayed for enzymic transfer of sulphate from p-NPS (G.M. 8) to phenol using PAP as co-factor (37) (Expt. 26).

$$\text{p-NPS} + \text{PAP} \longrightarrow \text{p-NP} + \text{PAPS}$$

$$\text{PAPS} + C_6H_5OH \longrightarrow \text{PAP} + C_6H_5OSO_3H.$$

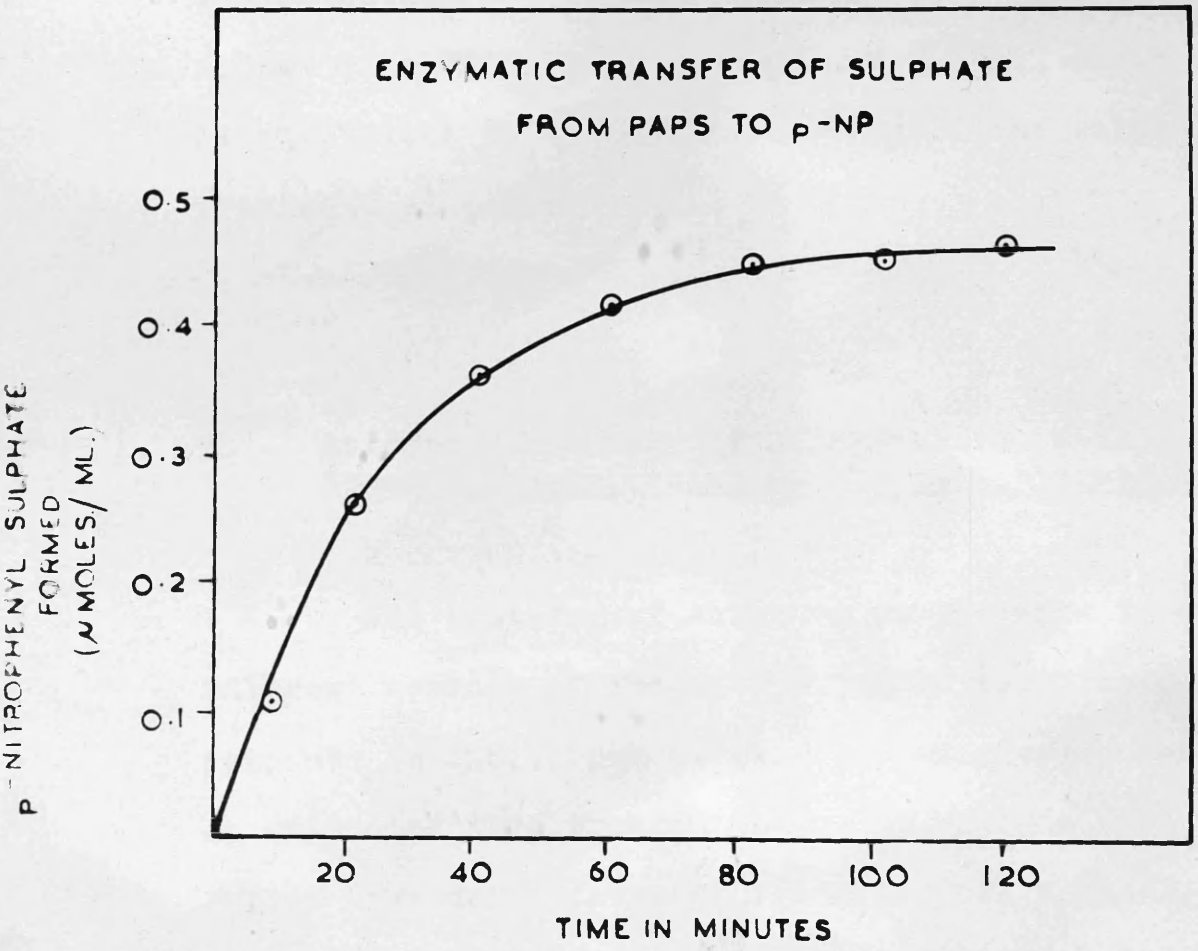

ENZYMATIC TRANSFER OF SULPHATE
FROM PAPS TO p-NP
p-NITROPHENYL SULPHATE FORMED (μMOLES/ML.)
0·5
0·4
0·3
0·2
0·1
20
40
60
80
100
120
TIME IN MINUTES

FIG 15

Phenol conjugation was followed by the release of p-NP which was determined using an EEL photoelectric colorimeter (Filter 601). The amount of p-NP released was 40 mμM per ml. which is equivalent to a transfer of 5.2% of the sulphate available as p-NPS.

3. Attempted transfer of sulphate from p-NPS to some low-molecular weight carbohydrates.

The transfer of sulphate to tyrosine is of interest because of the occurrence of tyrosine-O-sulphate in fibrinogen (174). In an enzymic investigation of this problem Segal and Mologne (39) report that derivatives of tyrosine with a blocked carboxyl group and free amino group, are sulphated by PAPS with crude rat liver preparations as the enzyme source. Whether this activity is a non-specific function of phenol sulphuryltransferase or whether it reflects a step in the biosynthesis of fibrinogen, is not clear.

The enzyme phenol sulphuryltransferase appears to have a rather broad specificity (see Introduction this section), if indeed it is only one enzyme. The easy reversibility of the p-NP transfer reaction has led to the use of p-NPS as a sulphurylating agent for other phenols, using catalytic amounts of PAP (37). This system is particularly convenient because of the ease with which the release of p-NP can be followed. There has,however, been no clear demonstration that low molecular weight materials other than phenols can serve as secondary acceptors in this system, even though, in principle there appears no reason that this should not be true.

A series of low-molecular weight carbo-:hydrates were assayed as possible acceptors using p-NPS as sulphate donor, the rat liver preparation and PAP as co-factors (Expt. 27). The results are shown in Table 3 and are typical of those obtained when the experiment was repeated on several occas-:ions using freshly prepared extracts.

TABLE 3.

Attempted transfer of sulphate to some low-molecular weight carbohydrates.

CARBOHYDRATE	EEL Readings DIGEST	CONTROL.
1. sucrose	1.31	1.30
2. maltose	1.31	1.31
3. lactose	1.30	1.30
4. D-galactose	1.29	1.30
5. D-mannose	1.30	1.32
6. D-glucose	1.32	1.30
7. D-glucosamine	1.33	1.30
8. D-galactosamine	1.32	1.28
9. D-glucuronic acid	1.32	1.29
10. D-galacturonic acid	1.30	1.30
11. D-arabinose	1.30	1.30
12. L-fucose	1.30	1.31
13. D-ribose	1.29	1.30
14. L-rhamnose	1.30	1.30

Transfer of sulphate from p-NPS would release p-NP which would result in an increase in the optical density at 400 mμ. The results tabul-:ated above fail to indicate any sulphate transfer to these low-molecular weight materials.

The enzyme extract used in the above experiment was assayed for transfer of sulphate from p-NPS to phenol using PAP as co-factor(the conditions used were as Expt. 26). The amount of sulphate transferred was 4.9% of that available as p-NPS thus indicating that the extract was active with regard to transfer of sulphate to a phenol.

4. PAP as a possible co-factor in the transfer of sulphate from heparin to p-NP.

Gregory and Lipmann (37) as mentioned earlier have demonstrated that the transfer of sulph-:ate from p-NPS to phenols is dependant on the pres-:ence of PAP. Wortman (56) has demonstrated the presence of phenol and mucopolysaccharide sulphuryl-

:transferase activities in beef cornea epithelial extract. He applied the phenol sulphuryltransfer-:ase reaction (p-NPS^{35} + PAP $\longrightarrow$ PAPS^{35} + p-NP) so that it acted as a feeder system and thus supplied SO_4^{2-} as PAPS for subsequent transfer to mucopoly-:saccharide. Hence, the transfer of sulphate was followed spectrophotometrically by the appearance of p-NP from p-NPS. The absence of PAP and corneal mucopolysaccharide was shown to be rate limiting in the above reaction.

It is known (35) that particle-free rat liver preparations contain enzymes which can transfer sulphate from PAPS to phenols, phenolic steroids, non-phenolic steroids, arylamines, mono - and dihyd-:ric alcohols as well as to endogenous sulphate acceptors in liver. It was of some interest, there-:fore, at this stage and in view of the work which was later to be attempted (Part II, Section II - sulphate transfer to some polysaccharides) to invest-:igate the possible transfer of sulphate from a polymeric sulphated material (e.g. heparin) to a

phenol (e.g. p-NP) using PAP as a possible co-factor.

Initial experiments indicated a decrease in the concentration of the yellow coloured anion of p-NP and hence formation of a p-NP conjugate, but it was also observed that some conjugation of the phenol took place in control experiments which did not contain any added PAP. In order to achieve a better ratio of p-NPS formation and to facilitate the determination of the degree of sulphate transfer, the concentration of p-NP was scaled down in subse-:quent experiments and a series of controls (see below) were set up which would ensure the correct interpretation of the results obtained. The exper-:iment described here is typical of many carried out.

Heparin was incubated (Expt. 28) with p-NP, PAP and the rat liver extract using an adequate series of controls (Table 4). The assay is based on the quantitative measurement of p-NP. Thus, the differ-:ence between control 4 (C4) and the blank (C5)

TABLE 4.

Controls used in the enzymic transfer of sulphate from heparin to p-NP.

	TEST	CONTROLS.				
		1	2	3	4	5
PAP	+	-	+	+	+	+
p-NP	+	+	+	+	B	-
Heparin	+	+	-	+	+	+
Enzyme	+	+	+	A	+	+
	A = Enzyme denatured at commencement of reaction time (zero). B = p-NP solution added immediately before deproteinization.					

readings at 400 mμ (EEL Spectra) represents the amount of p-NP (0.6 μM) used (Expt. 28). Expt. C3 indicates that the reaction is not spontaneous but dependant on the presence of the enzyme while Expt. C2 would indicate transfer of sulphate from any endogenous donors to p-NP and/or the dependance of the transfer reaction on the polysaccharide, heparin. Expt. C1 should indicate the role the co-factor PAP plays in the reaction. The difference therefore between the extinction (C4 - C5) minus that between the test and Expt. C5 proportionally represents the amount of p-NP which has been sulphated. The result is tabulated in Table 5.

TABLE 5.

Transfer of sulphate from heparin to p-NP.

EEL Spectra Readings (at 400 mμ).

Test	Controls.				
	1	2	3	4	5
1.73	1.80	1.89	2.06	2.1	0.0

The amount of p-NPS formed in the test was therefore 106 mμM per 0.6 μM p-NP, which is equivalent to a transfer of 2.84% of the sulphate available as heparin. The comparatively low reading of Expt. C1 and hence conjugation of sulphate in this control (86 mμM) could possibly be accounted for by virtue of the existence of PAP in the enzyme preparation. It has been shown for example (37) that rabbit liver contains 18 mμM of PAP per grm. of wet weight and Wortman (56) has estimated that beef cornea epithelial extract contains at least 68 mμM PAP per grm. of wet weight. In view of these facts therefore it is plausible that endogenous PAP may account for the conjugation of p-NP in this control.

Expt. C2 which did not contain heparin shows a conjugation of p-NP equivalent to 60 mμM per 0.6 μM p-NP. Assuming the presence of endogenous PAP, the conjugation in this control can only be ascribed to the presence of one or more sulphated

substances in the liver extract which act as sulphate donors in the system. This material is however present to the same extent in the other controls.

The trend thus demonstrated by the results of this experiment indicate

1) that PAP does stimulate transfer of sulphate from heparin to p-NP.

2) that the dependence of the transfer reaction on PAP is not as pronounced as might be expected, because (it is assumed) of the presence of endogenous PAP in the enzyme preparation. However, added PAP does increase the rate of transfer (compare test and C1) and

3) that the reaction is dependent on the presence of heparin and hence heparin is a donor of sulphate to p-NP in this system.

S E C T I O N II.

Sulphate transfer to some polysaccharides.

I. Introduction (See also General Introduction)

D'Abramo and Lipmann (118) were the first to demonstrate that an extract prepared from embryonic chick cartilage would catalyze transfer of sulphate from PAPS to a polysaccharide, chondroitin sulphate. All other mucopolysaccharide sulphuryltransferases investigated to date have been shown to utilize PAPS as the sulphate donor.

The discovery of uridine diphosphate-N-acetylgalactosamine sulphate in 1955 by Strominger (175) led to speculation that the sulphurylation step in chondroitin sulphate biosynthesis takes place at the nucleotide level. Subsequent work however has shown this assumption to be incorrect. Suzuki and Strominger (120) found no incorporation of uridined-:iphosphate-N-acetylgalactosamine sulphate into muco-:polysaccharide in extracts of the isthmus region of hen oviduct, where the incorporation of sulphate from

PAPS was demonstrated. The uridine compound is a potential intermediate normally present in this tissue. To date the function of this sulphated sugar nucleo-:tide is unknown. In addition Korn (176) was unable to find any nucleotide linked sugar sulphates in mouse mast cell tumour extracts, where such low molecular weight intermediates might exist if they were involved in the active synthesis of heparin, which is a charac-:teristic of the tissue.

Using an extract of the isthmus of hen oviduct Suzuki and Strominger (120-122) demonstrated incorporation of sulphate from PAPS^{35} to various acceptors such as the chondroitin sulphates A, B and C and a heparitin type sulphated heptasaccharide. Other acceptors were oligosaccharides obtained by enzymic degradation of chondroitin and chondroitin sulphate A and also acetylgalactosamine and acetylgal-:actosamine sulphate which were converted to the mono- and disulphates respectively. The enzymatically formed mono-sulphate was shown (123) to be the 4-sulphate, the position the sulphate group occupies in chondroit-:in sulphates A and B. It is interesting to note

that the rate of sulphate transfer to the above acceptors increased with increasing chain length of acceptor.

Adams (177) investigated the effect of the addition of various mucopolysaccharides on the biosynthesis of chondroitin sulphate in a particle free enzyme extract of chick embryo condyles. The enzyme was incubated with ATP and magnesium chlor-:ide in the presence of added mucopolysaccharide and $Na_2S^{35}O_4$. The activity of the chondroitin sulphate formed was then assayed. Hyaluronic was without effect; umbilical cord chondroitin sulphate C and its protein complex stimulated incorporation of sulphate to the same extent. Bovine trachea chondroitin sulphate A had a low degree of stimulat-:ion compared with chondroitin sulphate C.

In an investigation of the role of vitamin A in mucopolysaccharide formation Wolf and co-workers have obtained evidence that the incorporation of sulphate is dependant upon the presence of the vitamin.

The addition of vitamin A was shown to increase sulphate incorporation in rat colon segments and in homogenates from vitamin A-deficient rats (180). The defect in the preparations (181) was traced to an enzyme system not specifically con-:cerned with the incorporation of hexose precursors into the polysaccharide chains (182) but attribut-:able to diminished synthesis of PAPS. This condition was rectified by the addition of vitamin A to the incubation medium. These studies are of considerable interest in that they may eventually lead to the biochemical explanation for the well known lesions in mucous membranes, more specifically in mucopolysaccharide formation, that are observed in vitamin A deficiency.

As with other types of sulphuryltransfer-:ases one of the principal questions to be answered in relation to the polysaccharide sulphurylating enzymes is the number of enzymes present in a given tissue and the substrate specificity of these enzymes. In general, it is found that extracts

from tissues such as embryonic tissue and hen oviduct will catalyze the transfer of sulphate from PAPS to a wide range of oligosaccharides and polysaccharides (120-122,177). The lack of pure chemically characterized substrates adds to the difficulties in this field.

Sulphuryltransferases with specificity towards certain mucopolysaccharides have been detected and studied. Thus, an enzyme from rabbit skin has been purified (124) and shown to have greater activity with chondroitin sulphate B than with either chondroitin sulphates A or C. The fact that only one sulphate group was introduced per five or six disaccharide units together with information from turnover studies (183) led the authors to consider the possibility that an oligo-:saccharide or other low molecular weight substance might be the sulphate acceptor in vivo. It is interesting to note that uridine triphosphate stim-:ulated the sulphation of chondroitin sulphate B.

Suzuki et al. (184) have separated specific mucopolysaccharide sulphuryltransferases by chromatography of hen oviduct extracts on diethylaminoethylcellulose. The heparitin sul-:phuryltransferase was obtained relatively free of other enzymes but the remaining sulphuryltransfer-:ases were only partially separated from each other.

Although a considerable number of studies have been made on the biological effects of heparin, comparatively little is known concerning its meta-:bolism. The utilization of exogenous inorganic sulphate in the biosynthesis of heparin was demon-:strated by the isolation of S^{35}-heparin from the livers of dogs previously injected with $Na_2S^{35}O_4$ (127) and there is suggestive evidence for the incorporation of sulphate in rat liver slices (187) although the identification of the radioactive product as heparin was not rigorously established. Autoradiographic experiments by Jorpes et al. (128) have shown that inorganic sulphate is taken up into

mast cells which are known to contain a high concentration of heparin and other mucopolysaccharides. (Mast cells are generally located in connective tissue, in the vicinity of capillaries, in the walls of blood vessels and are especially plentiful in liver and lung tissue). Korn (129) using mouse mast cell tumor sliceshas demonstrated incorporation of C^{14}-glucose and S^{35}-sulphate into a mucopolysaccharide which was indistinguishable from heparin. In a later paper (133) this worker presented evidence which indicates that the soluble fraction of mast cell tumor homogenates contains the enzymes necessary to synthesize PAPS from ATP and SO_4^{2-}. Incorporation of sulphate from PAPS into the heparin molecule was also demonstrated. It was not shown however whether all of the SO_4^{2-} groups were added at this stage of polymerization or whether the radioactive sulphate which was incorporated occupied normal sites in the heparin molecule. Using slices of mouse mast cell tumors (185) Korn has demonstrated incorporation of S^{35} sulphate into both amide and ester positions.

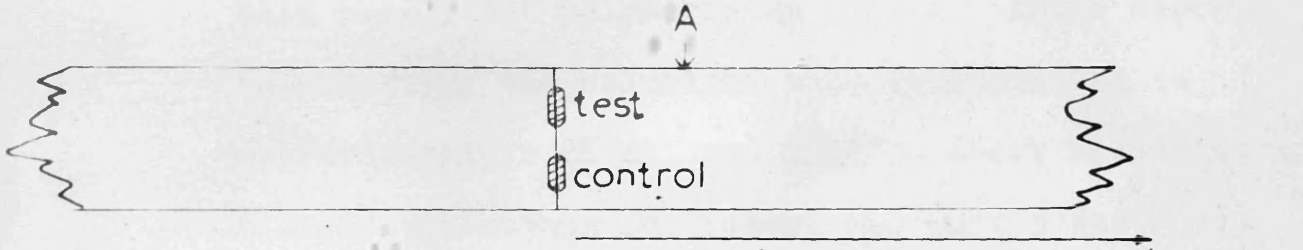
2. Preliminary experiments and attempts to purify the polysaccharide material after incubation.

A thorough study of the mechanism of biosynthesis of heparin offers several interesting prospects. A molecule of heparin is composed of approximately 25 glucosamine residues, 25 glucuron-:ic acid residues and 75 sulphate residues. These are united in a sequence, which has yet to be finally determined, by five known co-valent bonds, Fig. 4, (one glucosaminidic, one glucuronidic, one sulphamide and two O-sulphate ester (12). Thus, many alternate pathways of the biosynthesis of heparin may be envisaged. Two possible mechanisms are: (1) the initial formation of sulphated hexose derivatives which would then be polymerized and (2) the initial synthesis of a non-sulphated muco-:polysaccharide which would then serve as a sulphate acceptor thus forming heparin. However, the possibility also exists that sulphation may occur at some intermediate stage of complexity. It was, therefore, of some considerable interest to invest-

:igate possible transfer of sulphate to the partially desulphated heparin we had succeeded in preparing (Part I Section II). Such an investig-:ation would determine whether this polymeric material was capable of acting as a sulphate acceptor. The main object therefore of this section of the work was to confirm or otherwise the transfer of sulphate from PAPS to desulphated heparin and not an investigation of the enzyme.

Desulphated heparin was incubated with PAPS, KF and rat liver extract (as prepared in Expt. 24) in a phosphate buffer (Expt. 29) at 37° for 3 hours. A control experiment which did not contain any PAPS was also incubated. It was now necessary to determine whether any sulphate had been trans-:ferred to the polymer and this required first the isolation of the polysaccharide in a pure form. Isolation of a polysaccharide by alcohol precipitat-:ion or detergents followed by appropriate washing or dialysis is limited by its requirement for relat-:ively large quantities of the sample and as the

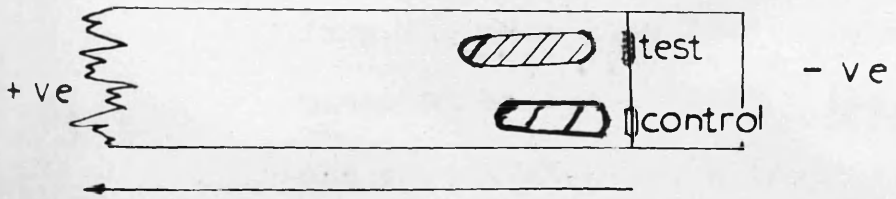
PURIFICATION OF DESULPHATED HEPARIN FROM ENZYME DIGESTS



Paper irrigated along this direction with i butyric acid / 0·5N NH_3 (5 : 3 V/V)

Removes low M.W substances

Paper cut at point A.



Electrophoresis carried out

Polysaccharides migrate in direction of arrow.

FIG. 16

incubation digests contain only 100 µg of poly-:saccharide such a method was not recommendable. However, the paper chromatographic immobility of mucopolysaccharides in several solvents is well known (120) and use was made of this property to help purify the polymeric material. After deprot-:einization, the solutions were concentrated to approximately 0.05 ml. and applied about midway to a strip of Whatman No1 paper, 80 cms x 9 cms., Fig. 16. The paper was then developed by the descend-:ing fashion in the solvent system, isobutyric acid/ 0.5N ammonia (5:3 ^{v}/v) for a period of 48 hours. This procedure removed low molecular weight compounds from the base line leaving the polysaccharide material at the origin. The paper was dried and cut across at point A (approx. 6 - 8 cms. from the starting line) and then submitted to electrophoresis in 0.05 M phosphate buffer pH 7.0, 700 V for 2 hours. The electrophoretogram was dried and stained with Azure A (G.M. 2) to detect the polysaccharides. The rate of movement of a polysaccharide is partly

dependant on the ionizable groups present. A greater movement (towards the anode) of the polysaccharide material in the "test" compared with that in the control, would thus seem to indicate that transfer of sulphate had occured. The results of applying this technique to desulphated heparin are reproduced in Fig. 16 where an increase in mobility was observed. However, it should be mentioned that the polysaccharide material was not detectable as a discrete spot but rather as a streak.

When experiments were carried out (Expt. 30) using (1) a sulphated polysaccharide from the red seaweed Furcellaria fastigitata, (2) a sulphated polysaccharide from the red seaweed Chondrus crispus and (3) desulphated chondroitin sulphate, slight increase in mobility was noted with the Furcellaria polysaccharide but with the other two polysaccharides little or no effect was observed.

The results obtainable using this technique

must be regarded as of qualitative value only. Staining with Azure A is not specific to acidic polysaccharides only. It was observed, for example, that this dye will react with other materials e.g. even a dilute solution of a salt like KCl when spotted on chromatographic paper produces a blue colour (with Azure A) indistin-:guishable from that given by polymeric material. Thus the effectivness of the method used above to purify the polysaccharides was suspect and despite the fact that the chromatograms were given prolonged development in the isobutyric acid/0.5N ammonia solvent (to remove low-molecular weight materials) it should be remembered that a mere trace of impurity remaining at the origin would be sufficient to yield erroneous results on submission of the paper to electrophoresis and subsequent staining with Azure A. Final confirmation of sulphate transfer would require a direct sulphate analysis.

A method was sought whereby the purified polysaccharide material could be recovered for subsequent sulphate determination. Initially it was thought that the polymeric material could be eluted from the chromatograms after developing in the isobutyric acid /0.5N ammonia solvent and then submitted to a further purification procedure. However it was shown (Expt. 31) that the results obtained on the elution of desulphated heparin with H_2O from a strip of Whatman No. 1 paper were unsat-:isfactory. The recovery of polysaccharide varied between 40 and 48%. It is assumed that the polymer was adsorbed on the paper to an extent that did not facilitate its complete recovery by eluting with water.

3. Purification of polysaccharide material by gel filtration using Sephadex 6-25.

Developments in chemical and biochemical

research in the last decade have created an increasing demand for new preparative and analyt-:ical methods. Thus methods such as paper chromatography, thin-layer chromatography, ion exchange chromatography, paper and starch gel-electrophoresis have become widely used techniques.

A new separation method known as gel filtration has been developed from observations of the behaviour of different substances filtered through starch columns (188). The effect obtained was a sort of molecular sieving, whereby molecules of different size could be separated. As a result of these observations a material called Sephadex (Pharmacia, Uppsala, Sweden) has been developed which is highly suitable as a bed material for separation in water solution.

Sephadex is a hydrophilic, insoluble granular material made by cross-linking the poly-:saccharide dextran. Thus, it consists of a three-dimensional network of polysaccharide chains, and

is essentially non-ionic in character as distinct from an anion or cation exchange resin. Sephadex is made with different degrees of cross-linkage which determine the porosity of the network. Thus a high degree of cross-linkage gives a compact structure with low porosity and a low cross-linkage gives a highly porous structure.

Sephadex has a great affinity for water; when placed in an aqueous solution it swells con-:siderably and forms gel grains, the degree of swelling being determined by the porosity of the network. Thus, in contact with water, a gel with a definite cross-linkage will always hold a definite quantity of water.

If Sephadex is allowed to swell in a salt solution, the solute can diffuse freely through the network structure in the gel grains. For solutes with moderately large molecular dimensions the diffusion through the grains will be restricted according to the porosity of the network. Big

INITIAL ATTEMPT TO SEPARATE POLYMERIC FROM LOW MOLECULAR WEIGHT MATERIAL ON SEPHADEX G 25

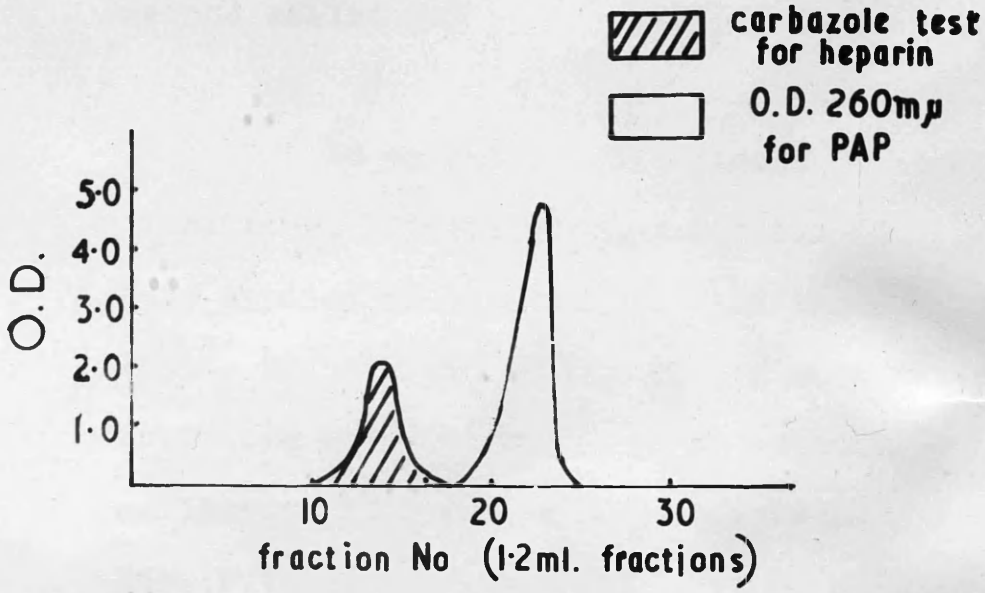


FIG 17

molecules are completely prevented from entering the gel matrix.

This fundamental property of Sephadex to exclude solutes of large molecular size and to be accessible for diffusion to molecules of smaller dimension, forms the basis for the separation method called gel filtration (189).

In an initial experiment to standardize conditions, heparin (1 mg.) and PAP (1.5 mgs.) were applied to a column (2 x 12 cms.) of Sephadex (Expt. 32) and the column eluted with water. Fractions were collected on an automatic fraction collector and analyzed for heparin (carbazole) and PAP (U.V. absorption at 260 mu). Resolution was effected (Fig. 17) but not of as high an order that would permit separation if a greater amount of impurity was present. It was found necessary to ensure that the upper level of the material (i.e. Sephadex) in the column was absolutely horiz-:ontal, otherwise considerable trailing occured on

SEPARATION OF POLYSACCHARIDE FROM A REACTION MIXTURE ON SEPHADEX G 25.

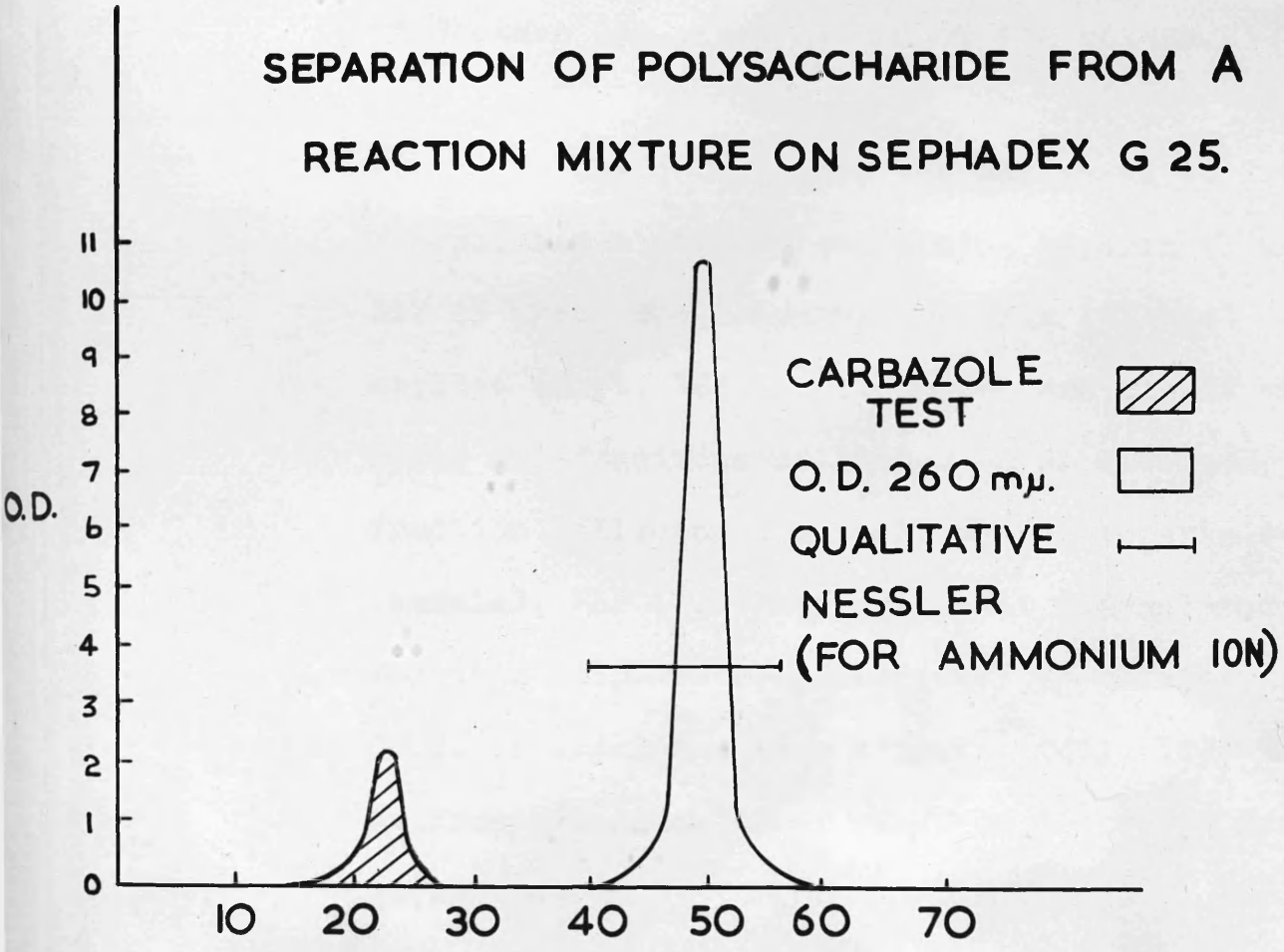


FIG 18

elution which resulted in inadequate separations. This was best overcome by placing a circular disk of Whatman 3MM paper on top of the column.

The column size was increased to 2.2 x 18 cms. and a mixture containing heparin (1 mg.), PAP (3 mgs.) and ammonium sulphate (25 mgs) applied (Expt. 38). The column was eluted with water and fractions collected on an automatic fraction collector and analyzed for heparin (carbazole), PAP (U.V. absorption at 260 mμ) and ammonium sulphate (qualitatively by Nesslerization). Complete resolution was effected (Fig. 18); the polysaccharide material was thus recovered free of impurities.

The excellent separation of solutes using gel filtration is indicated by the result obtained in the above experiment in which the polysaccharide composed only 3.45% (by weight) of the mixture applied to the column. The recovery of polysaccharide material was on average 96-98%. Thus a

method was available whereby the complete recovery of polymer in a purified form from incubation mixtures could be effected.

4. Further experiments on the transfer of sulphate to polysaccharides and subsequent sulphate analysis.

In all experiments reported hereafter the rat liver extracts used was the supernatant from the KCl/EDTA homogenate (Expt. 34). While the initial experiments were performed using similar preparations as used in the preceding section in the transfer of sulphate to p-NP (i.e. the protein fraction precipitating between 1.5 M and 2.3 M ammonium sulphate concentration) it was observed that rather variable activity was obtained with some extracts. As a study of the fractionation of the enzyme was not envisaged at this stage the KCl/EDTA homogenate was therefore used (Expt. 34).

PAPS (2.8μM) was incubated with de-
:sulphated heparin (1 mg.) and rat liver extract (Expt. 35). The polysaccharide was then isolated by gel filtration, the appropriate fractions con-
:taining the polymer were bulked, taken to dryness in a vacuum desiccator and a standard solution prepared by redissolving in water. A quantitative sulphate determination (G.M. 5) was then carried out on a known amount of polysaccharide. The ester sulphate was removed by acid hydrolysis and precipitated as 4- amino - 4'- chlorodiphenyl sulphate (197) G.M. 5. By using a standard sol-
:ution of 4-amino-4'-chlorodiphenyl (CAD) the amount of reagent combining with the sulphate was calculated by difference from the amount remaining inssolution. CAD has a maximum absorption at 254 mμ and by reference to a standard graph the amount of sulphate precipitated was therefore determined. (G.M. 5).

Table 6 shows the result obtained - an

TABLE 6

Transfer of sulphate to desulphated heparin.

	µgs polysacch-:aride used	O.D. 254mµ	Difference Blank — O.D. Reading.	≡ µgs SO_4^{2-}	µgs SO_4^{2-} in 100µgs polysacch-:aride.	SO_4^{2-} increase per 100µg polysacch-:aride.
Test	240	0.82	0.20	38	15.83	2.22 ugs.
Control	235	0.85	0.17	32	13.61	-
Blank	-	1.02	-		-	-

TABLE 7.

Transfer of Sulphate to desulphated heparin.

	µgs.polysaccharide.	O.D. 254mµ	Difference Blank — O.D. Reading.	= µgs SO_4^{2-}	µgs SO_4^{2-} in 100µgs polysaccharide.	SO_4^{2-} increase per 100 µgs polysaccharide.
Test	180	0.840	0.18	34	18.88	5.0 ugs.
Control	180	0.885	0.135	25	13.88	
Blank	-	1.02	-	-	-	

increase of 2.22µg sulphate per 100 µg. polysaccharide.

This experiment was repeated using a fresh liver extract and the incubation period extended to 4 hours (Expt. 36).

The polysaccharide material was isolated as before and a sulphate analysis carried out (Table 7) This extract was shown to be considerably more active than the preceding one, thus a transfer of 5 µg. per 100 µg. of polysaccharide was observed. In a subse-:quent experiment (Expt. 37) carrying a full series of controls, the concentration of donor (PAPS) was increased two-fold. Table 8 shows the composition of the controls which illustrates 1) that desulphated heparin is the sulphate acceptor, 2) that the reaction is catalyzed by the enzyme preparation and 3) that PAPS is the sulphate donor.

The results are shown in Table 9; transfer of sulphate to acceptor was 7.32µgs. per 100 µgs. of polysacchar-:ide thus increasing the sulphate content of the polymer from 13.68% to 21% (Fig. 19). While this

TABLE 8.

Composition of digests.

	Test	Controls		
		1	2	3
desulphated heparin	+	-	+	+
PAPS	+	+	+	-
Enzyme preparation	+	+	A	+
	A = protein denatured at commence-:ment of reaction.			

figure denotes a not inconsiderable increase in sulphate it still falls well short of the sulphate content of natural heparin which is of the order of 36-37%.

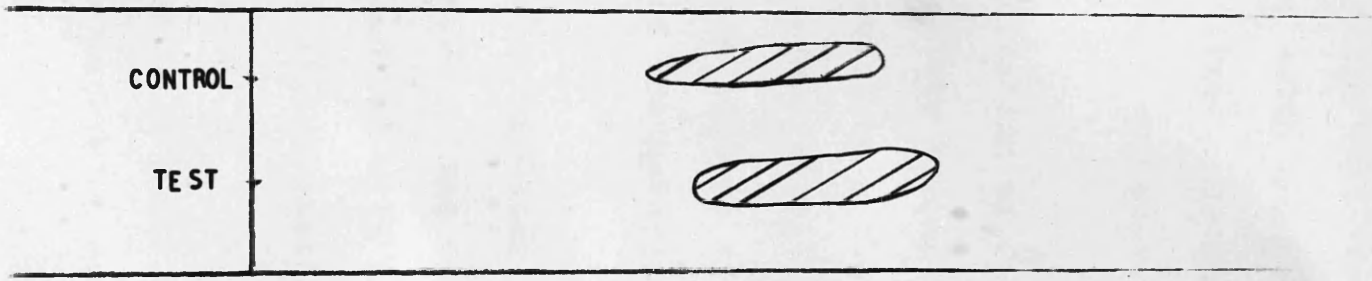
In view of the success of the above experiments using desulphated heparin it was there-:fore of considerable interest to substitute

TABLE 9.

Sulphate transfer to desulphated heparin.

	µgs polysacch-:aride used	O.D. 254mµ	Difference Blank — O.D. Reading. ≡ µgs SO_4^{2-}	µgs SO_4^{2-} per 100ug polysacch-:aride.	SO_4^{2-} increase per 100 µgs polysacch-:aride.
Test	200	0.795	0.225 42	21.0	7.32µgs
Control 1	-	1.01	0.01 2	-	-
2	190	0.88	0.14 26	13.68	-
3	195	0.875	0.145 27	13.8	-
Blank	-	1.02	- -	-	-

PAPER ELECTROPHORESIS, SHOWING THE ENZYMIC TRANSFER OF SULPHATE TO DESULPHATED HEPARIN.



0·05M. ACETATE BUFFER, pH 5·2, 500V/3 HOURS.

FIG. 19

another partially desulphated derivative of heparin e.g. one from which only the N-sulphate groups had been removed (Expt. 22) and to assay this material as a comparative acceptor.

Incubation of de N-sulphated heparin with PAPS and rat liver extract (Expt. 38) showed an increase in sulphate content of 5.89 µgrs per 100µgrs of polysaccharide (Table 10) thus yielding a polymer with a sulphate content of 31.82%. Electrophoresis of a sample of the material is shown in Fig. 20.

While a greater net transfer of sulphate was demonstrated in the case of desulphated heparin (Expt. 37) compared with de N-sulphated heparin (Expt. 38) it is interesting to note that from a comparative point of view and assuming that fully sulphated (natural) heparin contains 36% sulphate, the number of sites available for transfer to (i.e. acceptance of sulphate) in the case of de N-sulphated heparin are obviously considerably less than in the case of desulphated heparin. Thus it

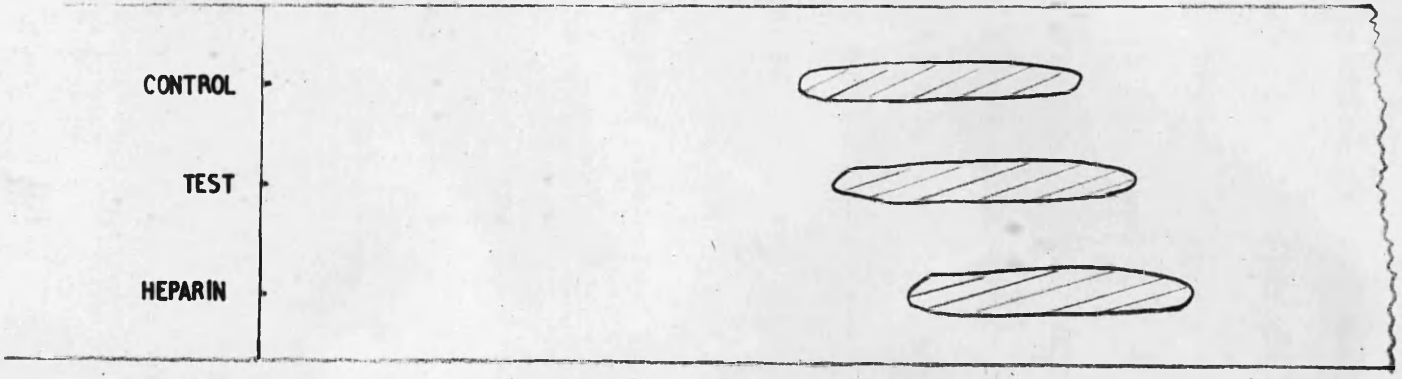
TABLE 10.

Transfer of sulphate to de N-sulphated heparin.

	µgs polysaccharide used.	O.D. 254mµ	Difference Blank — O.D. Reading.	≡ µgs SO_4^{2-}	µgs SO_4^{2-} in 100µgs polysaccharide.	SO_4^{2-} increase per 100µgs polysaccharide.
Test	220	0.64	0.37	70	31.82	5.89 µgs.
Control	216	0.712	0.298	56	25.93	-
Blank	-	1.01	-		-	-

FIG. 20.

PAPER ELECTROPHORESIS, SHOWING THE ENZYMIC TRANSFER OF SULPHATE TO N-DE-SULPHATED HEPARIN & COMPARISON WITH NATURAL HEPARIN.



0.05M PHOSPHATE BUFFER, 700V./2 HOURS.
(pH 7.0)

was shown that of the sites presumably available 58% were sulphated in the case of de N-sulphated heparin (Expt. 38) as compared with 32.7% in the case of desulphated heparin (Expt. 37). The same enzyme extract was used in both experiments. These results suggest different affinities by the enzyme for the two substrates which might be due to one or all of a combination of factors e.g. size and/or shape of the molecules, degree of sulphation and/or distribution of sulphate groups.

Further transfer experiments were carried out using the rat liver homogenate and the following materials as acceptors 1) desulphated chondroitin sulphate (Expt. 39), 2) a polysaccharide from Chon-:drus crispus (Expt. 40), 3) a polysaccharide from Furcellaria fastigitata (Expt. 41) and 4) hyaluronic acid (Expt. 42). The polysaccharides were isolated by gel filtration as before and analyzed for sulphate content (Table 11).

No indication of any sulphate transfer was

TABLE 11.

Attempted transfer of sulphate to some polysaccharides sulphate analysis using CAD.

Polysacch-:aride.		µgs poly-saccharide used	O.D. 254mµ	Difference Blank — O.D. Reading.	=µgs SO_4^{2-}	µgs SO_4^{2-} in 100µgs polysacch-:aride.	SO_4^{2-} increase per 100 µgs. polysacch-:aride.
desulphated chondroitin sulphate.	Test	850	0.850	0.135	25	2.94	1.32ugs.
	Control	800	0.915	0.07	13	1.62	
Chondrus crispus	Test	200	0.735	0.25	47.5	23.75	-
	Control	220	0.710	0.275	53	24	
Furcellaria fastigitata	Test	245	0.790	0.195	37	15.10	2.18ugs.
	Control	240	0.820	0.165	31	12.92	
Hyaluronic acid	Test	500	0.969	0.016	3.0	0.60	-
	Control	500	0.970	0.015	3.0	0.60	
	Blank	-	0.985	-	-		

TABLE 12.

Relative Activity of Enzyme Extracts towards different polysaccharides.

Polysaccharide	µgrs SO_4^{2-} transferred per 100µgrs polysaccharide.	Relative Activity.
desulphated heparin	7.3	100
de N-sulphated heparin	5.89	79
F.fastigitata polysaccharide	2.9	40
desulphated chondroitin sulphate	1.6	22
C.crispus polysaccharide	-	0
hyaluronic acid	-	0

discernable with the C. crispus and hyaluronic acid polysaccharides while a net gain of 1.32µgs of sul-:phate per 100 µgs desulphated chondroitin sulphate and 2.18µgs per 100 µgs of F. fastigitata was observed. While the amount of sulphate transferred in both instances is comparatively small the exper-:iment was repeated several times with the same general results. The amount transferred varied with the particular extract and from a series of six experiments, one of which failed to indicate any transfer in the case of F. fastigitata polysaccharide, the sulphate transfer in the other five ranged from 1.8 to 2.9 µgs (per 100 µgs polysaccharide). Desul-:phated chondroitin sulphate was active in all six extracts and the range of transfer varied between 1.0 µgs and 1.6 µgs (per 100 µgs polysaccharide). Thus, these results indicate a much lower activity with these two polysaccharides than with desulphated heparin. The activity of the enzyme preparation towards the differ-:ent polysaccharides assayed in this system is shown in Table 12. The activity towards de-sulphated heparin was taken

as 100 and using the highest sulphate transfer obtain-:ed with the other polysaccharides the relative activ-:ities were thus calculated.

A purification of the enzyme extract used in the series of experiments described above should undoubtedly reveal further characteristics of the system. Thus, a fractionation procedure might indicate several enzymes with specificity towards individual polysaccharides or alternatively a non-specific sulphuryltransferase. Fractionation of the extract should also yield preparations with greater affinity towards desulphated chondroitin sulphate and towards the sulphated polysaccharide from F.fastigit-:ata. Transfer of sulphate by the rat enzyme system from PAPS to the latter material is undoubtedly of special interest and further investigation should add greater detail to this observation. A much higher transfer of sulphate might be obtained for example if the polysaccharide was chemically desulphated and then investigated as an acceptor.

EXPERIMENTAL SECTION.

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15. Analysis of A 2'(3'):5' DP.

16. Separation of A 2':5'DP and A 3':5'DP by ion exchange-chromatography.

17. Preparation of Adenosine 3'phosphate 5'sulphatophosphate (PAPS).

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18. Preparation of potassium chondroitin sulphate.

19. Desulphation of potassium chondroitin sulphate.

20. Purification of Heparin.

21. Desulphation of heparin.

22. De-N-sulphation of heparin.

23. Isolation and Purification of Hyalur: :onic Acid.

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39. Enzymic transfer of sulphate to desulphated chondroitin sulphate.

40. Attempted transfer of sulphate to a polysaccharide from Chondrus crispus.

Expt. 41. Enzymic transfer of sulphate to a polysaccharide from Furcellaria fastigitata.

42. Attempted Enzymic transfer of sulphate to hyaluronic acid.

General Methods.

G.M. 1. The determination of phosphorus as inorganic phosphate.

2. Chromatography and electrophoresis on paper; detection of compounds on paper.

3. Preparation of pyridine sulphurtrioxide.

4. Preparation of methanolic-HCl.

5. Sulphate Analysis.

6. Colorimetric analysis of polysaccharides.

7. Purification of charcoal for adsorption chromatography of nucleotides.

8. Preparation of p-nitrophenyl sulphate (p-NPS).

9. Preparation of 3' Nucleotidase from rye-grass.

10. Estimation of Protein (Biuret Method).

11. Determination of ammonia by Nessler's reagent.

PART I. SECTION I.

EXPERIMENT I.

Preparation of dibenzyl phosphite.

A solution of 261 ml. of freshly distilled phosphorus trichloride (B.P. 75-76°) in dry benzene (2.25 l.) was cooled to 0° and a mixture of benzyl alcohol (616 ml.) and diethylaniline (960 ml.) previously cooled to 0° was added dropwise with continuous stirring. The addition was carried out at such a rate that the reaction temperature did not rise above 5° and with efficient cooling using an ice-salt mixture was complete in approximately 2½ hours. Stirring was continued for a further 1½ hours and then water (1 L.) was added and the phases allowed to separate. The organic phase was washed successively with water (3 x 1 L. aliquots), 3 N ammonia (3 x 1 L. aliquots) and again with water (3 x 1 L. amounts) and then dried with sodium sulphate overnight. The solution was filtered on a Buchner funnel and the filtrate concentrated under reduced pressure on a boiling water bath until all the benzyl chloride was

removed. The residue set to a white solid mass after being stored in the cold (-14°) for 5 - 7 days. Yield: 232 grs.

Dibenzyl phosphite was further purified as follows: gaseous ammonia was passed into the crude material (150 grs) for five minutes. The impurities which separated out were removed by fil-:tration. N-methylmorpholine (5% by weight i.e. $3\frac{1}{2}$ grs.) was added to the filtrate which was dis-:tilled immediately in an atmosphere of nitrogen at a pressure of 10^{-3}mm. on an oil bath, (bath temper-:ature range $200-210^{\circ}$; temperature range inside distilling flask 175° - 185°). The first 3 - 5 ml. were rejected and the remainder collected until most of the material had distilled or until the material in the distilling flask showed signs of decomposition (a sudden darkening in colour) - in which case the system was immediately flooded with nitrogen, the oil bath removed and the vacuum system turned off. Yield of dibenzyl phosphite - 32 grs. M.P. $18-19^{\circ}$.

EXPERIMENT 2.

Preparation of dibenzylphosphorochloridate.

Dibenzylphosphite (26 grs.) was dissolved in dry benzene (400 ml.) and N-chlorosuccinimide (13.6 grs.) added and the mixture shaken until the latter had dissolved. After 2 hours the succinimide (C.9.8 grs. dry weight) which had separated out was removed by filtration. Benzene was removed by distillation under reduced pressure on a water bath (temperature 25-28°) and the resultant oil was used directly in phosphorylation reactions.

EXPERIMENT 3.

Attempt to phosphorylate adenosine.

Adenosine (5.0 grs. L. Light & Co.) dried at 110°/1 mm. for 60 hours was dissolved in boiling anhydrous pyridine (300 ml.) and then cooled to -30°

before adding to dibenzylphosphorochloridate (from 26 grs. of dibenzylphosphite). The reaction mixture was kept at -30° for 30 minutes and then allowed to rise to 0° and maintained at this tem-:perature (±2°) for 18 hours. 2-ethoxyethanol (13 ml.) was added to the mixture and the small amount of pyridine-hydrochloride which had separated after 30 minutes was filtered off and the filtrate concentrated in vacuo on a water-bath (temperature 30°) to an oil. After addition of a further 100 ml. of 2-ethoxyethanol and again concentrating, the syrup was dissolved in 2-ethoxyethanol (100 ml.) containing anhydrous lithium chloride (12.0 grs.) and the solution heated at 100° for 3 hours with exclusion of moisture.

Addition of ether (500 ml.) to the cooled solution (0°) precipitated a gum which solidified when shaken with ethanolic-acetone ((1:3 v/v) 400ml.). The material was centrifuged and the solid washed thrice with further ethanolic-acetone (100 ml. portions) and dried to give a light cream coloured

powder I in a yield of 4.00grs. The yield at this stage is only 32% of that quoted.

Concentration of the original ethanolic-acetone solution above - used in conversion of the gum to a solid - gave a gummy material which was redissolved in 2-ethoxyethanol (100 ml.) containing lithium chloride (12.0 grs.) and heated at 100° for 3 hours and then cooled and treated with ether (500 ml.) followed by ethanolic-acetone as before to give 3.5 grs. of product II. The ethanolic-acetone supernatant from above was again concentrated and worked up as above to give 1½ grs. of material III. The three precipitates were combined to give product A (9.0 grs.).

The ethanolic-acetone supernatant after the third attempted debenzylation above still yielded a gum B on concentrating in vacuo. Submission of a small portion of this gum to paper electrophoresis (0.1 M ammonium acetate buffer pH 6.0, 600V/20mA/1hr.) indicated an ultra violet absorbing material which

moved slightly (C.2.5 cms.) towards the cathode as might be expected of the fully tetrabenzylated derivative of adenosine i.e. adenosine 2'(3') dibenzylphosphate-5' dibenzylphosphate.

The combined precipitates (A above) were treated with N-NaOH (200 ml.) at room temperature to dispose of any cyclic phosphates. After 40 hours the solution was neutralized to pH7 with dilute HCl, extracted with ether (2 x 50 ml. aliquots) and con-:centrated in vacuo to approximately 150 ml. The solution was passed through a column (20 x 5 cms.) of Dowex -50 (x4), lithium form resin which was washed with water (500 ml.) and the eluate and wash-:ings concentrated in vacuo. The residual syrup was shaken with ethanolic-acetone (1:4 v/v, 500 ml.) and the resulting solid collected by centrifugation and dried to give a white powder (4.1 grs.). This material was dissolved in water (150 ml.) containing glacial acetic acid (2.0 ml.); palladium chloride (100 mgs.) was added and the whole hydrogenated. On completion of the uptake of hydrogen (380 ml. in 4½

hours) Fig. 6, the catalyst was removed by fil-:tration and the filtrate shown to contain 0.285 grs. of nucleotide material (based on U.V. absorpt-:ion at 260 mμ and calculated as adenosine 2'(3'):5 diphosphate). The filtrate was adjusted to pH 8.0 with ammonia, diluted to 500 ml. with distilled water and applied to a column (20 x 2 cms.) of Dowex -2 (x8). 200-400 mesh, chloride form resin. The column was washed with water (300 ml.) and then eluted with 0.003 N HCl to remove monophosphates (0.1 gr.); 25 ml. fractions were collected and the optical density of each fraction at 260 mμ was read on a Unicam spectrophotometer. Diphosphates (0.08 gr.) were then eluted with 0.03N HCl.

EXPERIMENT 4.

Attempt to monodebenzylate adenosine derivative using potassium acetate.

To a solution of potassium acetate (anhydrous 2.0grs.) dissolved in 2-ethoxyethanol

was added a portion (1.5 grs.) of the gum (B) adenosine 2'(3') dibenzylphosphate-5' dibenzyl-:phosphate obtained in the previous experiment and the whole heated at 100° for three hours with exclusion of moisture.

Analysis by paper electrophoresis (0.1 M ammonium acetate buffer pH 6.0, 600V/20mA/1hr.) failed to indicate that any of the material had been debenzylated.

EXPERIMENT 5.

Attempted monodebenzylation using N-methylmorpholine.

Adenosine 2'(3') dibenzylphosphate -5' dibenzylphosphate (1.5 grs.) was heated in freshly distilled N-methylmorpholine (30 ml.) in a stoppered flask at 100° for 1 hour. Solvent was removed by evaporation under reduced pressure and a portion of the residue dissolved in water and an aliquot

submitted to paper electrophoresis. (as described in Expt. 4).

EXPERIMENT 6.

An attempt to hydrogenate the tetrabenzylated phosphate derivative of adenosine.

Adenosine 2'(3'): dibenzylphosphate -5' dibenzylphosphate (1.5 grs.) was dissolved in distilled water (30 ml.) containing 0.5 ml. of glacial acetic acid. Palladium chloride (20 mgs.) was added and the flask attached to the hydrogenation apparatus. The material failed to absorb any hydrogen.

EXPERIMENT 7.

Preparation of 2-cyanoethyl phosphate.

Freshly distilled phosphorus oxychloride (18.4 ml. B.P. 105^{o}) was mixed with anhydrous ether

(200 ml.) in a three necked flask and cooled to -15^{o}. A mixture of anhydrous pyridine (16.1 ml.) and hydracrylonitrile (14.2 grs.), previously cooled to -15^{o} was added dropwise with vigorous stirring, care being taken to exclude moisture from the reaction mixture. The addition took about 1 hour to complete and the temperature in the flask was maintained below -10^{o}. The mixture was then stirred for an additional hour at -10^{o}. Pyridinehydrochloride precipitated from the commencement of the addition of pyridine and hydracrylonitrile but no effort was made to remove it.

The contents of the reaction flask were poured with stirring into a mixture of water (750 ml.) pyridine (80 ml.) and ice (300 grs.) and then a solution of 100 grs. of barium acetate in water (300 ml.) was added. After two hours the barium phosphate which had precipitated was filtered on a Buchner funnel. To the clear filtrate 2 volumes of 95% ethanol were added slowly with stirring. Gleaming platelettes of the barium salt separated; these were collected by centrifugation after standing 1 hour at

0^{o}. The crystals were washed successively with 50% ethanol, 95% ethanol and then dried in a vacuum desiccator.

A standard solution containing 1 m.mole/ml. was prepared by dissolving the dried barium salt (16.1 grs.) in water (50 ml.) to which Dowex-50 (H^{+}) was added to aid solution. The material was applied to a column of Dowex -50 (H^{+}) resin (8cms. x 4) and washed with distilled water to pH 7. Effluent and washings were combined and pyridine (20 ml.) added and the solution concentrated in vacuo on a water-bath (temperature 30^{o}) to 20 ml. (approx.). This solution was transferred to a graduated flask (50 ml.) and diluted to the mark with pyridine.

EXPERIMENT 6.

Initial attempt to phosphorylate adenosine using 2-cyanoethylphosphate.

Adenosine (1 m.mole, 267 mgs.) dried at

110°/1 mm. for 60 hours, was dissolved in boiling anhydrous pyridine and then cooled to room temper-:ature. CEP (3 m.moles) was added and the mixture concentrated in vacuo on a water bath (temperature 25°) to an oil. A further 15 ml. of pyridine was added and the mixture again concentrated. This procedure was repeated three times. Finally the oil was dissolved in anhydrous pyridine (15 ml.) and dicyclohexylcarbodiimide (DCC, 2.0 grs.) added. The mixture was shaken vigorously in a stoppered flask and allowed to stand at room temperature. After a few minutes dicyclohexylurea (DCU) began to settle out as a flocculent precipitate. Aliquots (0.1 ml.) of the mixture were removed after set intervals (e.g. 1, 4, 8, 12, 24 hours) to study the progress of the reaction. Each aliquot was treated with water (0.1 ml.) and concentrated ammonia (0.2 ml.) added and the solution heated at 60° for 1 hour. The supernatant, after centrifugation, was submitted to paper electrophoresis (M/50 phosphate buffer pH 7.5 600 V/1 hour).

After 48 hours water (15 ml.) was added to the reaction mixture and after 30 minutes DCU was filtered on a Buchner funnel. Concentrated ammonia (30 ml.) was added to the filtrate and the solution heated at 60° for 1 hour and concentrated to dryness in vacuo on a water bath at 35°. Water (25 ml.) was added to the white solid thus obtained and after vigorous shaking any remaining DCU was filtered off and the precipitate washed with a further 25 ml. water. The filtrate and washings were combined and the solution (containing 259 mgs. of nucleotide material) concentrated in vacuo to 10 ml. (bath temperature 30°) and then freeze dried to give a white solid powder. This material was exam-:ined by paper electrophoresis and paper chromatog-:raphy, using the following controls; Adenosine, A 3'P, A 5'P and A DP. Paper electrophoresis (M/50 phosphate buffer, pH 7.5 600 V/1 hr.) showed a major component (Rf 8.6 cms.) comparable with A 5'P (Rf 8.7 cms.) and two minor components, (1) Rf 12.8 cms. as might be expected of a diphosphate and (2) some material which remained on the starting point and

hence was likely thought to be unchanged adenosine.

Ascending paper chromatography in the following systems (1) n-propylalcohol: ammonia: water (6:3:1 $^{v}/v$) and (2) saturated ammonium sulphate: 0.1 M ammonium acetate: isopropylalcohol (79:19:2 $^{v}/v$) using the same controls as above again indicated a monophosphate as the major component.

EXPERIMENT 9.

Investigation of the alkaline lability of adenosine 3'-phosphate and adenosine 5'-phosphate.

The respective nucleotides (10 mgs.) were dissolved in water (1.0 ml.) and concentrated ammonia (1.0 ml.) added to each. The solutions were heated in a water bath at 60^{o} for 1 hour. Samples were examined by paper electrophoresis, paper chromatog-:raphy (as used in Expt. 8) and for release of inor-:ganic phosphate (G.M.1). There was no evidence to suggest any hydrolysis of phosphate in either

A 3'P or A 5'P.

EXPERIMENT 10.

The effect of increasing the ratio of phosphorylating agent (CEP) to adenosine.

Adenosine (4 m.moles, 1.068 grs.), dried 110°/1mm. for 60 hours was dissolved in boiling an-:hydrous pyridine (60 ml.) and then cooled to room temperature. CEP (16 m. moles) was added and the solution concentrated in vacuo, followed by further addition of pyridine and concentrating as described in Expt. 8. Finally, anhydrous pyridine (60 ml.) and DCC (13.056 grs., 64 m.moles) were added and the mixture allowed to stand at room temperature. Ali-:quots were withdrawn at set invervals and again examined by paper electrophoresis (as Expt. 8). After 24 hours the major product corresponded to a diphos-:phate. Water (60 ml.) was added to the reaction mixture; the solution filtered after 30 minutes and the filtrate treated with one volume of concentrated

ammonia at 60° for one hour. The solution was then concentrated to dryness in vacuo on a water bath (35°).

Water (60 ml.) was added to the white solid and the whole shaken vigorously. DCU was filtered on a Buchner funnel and the precipitate washed with a further 20 ml. water. The filtrate and washings were combined and diluted to 800 ml. with water, adjusted to pH 8.0 with N/100 ammonia and applied to a column (3.2 x 16 cms.) of Dowex-2 (x8).chloride form, resin. The column was washed with water (1 L.) Monophosphates were eluted with 0.003 N HCl; 100 ml. fractions were collected and each fraction read at 260 mμ on a Unicam spectro-:photometer. Diphosphates were eluted with 0.03 N HCl (Fig. 7).

EXPERIMENT 11.

The effect of increasing the temperature on the yield of adenosine 2'(3'):5'diphosphate.

Adenosine (4 m.moles) was phosphorylated

at 37° (all other details as Expt. 10) and after 26 hours the solution was worked up as previously described, applied to a column of Dowex -2 (x8) chloride form resin, 3.2 x 16 cms. and the nucleo-:tides eluted (Fig. 8).

EXPERIMENT 12.

Isolation of nucleotides as the calcium salt.

The appropriate fractions (Fractions 52-89, Peak II Expt. 10) were bulked and neutralized to pH 7.0 with a suspension of calcium hydroxide and then concentrated in vacuo on a rotary evapor-:ator (temperature 35°) to a small volume (50 ml.) and then freeze dried. The white solid was then washed with ethanolic-ether (1:1 v/v, 50 ml. port:-:ions) until the supernatant was free of chloride ions and then finally with 3 x 50 ml. aliquots of anhydrous ether. The solid was collected by centrifugation and air dried in vacuo to yield 1.7

grs. of product. The monophosphate was isolated in a similar manner (0.52 gr.).

EXPERIMENT 13.

Purification of nucleotide material by charcoal chromatography.

700 mgs. of the calcium salt of A 2' (3'):5'DP was dissolved in 200 ml. of 0.01 N HCl and applied to a column (6 x 3.5 cms.) of activated charcoal (see G.M. 7). The column was washed with water to pH 7 and then with 400 ml. of 0.01 N sodium bicarbonate and finally the nucleotide material was eluted with 50% aqueous ethanol containing 2% ammonia. The eluate containing the nucleotide material was concentrated to dryness on a rotary evaporator (35°). The solid was washed with ethan-:olic-ether (1:4 v/v), 3 x 100 ml. portions, then with ether, 3 x 50 ml. portions and air dried in vacuo to yield an off-white coloured powder (252 mgs.). The purity of this product was estimated by U.V. absorpt-:ion at 260 mμ, and shown to be 97%.

EXPERIMENT 14.

Isolation of A 2'(3'):5'DP as the lithium salt.

Fractions 79-120, Peak II, Expt. 11 were bulked and adjusted to pH 8.0 with ammonia. The volume was diluted three times with water and the solution applied to a column (2 x 3.2 cms.) of Dowex -2 (x8), chloride form, resin. The column was washed with water (1 L.) and then eluted with 0.06 N HCl. Fractions (100 ml.) were collected and read on a Unicam spectrophotometer at 260 mu. The appropriate fractions were bulked, adjusted to pH 6.5 with a solution of lithium hydroxide and the solution concentrated in vacuo (35^{o}) to dryness. The solid was stirred with 150 ml. methanolic-acetone (1:5 $^{v}/_{v}$); the nucleotide material was collected by centrifugation and washed repeatedly with methanolic-acetone until the supernatant was free of chloride ions and air dried in vacuo to give a white solid (0.84 gr., 44% yield based on adenosine). The purity of this material, estimated by U.V. absorption at

260 mμ., was 98%.A 5'-P (0.6 gr.) was isolated in a similar manner.

EXPERIMENT 15.

Analysis of A 2'(3'): 5'DP.

(a) Ultra violet spectrum.

A 2'(3') 5 P was converted to the free acid by passing a solution of the calcium salt through a column of Dowex -50 (x4), hydrogen form resin and diluting to a standard volume (25 ml.). 1 ml. of this solution was diluted to 25 ml. with N/10 HCl and the ultraviolet spectrum recorded (Unicam spectrophotometer) in the region 220-290 mμ. (Fig. 10).

(b) Phosphorus analysis.

1) Phosphorus analysis (G.M. 1) on 0.58 mgs of the

free acid of A 2'(3'):5'DP as estimated by U.V. absorption showed a total phosphorus content of 80 µgrs. (theoretical 81 µgrs) thus indicating an adenine to phosphorus ratio of 1:1.97.

2) Hydrolysis of 0.58 mgs of the free acid of A 2'(3'):5'DP in 1N HCl for 30 minutes at 100° showed a release of 39 µgrs of phosphorus (48.1% of the total phosphorus present).

3) Hydrolysis of A 2'(3,):5'DP (0.58 mgs of the free acid) using 3' nucleotidase (G.M.9) showed a release of 39.7 µgrs of phosphorus (49% of the easily hydrolyzable phosphorus (i.e. the phosphor-:us released by IN HCl) or 24.5% of the total phosphorus present). This demonstrates that the two isomers are present in approximately equal amounts.

(c) Paper electrophoresis and paper chromat-:ography of A 2'(3'):5'DP.

A 2'(3'):5'DP was examined by paper elec-

:trophoresis and paper chromatography (G.M. 2). The results are shown in Table 2.

EXPERIMENT 16.

Separation of A 2':5'DP and A 3':5'DP by ion-exchange chromatography.

The lithium salt of A 2'(3'):5'DP (26 mgs) was dissolved in water (250 ml.) and applied to a column (1 x 65 cms.) of Dowex -1 (x2), chloride form resin. The column was washed with water (200 ml.). Gradient elution was then carried out using 0.03 M $CaCl_2$ and 0.0045 N HCl (5 L.) in the mixing chamber and 0.07 M $CaCl_2$ and 0.007 N HCl (2 L.) in the reservoir. Fractions (25 ml.) were collected at an elution rate of 1 ml./2½ mins. and the optical density of each fraction read on a Unicam spectrophotometer at 260 mμ. Two peaks were observed; Peak I corres-:ponding to A 2':5' DP and Peak II corresponding to A 3':5' DP.

The appropriate fractions were bulked and applied to a column (2 x 6 cms.) of activated char-:coal which was then washed with water to pH 7.0. Nucleotide material was eluted with 50% aqueous ethanol containing 2% ammonia. The eluate was con-:centrated to a small volume (10 ml.) and passed through a column (1 x 4 cms.) of Dowex -50 (lithium form) resin. The column was washed with a further 20 ml. of water and the eluate and washings combined and freeze dried to give an off-white powder. Recovery of A 2':5'DP, 10 mgs. and A 3':5'DP, 11 mgs.

Digestion of A3':5'DP with 3'nucleotidase (G.M. 9) released 96% of the 3'-phosphate. A 2':5'DP was inactive toward this enzyme.

EXPERIMENT 17.

Preparation of Adenosine 3' phosphate 5' sulphatophosphate (PAPS).

Pyridine-sulphur trioxide (G.M. 3) 88 mgs.

was added with stirring to a solution of the lithium salt of A 3':5' DP (23 mgs.) and sodium bicarbonate (132 mgs.) in water (1.3 ml.). The reaction was carried out at 45°. After 40 minutes the solution was cooled and diluted with ice-water (50 ml.), adjusted to pH 6.0 with N formic acid and passed through a column (2 x 8 cms.) of Norit A Charcoal-celite 535 (4:3 by weight). The column was washed with water (60 ml.) and the nucleotides eluted with 50% aqueous ethanol containing 2% ammonia. The eluate was applied to a column (2 x 8 cms.) of Dowex -1 (x2) chloride form, resin which was then washed with water (200 ml.). Gradient elution was carried out using 1.95 N lithium chloride (666 ml.) as eluent in the reservoir and water (1 L.) in the mixing flask. Fractions (25 ml.) were collected and the optical density of each fraction read on a Unicam spectrophotometer at 260 mμ. The fractions containing APS were bulked and readsorbed on a column (1 x 4 cms.) of Norit A charcoal-celite 535 (4:3 by weight) as described above. The ethanolic-ammonia eluate was concentrated in vacuo on a water

bath (32-34°). The concentrate (5 ml.) was passed through a column (1 x 1.5 cms.) of Dowex-50, lithium form, resin, and the column washed with a further 5 ml. water. The eluate and washings were combined and freeze dried to yield a light brown coloured solid (2.4 mgs.) which was stored at -14°.

The fractions containing A 3':5'DP were bulked and isolated in a similar manner. Recovery 17 mgs.

PART I. SECTION II.

EXPERIMENT 18.

Preparation of potassium chondroitin sulphate.

Chondroitin sulphate (3.0 grs. L. Light & Co.) was dissolved in water (50 ml.) and applied to a column (3.4 x 10 cms.) of Dowex -50, H^+ form, resin. The column was washed with water to pH 7.0, the eluate and washings were combined and adjusted to pH 7.0 with a solution of N/10 KOH. The solution was concentrated in vacuo to 40 ml. on a rotary evaporator. Addition of ethanol (2 volumes) yielded a white precipitate of the potassium salt which was recovered by centrifugation, washed with 95% ethanol (2 x 30 ml. aliquots) and air dried to give 2.1 grs. of product.

EXPERIMENT 19.

Desulphation of potassium chondroitin sulphate.

Finely powdered, well dried potassium

chondroitin sulphate (1.25 grs.), SO_4^{2-} content 15.3% (G.M. 5) was shaken with 0.063 N acid methanol (210 ml., G.M. 4) for 24 hours at room temperature The mixture was centrifuged and the insoluble residue was shaken for two further days with fresh methanolic -HCl added at the end of each 24 hour period.

The insoluble residue was dissolved in water (20 ml.) and dialyzed against cold running water for 24 hours. The product was precipitated by the addition of ethanol (200 ml.), separated by centrifugation, washed with ethanol (2 x 20 ml. aliquots) and ether (2 x 20 ml. aliquots) and air dried in vacuo. (0.712 grs. of the methyl ester of desulphated chondroitin sulphate).

The methyl ester (500 mgs.) was treated with 0.1 N NaOH at room temperature for 48 hours. The solution was acidified and dialyzed against cold distilled water, adjusted to pH 7.0 with N/10 NaOH and the sodium salt of desulphated chondroitin

sulphate precipitated by the addition of five volumes of ethanol. The solid was collected by centrifugation, washed with ethanol (3 x 20 ml. aliquots) and ether (3 x 20 ml. aliquots) and air dried in vacuo to yield 0.455 gr. of a white powder (SO_4^{2-} content 1.5%, G.M. 5).

EXPERIMENT 20.

Purification of Heparin (168).

Heparin (L.Light & Co., 2.0 grs.) was dissolved in distilled water (80 ml.). Barium acetate (26.5 ml. of a 5% solution) was added and the whole allowed to stand at room temperature for 3 days. The solution was clarified by centrifug-:ation, 20,000 xg. The supernatant was heated to 65° and glacial acetic acid (20 ml.) added and the solution allowed to cool to room temperature. The crystalline material was removed by centrifugation and washed successively with 80% acetic acid, glacial

acetic acid, 95% ethanol and finally ether before air drying to yield 1.62 grs. of a white solid. The barium salt of heparin (1.0 gr.) was dissolved in water (50 ml.) and passed through a column (3.2 x 6 cms.) of Amberlite IR 120, H^+ form, resin and the column washed with water to pH 7.0. The eluate and washings were combined and adjusted to pH 7.0 with 0.1N KOH. The solution was concentrat-:ed to 40 ml. on a rotary evaporator. Addition of ethanol (6 volumes) precipitated potassium heparin-:ate which was collected by centrifugation after standing at $0-2^0$ for 14 hours. The product was washed with ethanol (3 x 20 ml. aliquots) and ether (3 x 20 ml. aliquots) and air dried to give a white powder, 0.606 gr. (SO_4^{2-} content 36.5%).

EXPERIMENT 21.

Desulphation of heparin.

Finely powdered, well dried (in vacuo

over P_2O_5, 24 hours) potassium heparinate (1.0gr.) was shaken, for 24 hours at room temperature with methanolic-HCl (160 ml.). The mixture was cen-:trifuged and the residue was shaken for three further 24 hour periods with fresh methanolic-HCl added each day. A small portion of the insoluble polysaccharide was removed, for subsequent sulphate analysis, at the end of each 24 hour period.

After the fourth day the mixture was centrifuged and the insoluble material washed several times with methanol and then dissolved in water (25 ml.). The polysaccharide was precipit-:ated by the addition of ethanol (120 ml.). The precipitate was collected by centrifugation, washed with ethanol (2 x 30 ml. aliquots), ether (2 x 30 ml. aliquots) and finally air dried in vacuo.

EXPERIMENT 22.

De-N-sulphation of heparin (186).

Sodium heparinate (0.5 gr.) was diss-

:olved in 0.04 N HCl and the solution heated on a boiling water bath for 3 hours and then dialyzed against repeated changes of distilled water for 24 hours. The pH was adjusted to 7.0 with N/100 NaOH and the de-N-sulphated polysaccharide precip-:itated by the addition of 95% ethanol (3 volumes). The precipitate was recovered by centrifugation, washed with ethanol, ether and dried to give 0.29 grs. of product, SO_4^{2-} content 27%. (G.M. 5).

EXPERIMENT 23.

Isolation and Purification of Hyaluronic Acid.

Pigskin was obtained fresh, from the slaughter house, cooled to 0°. The skin was scraped as free of fat as possible, cut into small sections and passed twice through a power driven meat grinder. The ground skin (1.1 Kg.) was sus-:pended in water (2 L.) and acidified to pH 1.5 with dilute HCl. Pepsin (3.5 grs., B.D.H.) was

stirred in; the suspension covered with a layer of toluene and incubated at 37° for 40 hours with occasional stirring. Most of the toluene was removed and the pH adjusted to 7.5 with 5 N NaOH. Trypsin (3.0 grs., B.D.H.) and a fresh layer of toluene were added and the mixture incubated at 37° for 36 hours. Most of the toluene was removed and the material filtered through cheese cloth and then glass wool. To the cooled fil-:trate, calcium acetate was added to 2.5% and acetic acid to 0.25 N followed by 1.25 volumes of 95% ethanol. The mixture was kept at 0-4° for 14 hours and then centrifuged.

The precipitate was washed several times with ethanol (95%) and suspended in 200 ml. of 5% sodium acetate: 0.5 N acetic acid solution. The solution was shaken successively with 3 x 80 ml. aliquots of chloroform: n-butanol solution (9:1 v/v). The aqueous supernatant was then treated with 1.6 volumes of ethanol. The precipitate was collected by centrifugation and suspended in 180ml.

of 5% sodium acetate: 0.5 N acetic acid solution which was clarified by centrifugation. The crude polysaccharide mixture was again precipit-:ated by the addition of ethanol (1.6 volumes), dissolved in water (150 ml.), stirred with 30 ml. Amberlite IR-120 (H^+) form, resin and filtered. Filtrate and washings were combined and made to 5% with respect to calcium acetate, 0.5 N with respect to acetic acid and the polysaccharide material precipitated with an equal volume of 95% ethanol. After standing at 0-4° for 14 hours the precipitate was collected by centrifugation and washed with 95% ethanol (3 x 50 ml. aliquots) anhydrous ether (3 x 50 ml. aliquots) and air dried to yield a white powder (1.259 grs.)

The precipitate was dissolved in water (95 ml.), centrifuged at 17,000 x g for 1 hour. To the supernatant calcium acetate was added to 5% and acetic acid to 0.5 N followed by ethanol (27 ml.). The precipitate was collected by centrifugation after 24 hours at 0-4° and washed

with ethanol (3 x 25 ml.) and ether (3 x 25 ml. aliquots) and air dried to give a white powder (0.36 gr.). Analysis showed a sulphate content of 16.4% and an I.R. spectrum similar to that quoted for chondroitin sulphate B with peaks at 928 cm^{-1}, 852 cm^{-1} and 725 cm^{-1}.

The concentration of ethanol in the original supernatant (above) was raised from 21% to 30% by the addition of ethanol (16 ml.) and the resulting precipitate of hyaluronic acid collected by centrifugation after 24 hours at 0-4°. The solid was dissolved in calcium acetate - acetic acid solution (100 ml.) and reprecipitated at 28% ethanol saturation after 24 hours at 0°. After a further final reprecipitation the solid was washed with ethanol (3 x 50 ml. aliquots) and air dried to yield a white powder (0.32 gr.). Analysis (G.M. 5) showed a sulphate content of 0.5%.

EXPERIMENT 24.

The preparation of phenylsulphuryltransferase from rat liver (18).

Two fasted male rats were killed by a blow on the head, bled and the livers removed and immediately placed in ice water (total wet weight, 18.0grs.). The livers were sliced, 72 ml. of 0.15M KCl containing 0.001M EDTA (pH 7.2) added and the whole homogenized. All operations were carried out at 0 - 2°. The homogenate was filtered through glass wool to remove the superficial fatty layer and the filtrate centrifuged at 20,000 x g for 1½ hours. The supernatant was taken to 1.5 M ammonium sulphate concentration by the addition of 60 ml. of 3.75 M ammonium sulphate. The pH of a diluted sample (0.1M) of the ammonium sulphate solution was 7.2. The precipitate obtained on centrifugation (20,000 x g, 1 hour) was discarded and the supernatant (90 ml.) raised to 2.3 M ammonium sulphate concentration (by adding 72 ml. of 3.75 M ammonium sulphate) and the precipitate collected by centrifugation (20,000 x g,

45 minutes). The precipitate was dissolved in water (1.0 ml. per gram of original tissue). The extract was stored at -14° in 0.5 ml. aliquots. A protein determination (G.M. 10) on a portion of the extract showed a concentration of 3.2 mgs./0.1 ml. The stored preparation was active for 2 - 4 weeks.

EXPERIMENT 25.

Assay of PAPS using p-NP and rat liver preparation.

The composition of the digest was as follows -

0.4 ml. 0.1M phosphate buffer pH 7.2
0.1 ml. (5 µM) KF.
1.0 ml. (4.0 µM) p-NP.
2.0 ml. (4.2 µM) PAPS.
0.5 ml. enzyme solution (Expt. 24).

All components of the digest except the enzyme solution were mixed in a small test tube.

The enzyme was placed in a separate tube and both tubes held at 37° in a constant temperature water bath. The reaction was initiated by pouring the contents from one tube to the other. A control digest which did not contain any PAPS was also in-:cubated. Samples (0.4 ml.) were withdrawn at intervals, deproteinized by heating at 80° for 2 minutes, centrifuged and the supernatant diluted to 10 ml. with N/100 NaOH. The intensity of the colour produced was measured by means of an EEL photoelectric colorimeter (601 filter) and thus the rate of transfer of sulphate to p-NP was determined quantitatively by the decrease in absorption of the yellow coloured p-nitrophenol anion (Fig. 15).

EXPERIMENT 26.

Enzymic transfer of sulphate to phenol using p-NPS as donor and PAP as co-factor (37).

The following digest was composed -

0.5 ml. 0.1M phosphate buffer pH 7.2
0.2 ml. (1.0 μM) p-NPS.
0.2 ml. (1.0 μM) phenol.
0.1 ml. (1.0 mμM) PAP.
0.3 ml. enzyme solution (prepared as Expt.24)

A control digest which did not contain PAP was also incubated. The tubes were incubated at 37°. After 90 minutes 0.4 ml. aliquots were withdrawn, deproteinized by heating at 80° for 2 minutes and the supernatant diluted to 10.0 ml. with N/100 NaOH and the optical density read on an EEL photoelectric colorimeter (filter 601). The differ-:ence between the digest and control readings, 2.4 - 1.28 = 1.12, represents (from the standard graph for p-NP) an amount equal to 16 mμM of p-NP. Thus the amount of p-NP released or the amount of phenylsulph-:ate formed per 1.0 ml. of digest is 40 mμM.

EXPERIMENT 27.

Attempted transfer of sulphate from p-NPS to some low-molecular carbohydrates.

The composition of the digests was as

follows -

0.5 ml. 0.1M phosphate buffer pH 7.2.
0.2 ml. 4.0 µM p-NPS.
0.2 ml. carbohydrate (0.5 mg.)
0.1 ml. 1.0 mµM PAP.
0.3 ml. enzyme solution (As Expt. 24).

Incubation was carried out at 37° for 2 hours. A control digest which did not contain any PAP was also incubated. 0.4 ml. aliquots were withdrawn, deproteinized by heating at 80° for 2 minutes, centrifuged and the supernatant diluted to 10.0 ml. with N/100 NaOH, and read on an EEL photoelectric colorimeter (filter 601). The follow-:ing sugars were assayed (Table 3), sucrose, maltose, lactose, D-galactose, D-mannose, D-glucose, D-glucos-:amine, D-galactosamine, D-glucuronic acid, D-galactur-:onic acid, D-arabinose, L-fucose, D-ribose and L-rhamnose.

EXPERIMENT 28./

EXPERIMENT 28.

PAP as a possible co-factor in the transfer of sulphate from heparin to p-NP.

The composition of the digest was as follows -

0.3 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. (5 μM) KF.
0.1 ml. 0.8 mμM PAP
0.1 ml. 0.6 μM p-NP.
0.1 ml. (1 mg.) heparin solution.
0.3 ml. enzyme solution (prepared as Expt.24).

A series of five controls were set up, similar to the above except that,

Control 1 contained no PAP.
Control 2 contained no heparin.
Control 3 The enzyme was denatured at the commencement of reaction.
Control 4 the p-NP solution was added immed-:iately before deproteinization.
Control 5 contained no p-NP.

The tubes were incubated at 37°. After

90 minutes 0.1 ml. aliquots were withdrawn and diluted to 1.0 ml. with N/100 NaOH and read on an EEL spectra at 400 mμ. The results are shown in Table 5.

EXPERIMENT 29.

Attempted transfer of sulphate to desulphated heparin.

The composition of the digest was as follows -

0.1 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. 5 μM KF.
0.1 ml. desulphated heparin solution (100μgs).
0.1 ml. 0.2 μM PAPS.
0.1 ml. enzyme solution (as Expt. 24).

A control which did not contain any PAPS was also incubated. The tubes were placed in a water-bath at 37°. After 3 hours the protein was

precipitated by heating at 80° for 2 minutes. The supernatants, after centrifugation, were concentrated to 0.05 ml. (approximately) in a vacuum desiccator and applied to a strip of Whatman No. 1 paper (80 x 9 cms.). The paper was developed in the solvent system isobutyric acid/0.5 N ammonia (5:3 v/v). After 48 hours the paper was removed, dried, and divided into two portions by cutting along a line 6 - 8 cms. from the starting line (Fig. 16). The portion of the chromatogram still containing the polysaccharide material was then submitted to electrophoresis in 0.05 M phosphate buffer pH 7.0 at 700 V for 2 hours. The paper was removed, dried in a current of air and stained with Azure A (G.M.2).

EXPERIMENT 30.

Attempted transfer of sulphate to some other polysaccharides.

The composition of the digest was as

follows -

0.1 ml. 0.1 M phosphate buffer pH 7.2.
0.1 ml. 5 µM KF.
0.1 ml. polysaccharide solution (100 µgs.)
0.1 ml. 0.2 µM PAPS.
0.1 ml. enzyme solution (as Expt. 24).

The following polysaccharides were assayed 1) a sulphated polysaccharide from Furcellaria fastigitata 2) a sulphated polysaccharide from Chondrus crispus and 3) desulphated chondroitin sulphate. Control experiments did not contain any PAPS. The tubes were incubated at 37° for 3 hours and worked up in a similar manner as described in the previous experiment (Expt. 29) for desulphated heparin.

EXPERIMENT 31.

Attempt to elute desulphated heparin from Whatman No. 1 paper.

100 µgs. desulphated heparin were spotted

on a strip of Whatman No. 1 paper (8 x 30 cms.) about 10 cms. from one end and then developed in the isobutyric acid - 0.5 N ammonia (5:3 $^{v}/_{v}$) solvent for 48 hours. The paper was removed, dried and an area around the base line, 3.0cms. x 8 cms., cut out. This strip was eluted with water (5 ml.) and shown to contain 46 µgs of desulphated heparin - analysis carried out by the carbazole reaction (G.M. 6). Further elution with water (5 ml.) removed only a trace of polymer.

EXPERIMENT 32.

Attempt to separate heparin from PAP on Sephadex G - 25.

Heparin (1 mg.) and PAP (1.5 mgs.) in 1.0 ml. of water were applied to a column (2 x 12cms.) of Sephadex G - 25, which was prepared as follows - water was added to Sephadex G - 25 and after standing for some hours the slurry was poured into a column

(2 x 12 cms.) and allowed to settle under gravity. A circular disk of Whatman 3 MM paper was placed on top of the column before applying the solutes. The column was eluted with water and fractions (1.2 ml.) collected using an automatic fraction collector. Each fraction was analyzed for heparin (carbazole reaction, (G.M. 6))and PAP (U.V. absorption at 260mμ).

EXPERIMENT 33.

Separation of heparin from other low-molecular materials by gel filtration on Sephadex G-25.

To a column (2.2 x 18 cms.) of Sephadex G-25 the upper layer of which was covered with a disk of Whatman 3 MM paper, was added heparin (1 mg.), PAP (3 mgs.) and ammonium sulphate (25 mgs.) in 2.0ml. water. The column was eluted with water and fract-:ions (1.0 ml.) collected on an automatic fraction collector. Each fraction was analyzed for heparin (carbazole reaction, G.M. 6), PAP (U.V. absorption at

260 mμ) and ammonium ion (by Nessler's reaction. G.M. 11).

EXPERIMENT 34.

The preparation of an enzyme extract from rat liver.

The livers of fasted male rats were removed after the animals were killed by a blow on the head and bled. The livers were immediately placed in ice water. On cooling to 0 - 2° they were homogenized in 0.15 M KCl containing 0.001M EDTA pH 7.2 (2 ml. per grm. (wet weight) of liver). The homogenate was filtered through glass wool and the filtrate centrifuged at 20,000 x g for 2 hours at 0 - 2°. The supernatant liquid was stored frozen in 1.0 ml. aliquots at -14°.

EXPERIMENT 35./

EXPERIMENT 35.

Enzymic transfer of sulphate to desulphated heparin: quantitative sulphate determinations.

The composition of the digest (Test) was as follows -

0.2 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. 5 μM KF.
0.1 ml. 1 mg. desulphated heparin.
1.5 ml. 2.8 μM PAPS.
0.3 ml. enzyme solution (as Expt. 34).

A control experiment carried out simultaneously was identical in every respect but did not contain any PAPS. The tubes were incubated at 37° for 3 hours. After deproteinization (80° for 2 minutes) and centrifugation the supernatants were applied to columns (2.2 x 18 cms.) of Sephadex G-25. The columns were eluted with water, 1.0 ml. fractions being collected using an automatic fraction collector Each fraction was assayed by the carbazole method (G.M. 6) for desulphated heparin. The appropriate

fractions containing the polysaccharide (Test - fractions 21 - 27; control - fractions 23 - 29) were bulked and taken to dryness in a vacuum desic-:cator and then redissolved in 0.9 ml. water. A carbazole (G.M. 6) assay on an aliquot (0.1 ml.) of the solutions showed that the "Test" contained 96µgs per 0.1 ml. (a recovery of 96%) and the "control" contained 94 µgs per 0.1 ml. (94% recovery).

Sulphate analysis (G.M. 5) was then carried out, in duplicate, on 0.25 ml. aliquots of these solutions (Table 6).

EXPERIMENT 36.

Enzymic transfer of sulphate to desulphated heparin using a fresh liver extract.

The composition of the digest was as follows -

0.2 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. 5 μM KF
0.1 ml. 1 mg. desulphated heparin.
1.5 ml. 2.8 μM PAPS.
0.3 ml. enzyme solution (as Expt. 34).

The control experiment was identical but did not contain any PAPS. The tubes were incubated at 37° for 4 hours. After deproteinization by heating at 80° for 2 minutes, the material was centrifuged and the supernatants applied to columns of Sephadex G-25 (2.2 x 18 cms.) and isolated as described in Experiment 35 and sulphate analyses (G.M. 5) subsequently carried out (Table 7).

EXPERIMENT 37.

Enzymic transfer of sulphate to desulphated heparin using an increased PAPS concentration in the digest.

The following digest was composed -

0.2 ml. 0.1M phosphate buffer pH 7.2
0.1 ml. 5 μM KF.
0.1 ml. 1.0 mg. desulphated heparin.
2.0 ml. 5.6 μM PAPS.
0.5 ml. enzyme solution (as Expt. 34).

The following controls were also incubated (Table 8). -

Control 1 did not contain any desulphated heparin

Control 2 did not contain any active enzyme. This was replaced by an equal volume of heat denatured enzyme solution.

Control 3 did not contain any PAPS.

All other constituents of the controls were as in the "Test" digest. The tubes were incubated at 37° for 4 hours. After deproteinization (80° for 2 minutes) followed by centrifugation the supernatants were applied to columns of Sephadex G-25, (2.2.x 18.0 cms.) and isolated as described before (Expt. 35). After taking to dryness the polysaccharide material was redissolved in water

(1.0 ml.) the amount of material present estimated and sulphate analyses carried out on known amounts of polysaccharide (Table 9). No polysaccharide could be detected in the eluate from control 1 after apply-:ing to Sephadex.

EXPERIMENT 38.

Enzymic transfer of sulphate to de-N-sulphated heparin.

The composition of the digest was as follows -

0.2 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. 5 µM KF.
0.1 ml. 10mg. de N-sulphated heparin.
0.8 ml. 2.2 µM PAPS.
0.3 ml. enzyme solution (as Expt. 34).

A control was also incubated which did not contain any PAPS. The tubes were incubated at 37° for 4 hours, the contents deproteinized and the poly-

:saccharide material isolated by gel filtration as described in Expt. 35. Sulphate analyses were then carried out on known amounts of material (Table 10).

EXPERIMENT 39.

Enzymic transfer of sulphate to desulphated chondroitin sulphate.

The following digest was composed -

0.2 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. 5 μM KF.
0.1 ml. 1.0 mg. desulphated chondroitin sulphate
2.0 ml. 5.60 μM PAPS.
0.3 ml. enzyme solution (as Expt. 34).

A control which did not contain PAPS was also incubated. The tubes were placed in a water bath at 37° for 4 hours, deproteinized, the poly-:saccharides isolated by gel filtration as described

Expt. 35, and sulphate analyses carried out (Table 11).

EXPERIMENT 40.

Attempted transfer of sulphate to a polysaccharide from Chondrus crispus.

The composition of the digest was as follows -

0.2 ml. 0.1M phosphate buffer pH 7.2.
0.1 ml. 5 µM KF.
0.1 ml. 1.0 mg. polysaccharide.
2.0 ml. 5.6 µM PAPS.
0.3 ml. enzymic solution. (as Expt. 34)

A control which did not contain any PAPS was also incubated. The tubes were placed in a water bath at 37° for 4 hours, deproteinized, the polysaccharide isolated by gel filtration as described in Expt. 35 and sulphate analyses carried out (Table 11).

EXPERIMENT 41.

Enzymic transfer of sulphate to a polysaccharide from Furcellaria fastigitata.

The composition of the digest was similar to that in Expt. 40 except that the polysaccharide in this instance was the one obtained from F.fastig-:itata (0.1 ml. 1 mg.). A control lacking PAPS was also incubated. After 4 hours at 37°, the polysaccharide was isolated by gel filtration after deproteinization and sulphate analyses (G.M.5) carried out on known amounts of polysaccharide (Table 11).

EXPERIMENT 42.

Attempted enzymic transfer of sulphate to hyaluronic acid.

The composition of the digest was similar to that in Expt. 40 except that the polysaccharide

in this instance was hyaluronic acid (1 mg., 0.2 ml. Expt. 23). A control experiment which did not contain PAPS was also incubated. After 4 hours at 37° the digests were deproteinized by heating at 80° for 2 minutes and the polysaccharide isolated by gel filtration and sulphate analyses (G.M. 5) carried out on standard amounts of material (Table 11).

General Methods and Procedures.

(Abbreviated G.M.)

G.M.1. The determination of phosphorus as inorganic phosphate (191).

The method used was King's modification (191) of the method of Fiske and Subbarow (190).

Reagents.

5% aqueous ammonium molybdate.
60% perchloric acid.
A reducing agent containing 0.5gr. of 1-amino - 2-naphthol - 4- sulphonic acid, 30.0grs. of sodium bisulphite and 6.0 grs. of sodium sulphite in 250ml. water. This solution was stored in a well stoppered dark bottle.

To an aliquot (1.0ml.) of the test solution, containing not more than 60 µgrs. of phosphorus was added 1.2 ml. of 60% perchloric acid and the mixture heated strongly in a hard glass test tube until colourless (e.g. until all the organic matter was destroyed). To the cooled solution 5% ammonium

molybdate (1.0 ml.) and reducing agent (0.5 ml.) were added in that order. The solutions were diluted to 15.0 ml. with water and read on an EEL photoelectric colorimeter (filter ORI) after ten minutes. The phosphorus content was then determined from a calibration curve obtained by using standard solutions of dipotassium phosphate.

G.M. 2. Chromatography and electrophoresis on paper; detection of compounds on paper.

Whatman No. 1 chromatography paper was used unless otherwise stated. The method used was the descending technique of Martin (198), whenever an ascending technique was used it was mentioned in the text. The composition of the solvents used were as stated. The time of development was approximately 20 hours unless stated otherwise.

Paper electrophoresis was carried out in

an apparatus similar to that described by Markham and Smith (199). The buffers used, the voltage applied, together with the times of the run are mentioned in the text in the appropriate places. The dimensions of the paper strips were 9.0 cms x 45.0 cms. unless stated otherwise.

Detection of compounds. Nucleotides were located by inspection under ultra-violet light.

Acidic mucopolysaccharides were detected by staining with Azure A (200). An 0.05% solution in N/100 HCl was used. The strips were immersed in this solution for 30 seconds and then washed repeat-:edly in several changes of 50% aqueous ethanol which was 1% with respect to acetic acid. The acid muco-:polysaccharides appeared as blue spots on a pale blue background. It was found impossible to photo-:graph acid polysaccharides which had been stained with Azure A - diagrammatic representations are there-:fore included in this thesis.

G.M. 3. Preparation of pyridine sulphur-:trioxide (192).

A solution of pyridine (63.1 ml.) in

chloroform (350 ml.) in a three necked flask was equipped with a mechanical stirror and a dropping funnel containing chlorosulphonic acid (21.1 ml.). The flask was cooled to -5° with an ice-salt mixture, and then the acid added dropwise with stirring. The temperature was maintained below 0°. After the addition of chlorosulphonic acid was complete the mixture was stirred for a further 30 minutes. The solid pyridine sulphur-trioxide was filtered on a Buchner funnel and washed four times with 30 -40 ml. aliquots of ice-cold chloroform and then dried in vacuo to yield 25 grs. of product which was stored at -14°. It retained its activity for 10 - 12 weeks.

G.M. 4. <u>Preparation of methanolic-HCl. (167).</u>

Acetyl chloride (5.0 ml.) was added to 1 L. of methanol and the mixture allowed to stand in a stoppered flask at room temperature for 24 hours.

Aliquots (25 ml.) were then titrated with standard N/10 NaOH using phenolphthalein indicator and the normality of the HCl determined.

G.M. 5. Sulphate Analysis.

The method used was that of Letham and Jones (197).

Reagents.

0.19% 4- amino - 4' - chlorodiphenyl (which was 0.1% with respect to cetyltrimethylammonium bromide) in N/10 HCl.
Concentrated nitric acid.
Sodium chloride solution (0.6 mg. per 0.01 ml.water).

Procedure.

0.1 ml. polysaccharide solution containing not more than 80 µgrs. of sulphate was oxidized in a

sealed tube at 280° with concentrated nitric acid (0.3 ml.) and sodium chloride solution (0.01 ml.) for three hours. A blank digest (0.1 ml. H_2O + 0.3 ml. concentrated HNO_3 + 0.01 ml. sodium chlor-:ide solution) was also carried. The digests were concentrated to dryness. 0.4 ml. 5N HCl added to each tube and the solutions again taken to dryness. The residues were dissolved in 1.0 ml. water. To 0.2 ml. of these solutions, 0.3 ml. of water and 0.5 ml. of the 0.19% 4 - amino - 4' - chlorodiphenyl reagent (above) were added. After mixing the solut-:ions were allowed to stand at room temperature for 2 hours and then centrifuged at 20,000 x g for 15 minutes. (It was observed that only by centrifuging at this speed was complete removal of the precipit-:ated 4 - amino - 4' - chlorodiphenyl sulphate ensured; thus enhancing the reproducibility of the method).

Aliquots (0.1 ml.) of the supernatants were diluted to 10.0 ml. with N/10 HCl and read on a Unicam spectrophotometer at 254 mu. The difference in readings between the test and the blank solutions

represents proportionally the amount of sulphate which has combined with the reagent. The sulphate content was then determined from a calibration curve obtained by using standard solutions (0 - 100 µgs.) of sulphate.

G.M. 6. Colorimetric analysis of polysaccharides.

(1) Carbazole method (194).

The use of carbazole for the determination of uronic acids has been described by Dische (194) and this method has been used to estimate the follow-:ing uronic acid containing polysaccharides - heparin, desulphated heparin, de-N-sulphated heparin and desulphated chondroitin sulphate.

To 1.0 ml. of the solution containing not more than 100 µgs. of polysaccharide, concentrated sulphuric acid (6.0 ml.) was added while the tube was shaken under cold water. The mixture was then heated

in a vigorously boiling water bath for 20 minutes, cooled and 0.2 ml. of 0.1% carbazole added. The solution was allowed to stand at room temperature for 2 hours and then read on an EEL photoelectric colorimeter (filter 604). The polysaccharide con-:tent was then determined from a calibration curve obtained by using standard solutions of the poly-:saccharide.

(2) <u>phenol-sulphuric acid method (195).</u>

Carbohydrates react with phenol and sulphuric acid to give a stable yellow coloured chromogen. The method of Montgomery (195) was used to estimate hyaluronic acid and the sulphated polysaccharides from C.crispus and F. fastigitata. The procedure is simple and not as time consuming as the carbazole method.

To 0.1 ml. of the solution containing not more than 100 µgrs. of the polysaccharide, 2.0 ml. of 4% phenol was added followed by concentrated

sulphuric acid (5.0 ml.). The sulphuric acid was added from a pipette and the stream of acid directed at the liquid surface in order to obtain good mixing which is further ensured by shaking the tube after the addition was complete. During the addition of the acid and the subsequent shaking, the temperature of the solution rises spontaneously (to approximately 110°) and the heat so generated completes the develop-:ment of the chromogen.

The absorbancy was measured after 30 minutes using an EEL photoelectric colorimeter (filter 603). The polysaccharide concentration was then determined from a calibration curve obtained by using standard solutions of the polysaccharide.

G.M. 7. Purification of charcoal for adsorption chromatography of nucleotides.

The method used was that of Smith and Mills (201).

500 grs. of Norit A charcoal was refluxed with 2½ l. of 5 N HCl for 6 hours. After cooling it was filtered on a Buchner funnel and washed copiously with water until chloride free and then with distilled water (1 L.) and finally heated in an oven at 110° for 16 hours.

G.M. 8. Preparation of p-nitrophenyl sulphate (p-NPS).

The method used was that of Huggins and Smith (170) as modified by Gregory and Lipmann (37).

Dimethylaniline (47.0 ml.) was added to carbon disulphide (50 ml.) and the solution cooled to 0° in an ice salt mixture. Chlorosulphonic acid (9.1 ml.) was added dropwise with stirring. p-NP (13.9 grs.) M.P. 109-110°, was added quickly to the flask and the mixture stirred for 1 hour. After standing overnight at room temperature, 4 M KOH (100 ml.) was added to the solution and the bright yellow crystals of potassium p-NPS collected by

filtration after the flask had been heated in a water bath (80°) to remove last traces of carbon disulphide. The crystals were washed with 95% ethanol (50 ml.) and recrystallised several times from 80% ethanol to give small white needles which were dried in vacuo to yield 4.32 grs. of product. Spectrophotometric analysis (400 mμ) of a sample of the material indicated that the compound was free of p-NP.

G.M. 9. Preparation of 3' Nucleotidase from rye - grass.

An extract was prepared by the method of Shuster and Kaplan (193).

Domestic rye-grass (500 grs.) was placed in a shallow vessel containing enough water to keep the seed thoroughly wet for 3 days at room temperat-:ure. It was then homogenized with 1 L. of water in a Waring blendor. Blending was carried out for 3 minutes using small batches of seed. The homo-

:genate was squeezed through cheese cloth and the resulting juice used to blend subsequent portions of seed. The crude extract was centrifuged (5000 x g) to remove starch grains and cell debris.

In order to concentrate the extract solid ammonium sulphate was added to 90% saturation. The dark-brown precipitate which floated to the surface was removed by filtering and dissolved in 150 ml. water and the solution dialyzed against cold running water overnight. Any precipitated material was removed by centrifugation (5,000 x g.). Ammonium sulphate was again added to the supernatant to 90% saturation; the precipitated protein collected by centrifugation, dissolved in water (40 ml.)and dialyzed against running water overnight. The supernatant obtained after centrifugation was stored in 2.0 ml. aliquots at -14°.

The enzyme preparation was assayed as follows using A 2'(3') M.P. The composition of the digest was as follows - 0.1 M Tris-HCl buffer pH 7.5

(1.0 ml.), enzyme solution (0.3 ml.) and A 2' (3') M.P. (1.2 mgs., 0.4 ml.). A control digest which contained water (0.4 ml.) in place of the nucleotide was also carried. The tubes were incubated at 37° for 90 minutes and aliquots (0.4ml.) analyzed for release of phosphate (G.M. 1) after deproteinization by adding 0.2 ml. 20% trichloro-acetic acid; 46% of the total phosphorus of A 2' (3') M.P. was thus hydrolyzed.

The composition of the digests when assay-:ing other nucleotides was essentially the same as described above except that the appropriate nucleo-:tide was incubated in place of A 2'(3') M.P.

G.M. 10. Estimation of Protein (Biuret Method).

The method used was that of Gornall et al. (202).

Biuret Reagent: 1.5 grs. of cupric sulphate ($CuSO_4$.

$5H_2O$) and 6.0 grs. of sodium potassium tartarate ($NaK\ C_4H_4O_6 . 4H_2O$) were dissolved in water (500 ml.). 300 ml. of 10% sodium hydroxide was added with stirring and the mixture diluted to 1 L. with dis-:tilled water. The reagent was stored in a bottle lined with paraffin wax.

To 4.0 ml. of the protein solution contain-:ing from 0 - 10 mgs. of protein, 4.0 ml. of the biuret reagent was added with stirring. After stand-:ing at room temperature for 30 minutes the solution was read on an EEL photoelectric colorimeter (filter 605) against a blank consisting of 1.0 ml. of water plus 4.0 ml. of biuret reagent.

The concentration of protein was then determined from a calibration curve obtained by/stan-:dard solutions of crystallised egg albumin.

G.M. 11. Determination of ammonia by Nessler's reagent.

The reagent was prepared as follows (203).

50 grs. of potassium iodide was dissolved in 35 ml. of distilled water. A saturated solution of mercuric chloride was added dropwise until a slight precipitate appeared. 400 ml. of 36% sodium was added and the solution diluted to 1 L. with distilled water.

Qualitative analyses for ammonia were carried out by adding 0.05 ml. of the above reagent to 2.5 ml. of the test solutions. A yellow to brown colour is produced and a brown precipitate forms in more concentrated solutions of ammonia.

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