STUDIES ON

NUCLEOTIDES AND RELATED COMPOUNDS

IN PLANTS

THESIS

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PREFACE.

The experimental work described in this thesis was carried out in the Agricultural Chemistry Section of the Chemistry Department, University of Glasgow, under the supervision of Dr. W.R. Rees, from October 1959 to May 1965.

I gratefully acknowledge receipt of an Agricultural Research Studentship for one year from October 1959 to September 1960.

During this time and afterwards as a member of staff I availed myself of the facilities in the Chemistry Department as well as in the Agricultural Chemistry Section, thanks to the generosity of Professor J.M. Robertson, F.R.S.

I would like to record my thanks to Dr. W.R. Rees who directed this work through all its phases in an unselfish and inspiring manner. This task was not aided by frequent interruptions for lecturing and administrative duties which are unavoidable in the running of a small undergraduate teaching section.

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ABBREVIATIONS USED.

NOTE. All temperatures are expressed in degrees Centigrade.

RNA	- -	ribonucleic acid.
EDTA	- -	ethylenediaminetetra-acetic acid.
DCC	- -	dicyclohexylcarbodiimide.
DCU	- -	dicyclohexylurea.
CEP	- -	2-cyanoethyl phosphate.
DEAE-cellulose	-	diethylaminoethyl-cellulose.
tris	- -	2-amino-2-hydroxymethylpropane-1-3-diol.
AMP, A-5'-P	- -	adenosine-5'-phosphate.
ADP	- -	adenosine-5'-diphosphate.
UMP, U-5'-P	- -	uridine-5'-phosphate.
UDP	- -	uridine-5'-diphosphate.
GMP, G-5'-P	- -	guanosine-5'-phosphate.
GDP	- -	guanosine-5'-diphosphate.
CMP, C-5'-P	- -	cytidine-5'-phosphate.
CDP	- -	cytidine-5'-diphosphate.
NAD	- -	nicotinamide-adenine dinucleotide.
m.mole	- -	millimole.
µ.mole	- -	micromole.
kilo	- -	kilogram.
BDH	- -	The British Drug Houses Ltd.
H & W	- -	Hopkin and Williams Ltd.
Light	- -	L. Light and Co. Ltd.
PAPS	- -	adenosine-3'-phosphate-5'-sulphatophosphate.

GENERAL INTRODUCTION.

Investigations on glycosyl transfer reactions, in particular those which could conceivably be involved in polysaccharide synthesis can broadly be classified into three main groups.

In the first instance, many attempts have been made to induce hydrolytic enzymes to act reversibly by providing them with experimental conditions which would favour synthesis. Of many examples which could be quoted, the work of Bacon and Edelman (1) on a yeast invertase (Saccharomyces cerevisiae) is noteworthy in that light was thrown on possible routes to the biosynthesis of fructosans. In their work on yeast invertase, they were able to show that at high substrate sucrose concentrations, there were produced, in addition to the expected hydrolysis products glucose and fructose, a series of oligosaccharides. These were produced as the result of transglycosylations whereby the enzyme catalysed the transfer of fructose units from a sucrose donor molecule to a sucrose acceptor with the concomitant release of glucose from the donor. The isolation and characterisation of two trisaccharides viz. β-fructofuranosyl-2-1-β-fructofuranosyl-2-1-α-glucopyranoside (1-kestose) and β-fructofuranosyl-2-6-β-fructofuranosyl-2-1-α-glucopyranoside (6-kestose) demonstrated the ability of this enzyme

to synthesise both the levan and inulin type linkages. The formation of a further trisaccharide (neokestose) and on occasion tetrasaccharides gave further information concerning the specificity of this enzyme.

Similar work has been carried out on somewhat analogous enzyme systems. Thus it has been shown that cellobiases from several sources (2,3) are capable of being induced to form small amounts of cellodextrins under appropriate experimental conditions.

Attempts to induce the starch degrading amylases (4) to catalyse the transfer of glucosyl units to acceptors other than water have in general failed.

However, with the enzymes included in this group, even under optimum experimental conditions, hydrolysis appears to be the major role demonstrable in vitro. This does not of course preclude the possibility that in localised regions within cells their synthetic abilities might become manifest in an important way.

Certain members within this group possess characteristics which might entitle them to be included in the second category, which includes those enzymes which can be referred to as transglycosylases. These enzymes have the ability to catalyse the synthesis of a new glycosidic bond at the expense of the cleavage of another, both acts being envisaged as occurring simultaneously

(in some cases an enzyme sugar complex is postulated). Examples of such enzymes include amylomaltase (5) which catalyses the synthesis of maltodextrins from maltose; the so-called "amylase" of Bacillus macerans (6) which disproportionates linear sections of the starch complex into smaller maltodextrins and the Schardinger dextrins without hydrolysis; D-enzyme (7) which has a similar effect in that it redistributes maltosyl or larger radicals among short chain dextrins but does not attack maltose; and Q-enzyme (8) which converts amylose into amylopectin by the scission of an α-1-4 linkage at a point some twenty or so units from the non-reducing end of an amylose chain with its concomitant attachment to another amylose chain through an α-1-6 linkage. A number of other enzymes which can convert α-1-4 into α-1-6 linkages in an analogous fashion to Q-enzyme have been isolated from fungi (9), algae (10) and higher plants (11).

The enzymes of the second category are able to manifest their effects at low substrate concentrations and do not in general lead under any conditions to direct hydrolysis, which distinguishes them from members of the first group which give rise to hydrolysis or transglycosylation depending on the experimental conditions.

The third group comprises the phosphorylases which can be regarded as specialised types of transglycosylases in that the donor molecules for polymer formation are phosphorylated. The

first member of this group to be discovered was muscle phosphorylase (12) which in the presence of inorganic phosphate catalysed the partial degradation of glycogen to produce α-glucose-1-phosphate. The reverse synthetic reaction utilised α-glucose-1-phosphate, glucose moieties being transferred successively on to a "primer" molecule resulting in the eventual synthesis of a blue iodine staining linear polysaccharide of the amylose type with the simultaneous release of inorganic phosphate.

Plant phosphorylase systems were first discovered and investigated in detail by Hanes (13) and were found to act in a generally analogous manner to the muscle enzyme. These enzyme systems which result in the synthesis of amylose type polysaccharides are of widespread occurrence (14).

Whilst the reversibility of, for instance, potato phosphorylase is easily demonstrated, the incorporation into a digest of high concentrations of inorganic phosphate at a suitable pH can result in the complete degradation of amylose i.e. phosphorylase can be provided with experimental conditions which induce it to behave entirely as a degradative enzyme. As a consequence there is much current speculation concerning the precise role of phosphorylase in vivo and it has been suggested that it may play no role in starch synthesis. It is argued that the ratio of inorganic phosphate to glucose-1-phosphate in starch bearing tissues is

unfavourable for synthesis, as it is substantially greater than the equilibrium value for the enzyme system (15). However, as these ratios are derived mainly from an analysis of whole tissue (16, 17) it is not inconceivable that in localised regions the ratio may be more conducive to synthesis. Other evidence such as the effect of hormones (18) on phosphorylase activity tend to indicate a degradative role. At this stage it would appear premature to rule out completely the synthetic action of phosphorylase.

This route to polysaccharide synthesis no doubt appeared promising in cases other than that of starch and glycogen formation and attempts were doubtless made to discover analogous systems which could account for the synthesis of cellulose, xylans etc. No successes have so far been reported with respect to polysaccharide formation by analogous enzymes to phosphorylase, but three instances of disaccharides synthesised by this route can be quoted. Thus Hassid and Doudoroff (19) discovered a sucrose phosphorylase in Pseudomonas saccharophila adapted to utilise sucrose, which transfers the glucose moiety from α-glucose-1-phosphate on to fructose resulting in sucrose formation. The equilibrium of the reaction favours breakdown rather than synthesis. Maltose phosphorylase from Neisseria meningitides catalyses a similar reaction, but it is interesting to note that the donor molecule for the

synthesis of this α-1-4 linked glucose disaccharide is β-glucose-1-phosphate. By a similar inversion cellobiose phosphorylase, isolated from three separate microbial sources (21, 22, 23) catalysed the synthesis of cellobiose from α-glucose-1-phosphate and glucose as acceptor molecule.

Concepts such as these were the ones being considered prior to the work carried out by Leloir in his investigation on the interconversion of galactose and glucose by the yeast Saccharomyces fragilis (24). His studies on this interconversion resulted in the discovery of a heat stable cofactor, eventually characterised as uridine diphosphate glucose (UDP-glucose Fig. 1). The conversion is thought to involve an oxidation-reduction step at C-4 of the sugar moiety of the nucleotide as trace amounts of NAD are also required. The discovery of this cofactor UDP-glucose, which is in effect a "high energy" form of α-glucose-1-phosphate opened up the possibilities of its involvement in glucose transfer reactions. This conjecture was quickly verified in a spectacular fashion by Leloir and co-workers who demonstrated the existence of enzymes capable of utilising UDP-glucose in both trehalose and sucrose synthesis. The enzyme source for trehalose synthesis (25) was Saccharomyces fragilis and indeed yeasts contain free trehalose (26). The reaction proceeded by virtue of a transfer of glucose to a glucose-6-phosphate acceptor molecule, followed by phosphatase

action to produce trehalose.

UDP-glucose + glucose-6-phosphate → trehalose phosphate + UDP.

Trehalose phosphate → trehalose + inorganic phosphate.

Two separate enzyme systems found in wheat germ each capable of sucrose synthesis were also described at this time (27, 28). In each case, UDP-glucose was the glucosyl donor, but whereas fructose was the acceptor in one case, fructose-6-phosphate was required by the second enzyme. Thus the product in the latter case was sucrose phosphate, sucrose being released in an analogous fashion as in trehalose formation.

The first example of transglycosylation from a glycosyl nucleotide was reported by Dutton and Storey (29) who transferred glucuronic acid from a constituent in boiled liver extract. The active component was later identified as UDP-glucuronic acid, transfer occurring on to o-aminophenol, forming o-aminophenyl glucuronic acid. The enzyme source in this case was a liver protein fraction. Analogous examples of glycoside formation using plant enzymic systems soon followed. An enzyme from wheat germ (30) catalysed the transfer of glucose from UDP-glucose to quinol forming the well-known glycoside, arbutin. A further transfer of glucose from UDP-glucose to arbutin was brought about by an enzyme from the same source (31), forming quinol-β-gentiobioside.

These examples of the earlier discoveries have been selected in order to indicate that it became quickly apparent that UDP-glucose was a key intermediate in glycoside bond formation and also in certain sugar interconversions. They stimulated considerable efforts in the search for other related compounds from many biological sources. Details of this intensive research have been summarised by Neufeld and Hassid (32), Gabib (33), and Michelson (34).

In his initial studies on the isolation of UDP-glucose from yeast, Leloir (35, 36) had identified at least two other analogous compounds viz. guanosine diphosphate mannose (GDP-mannose) and uridine diphosphate N-acetylglucosamine (UDP-N-acetylglucosamine). The ion exchange techniques and the isolation procedures used, were perfected and applied to animal, plant, and microbial tissue extracts in many laboratories, in an intensive search for further members of this group of compounds.

Table 1 is a curtailed but representative list of the various nucleoside phosphate sugar compounds which have been found in nature.

This Table illustrates that entities such as UDP-glucose are present in almost every tissue examined whilst others as instanced by UDP-fructose appear to have a more restricted location.

A further point which emerges is that the nucleotides

TABLE 1.

NUCLEOTIDE.	SOURCE.
UDP-glucose.	Yeast(24), milk(37), penicillin(39), algae(38), mung beans(40), liver(41), barley(42) and bracken(43).
UDP-galactose.	Yeast(24), milk(37), mung beans(40), algae(38) and liver(41).
UDP-xylose.	Mung beans(40).
UDP-L-arabinose.	Mung beans(40).
UDP-glucuronic acid.	Milk(37), liver(41), mung beans(40), algae(38) and Pneumococcii(44).
UDP-galacturonic acid.	Pneumococcii(45) and plants(46).
UDP-N-acetylglucosamine.	Yeast(35), fungi(39), barley(42), hen oviduct(48), dahlias(47), bracken(43), milk(37), liver(41) and Pneumococcii(44).
UDP-N-acetylgalactosamine.	Hen oviduct(48), dahlias(47) and milk(37).
UDP-N-acetylglucosamine-6-phosphate.	Hen oviduct(49).
UDP-N-acetylglucosamine-6-phospho-β-galactoside.	Hen oviduct(49).
UDP-N-acetylgalactosamine-sulphate.	Hen oviduct(49).
UDP-N-acetylmuramic acid peptides.	Streptomyces aureus(50).
UDP-N-acetyllactosamine.	Milk(37).
UDP-N-acetyllactosamine-fucoside.	Milk(37).
UDP-fructose.	Dahlias(47).
UDP-L-rhamnose.	Pneumococcii(51).

TABLE 1. (contd.)

NUCLEOTIDE.	SOURCE.
UDP-dihydroxyacetone.	Pneumococcii(52).
UDP-colominic acid.	Escherichia coli(53).
CDP-ribitol.	Lactobacillii and Staphylococcii(54).
CDP-glycerol.	Lactobacillii(54).
CMP-N-acetylneuraminic acid.	Escherichia coli(55).
CDP-abequose.	Salmonella(56).
CDP-tyvelose.	Salmonella(56).
GDP-mannose.	Yeast(36), algae(38), Streptomyces griseus(57), milk(37) and hen oviduct(49).
GDP-glucose.	Streptomyces griseus(57) and milk(37).
GDP-fructose.	Streptomyces griseus(57).
GDP-colitose.	Escherichia coli(58).
GDP-aldoheptose.	Yeast(59).
GDP-L-galactose.	Algae(38) and milk(37).
GDP-L-fucose.	Aerobacter(60) and milk(37).
TDP-L-rhamnose.	Streptomyces griseus(61).
TDP-mannose.	Streptomyces griseus(61).
TDP-ribose.	Streptomyces griseus(61).
ADP-glucose.	Chlorella(62) and wheat endosperm(63).

thus far characterised have been predominantly of the uridine series although more recently members of the adenosine, cytidine, guanosine and thymidine series have been identified.

The fact that a sugar can be associated with more than one base (e.g. glucose has been isolated in the form of UDP-glucose, GDP-glucose and ADP-glucose) has ruled out the possibility of a direct sugar base relationship. Therefore it must be assumed that where a particular sugar is associated with more than one base, each form must have a different metabolic function in nature. However it should be noted that where the D and L forms of a sugar are present in the same tissue they are attached to different bases. (e.g. in the red algae where both D and L galactose are found, they are present in the nucleotide fraction as UDP-D-galactose and GDP-L-galactose(38).).

The concentrations of the various nucleotide sugar compounds found in nature vary enormously, in some cases there are only trace amounts approximately 0.1μ moles per kilo of fresh material, whereas in others values as high as 400 μ moles are obtained. However, the accumulation of uridine compounds (UDP-muramic acids) which occurs when cells of Staphylococcus aureus are treated with penicillin, can be quoted as a reason for not necessarily assuming that the existence of high concentrations of these compounds is evidence of high metabolic demand.

Also listed in Table 1 and worthy of further comment are examples of nucleoside diphosphates with oligosaccharide addends as opposed to monosaccharide units. Their existence opens the possibility that transfer of preformed oligosaccharide units from such donor molecules may occur, but no such instances have yet been reported.

The cytidine compounds (see Table 1) are of special interest. They first attained prominence as the result of the investigations of Kennedy and his associates (64) who isolated CDP-choline and CDP-ethanolamine from liver and showed that they were donor substrates for phospholipid synthesis by virtue of the transfer of choline phosphate and ethanolamine phosphate respectively to α-β-diglyceride acceptors with elimination of cytidine monophosphate. This atypical pattern has its counterpart in saccharide transfer reactions. Baddiley (65), in studies involving Lactobacillus arabinosus firstly isolated CDP-ribitol and CDP-glycerol, which aided the discovery of the teichoic acids from the same organism. In subsequent work carried out by Glaser (66), enzyme systems were isolated from Bacillus subtilus which brought about the complete synthesis of the glycerol and ribitol phosphate polymers from CDP-glycerol and CDP-ribitol respectively with the elimination of cytidine monophosphate as was the case with the phospholipid synthesising systems.

The discovery of cytidine 5' monophosphate-N-acetyl-neuraminic acid opens the possibility that monophosphate derivatives may be found to have donor properties analogous to the nucleoside diphosphate series. Recently a short report has appeared (67) claiming the synthesis of colominic acid, a polymer of N-acetyl-neuraminic acid, from C5'P-N-acetylneuraminic acid, using an extract from Escherichia coli as enzyme source.

As stated earlier, it cannot be assumed that the presence of substantial quantities as opposed to minor amounts of a nucleoside diphosphate sugar in a tissue is direct evidence of its greater involvement in a metabolic process within a tissue, but it is reasonable to suppose that the existence of any amount, however small, is in some sense meaningful in considerations of the way in which the polysaccharides of such a tissue might have been formed.

Table II is an attempt to correlate such facts as are available in the literature concerning the sugar composition of the polysaccharides of various tissues with the nucleoside diphosphate sugar composition of the same tissues.

As will be seen, in several cases the identical sugars are found in the nucleotides as are found in the polysaccharides. Where this is not so, known transformations could readily account for these apparent discrepancies.

Briefly, examples of these interconversions are:-

UDP-glucose	⟶	UDP-galactose	(68)
UDP-D-xylose	⟶	UDP-L-arabinose	(69)
UDP-glucuronic acid	⟶	UDP-galacturonic acid	(70)
UDP-glucosamine	⟶	UDP-galactosamine	(71)
UDP-N-acetylglucosamine	⟶	UDP-N-acetylgalactosamine	(72)
TDP-N-acetylglucosamine	⟶	TDP-N-acetylgalactosamine	(73)

These interconversions are all examples of epimerisation at C-4. They are all known to require catalytic amounts of NAD thus indicating that an oxidation-reduction step is involved.

Examples of other interconversions are the oxidation of the parent sugar to the uronic acid.

UDP-glucose	⟶	UDP-glucuronic acid	(74)
GDP-mannose	⟶	GDP-mannuronic acid	(75)

In these interconversions NAD is again required as an oxidant.

More complicated processes are involved in the formation of methylhexoses from hexoses.

GDP-D-mannose	⟶	GDP-L-fucose	(76)
TDP-D-glucose	⟶	TDP-L-rhamnose	(77)

These interconversions are multi-step in the sense that several reactions are involved, oxo-intermediates having been detected in both cases.

The compiling of Table 11 proved rather difficult, not so

TABLE 11.

SOURCE.	NUCLEOTIDES FOUND.	KNOWN POLYSACCHARIDES AND CONSTITUENT SUGARS.
Yeast. (Saccharomyces fragilis).	UDP-glucose(24), GDP-mannose(36), UDP-N-acetylglucosamine(35) and GDP-mannoheptulose(59).	Glycogen (glucose), glucan (glucose), chitin (N-acetylglucosamine) and mannan (mannose) (78).
Liver. (mainly chicken).	UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine and UDP-glucuronic acid (41).	Glycogen (glucose), hyaluronic acid (N-acetylglucosamine and glucuronic acid) and heparin (glucuronic acid and N-acetylglucosamine) (79).
Hen oviduct.	UDP-glucose, UDP-galactose, GDP-mannose, UDP-N-acetylgalactosamine, UDP-N-acetylgalactosamine-sulphate and UDP-N-acetylglucosamine-6-phosphoro-β-galactoside (48, 49).	Egg membrane (galactose, N-acetylglucosamine and phosphate) (49), hyaluronic acid (glucuronic acid and N-acetylglucosamine) and chondroitin sulphate (glucuronic acid, N-acetylgalactosamine and sulphate) (79).
Red Algae.	UDP-glucose, UDP-galactose, UDP-glucuronic acid, GDP-L-galactose, GDP-mannose and adenosine 3', 5' diphosphate (38).	Starch (glucose), cellulose (glucose) mannan (mannose) (81), galactan sulphate (D and L galactose and sulphate) (38).
Pneumococcii I.	UDP-galacturonic acid (45).	Polymer of galacturonic acid and N-acetylglucosamine (82).

TABLE 11. (contd).

SOURCE.	NUCLEOTIDES FOUND.	KNOWN POLYSACCHARIDES AND CONSTITUENT SUGARS.
Pneumococcii II and III.	UDP-glucose, UDP-glucuronic acid and UDP-N-acetylglucosamine (44).	Polymer of glucose and glucuronic acid (82).
Milk. (cow)	UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, GDP-mannose, GDP-glucose, GDP-galactose, GDP-L-fucose, UDP-N-acetyllactosamine & UDP-N-acetyllactosamine fucoside(37)	Lactose (glucose and galactose), N-acetyllactosamine (galactose and N-acetylglucosamine) and N-acetyllactosamine fucoside (N-acetylglucosamine, galactose and fucose) (80).
Penicillin.	UDP-glucose, UDP-N-acetylglucosamine, UDP-galactose and GDP-mannose (39).	Dextran (glucose), mannan (mannose), galactan (galactose) and chitin (N-acetylglucosamine) (78).
Salmonella.	UDP-glucose, UDP-galactose, UDP-rhamnose, CDP-tyvelose and CDP-abequose.(56).	Polymer of glucose, mannose, rhamnose galactose and dideoxyhexoses (82).
Mung beans.	UDP-glucose, UDP-galactose, UDP-xylose, UDP-L-arabinose and UDP-glucuronic acid (40).	Callose (glucose), cellulose (glucose, and hemicellulose (galactose, xylose, arabinose & glucuronic acid)(83).
Staphylococcus aureus.	UDP-N-acetylglucosamine, UDP-N-acetylmuramic acid and CDP-ribitol (50).	Chitin (N-acetylglucosamine), teichoic acid (ribitol and phosphate) and a muramic acid polymer (82, 83).

much due to the lack of corroborative evidence in favour of a nucleotide sugar-polysaccharide relationship, but due to the lack of comprehensive data. Typical results invoke the presence of a particular sugar in both the nucleotide and polysaccharide fractions without discussing the other sugar containing components of the system. In attempting to correlate nucleotide sugars with those present in polysaccharides most successes have been achieved with the cell wall polysaccharides, particularly those containing the less commonly occurring sugars. Due to their restricted location in nature they can be readily compared with the sugars of the corresponding nucleotide fraction with less fears of ambiguity. Thus, with Staphylococcus aureus a breakdown in cell wall structure and a build up of acid soluble nucleotides can be induced on treatment with penicillin (50). The acid soluble nucleotides were identified as UDP-N-acetylglucosamine, UDP-N-acetylmuramic acid and CDP-ribitol, the sugar components of which are all present in, and in the main confined to, bacterial cell walls. On the other hand the identification of a more common sugar viz. glucose in the nucleotide fraction does not lend itself so readily to a correlation of this kind due to the ubiquitous nature and variety of glucose polysaccharides found in living tissues. No attempt was made to be fully comprehensive in this Table as the prime intention was to draw comparisons between the sugars present in the two fractions. To do

this, representative examples had to be drawn from the voluminous literature available, otherwise the picture would have become too complicated. It should be stressed that when attempting to demonstrate a close relationship between the two fractions it is not essential, although obviously desirable, to have identical patterns of sugars in both cases. The lack of correlation between the two groups could be accounted for by the following possibilities. Nucleotide sugars can readily undergo interconversions, several of the more important of which have already been described (p. 15). Also it cannot be assumed that all the sugar components of polysaccharide mixtures have been identified. Recently the application of modern techniques to polysaccharide studies has led to the identification of significant amounts of sugars, the presence of which were not revealed by earlier studies. In several cases the identification of sugars in the nucleotide fraction has provided a clue to the existence of undetected polysaccharide material viz. the discovery of CDP-ribitol in Lactobacillus arabinosus (54) preceded the identification of the ribitol phosphate polymers (84).

Examination of the data presented in Table 11 makes it difficult to avoid the conclusion that there is a fairly direct relationship between the two classes of compounds. Insufficient data is available to make the case conclusive nor could it be argued with finality that the existence of an overwhelming amount of positive

correlation must force acceptance of the view that the nucleoside diphosphate sugar compounds must therefore be precursors of polysaccharides in tissues. A tenable alternative, at our present state of knowledge, might state that both these groups of compounds arise from some common metabolite as yet undiscovered, or else that the connection between these nucleotides and the polysaccharides is more remote than is suggested by the coincidence of their sugar content.

However, the view adopted in the present work has been that this close relationship is sufficiently suggestive of a metabolic connection to justify investigation and PART IV of this Thesis, separately introduced, develops the suggestion made in the above paragraph viz. that there may be other factors connecting these sugar containing nucleotides and polysaccharides in any fully integrated polysaccharide synthesising system.

The remainder of this Introduction is devoted to a more detailed consideration of the enzyme studies which have demonstrated a close connection between nucleotide-sugar compounds and polysaccharide synthesis.

In 1957 Glaser (85) reported the isolation of a cell free enzyme system from the cellulose forming bacterium Acetobacter xylinum which he claimed was capable of synthesising cellulose from UDP-glucose in presence of a cellodextrin primer. His

identification of the product was based mainly upon incorporation of radioactive glucose from the nucleotide donor and not on chemical studies. The amount of incorporation was only 1 - 2% which was not very satisfactory. The expected confirmatory publications did not appear. Hence, although it is possible that Glaser's system did in fact result in cellulose synthesis there is not sufficient evidence available to assess the validity of this earlier claim.

In a further report in the same year Glaser (86) claimed that an enzyme extract from Neurospora crassa acting upon radioactively labelled UDP-N-acetylglucosamine resulted in the incorporation of the N-acetylglucosamine into chitin provided as primer. There is some uncertainty concerning the definitive nature of these results as again the degree of incorporation was low. No confirmatory reports from other laboratories have appeared and Glaser has not extended his own observations on this system.

There appears to be no doubt, however, that a report by Hassid and co-workers in 1958 (87) of the isolation of a particulate enzyme system from mung beans which utilised UDP-glucose to synthesise an experimentally adequate amount of the polysaccharide callose, is more well founded. Callose is a β-1-3 linked polyglucan known to be present in mung beans and other plants particularly when damaged. The enzyme could be detached from the particulate material by treatment with digitonin and the synthesis of the polysaccharide initiated

by the addition of free glucose, which, however, did not appear to become incorporated into the final product. Sufficient polysaccharide was isolated to permit hydrolysis and identification of inter-sugar linkages and to allow for further proof of identity by enzymic means.

A similar β-1-3 linked glucose polymer referred to as paramylon occurs in granular form in Euglena. Recently the synthesis (88) of this polysaccharide has been reported. The results obtained were analogous to those for callose formation discussed above.

As mentioned earlier in this Introduction the work of Cori and subsequent investigators on glycogen synthesis (12) during the 1940's had indicated the presence in muscle and liver tissues of phosphorylase which acted on α-glucose-1-phosphate to produce a linear α-1-4 linked glucan which was then converted into glycogen by the so-called "branching factor". This explanation of glycogen synthesis has, however, been modified and extended as the result of the work of Leloir and Cardini (89) who have presented strong evidence that UDP-glucose is a glucose donor in further glycogen synthesis. The enzyme responsible for this action, referred to as glycogen synthetase, is bound to the tissue glycogen, tends to be unstable if removed from this association, but can be purified by adsorption on amylose. The outer chains of glycogen are extended

by transfers from UDP-glucose donor molecules. Maltodextrins may also act as acceptors in a similar way. The role of glucose-6-phosphate in facilitating these effects is obscure. On the basis of results obtained using hormones (90) and considerations of such factors as dissociation constants (15) and the location in tissues of the various enzymes (91) it has been suggested that in vivo, glycogen synthetase is responsible for glycogen synthesis and that the role of phosphorylase is confined to its breakdown.

This work with glycogen initiated an intensive search for a similar enzyme for synthesising starch. In 1960 Leloir (92) isolated a system capable of incorporating glucose from UDP-glucose into starch grains. The enzyme appeared to be attached to the actual grain and could not be removed in an active form. The same type of results as with glycogen were obtained, maltodextrins competing with the starch grain carbohydrate as acceptors of glucose from UDP-glucose. When maltotriose or maltotetraose were added only the next higher homologue was produced in each case i.e. only one unit transfers were obtained. Later Leloir (93) found that when testing a variety of glucose donors analogous to UDP-glucose in this system, ADP-glucose proved to be ten times as efficient.

Pottinger and Oliver (94) modified the technique of isolation of fresh starch grains as enzyme source and have claimed a 400 to 2,000 fold improvement in the rate of starch synthesis.

Their technique involved homogenising the potato tubers in a 0.5 molar sucrose buffer pH 7 in order, presumably, to prevent damage to the grains during the isolation procedure. The glucose donor in this work was UDP-glucose.

A more recent report by Leloir (95) has suggested that the enzyme may not after all be attached to the starch grain and may, in fact, be present in soluble form, the starch only being required as a specific acceptor. The report states that radioactive glucose from ADP-glucose could be transferred to the starch grain carbohydrates in low yield using a soluble extract from potatoes as enzyme source. The synthesising activity of the starch grain fraction could be reduced to a negligible level by treatment with hot methanol without affecting its acceptor properties.

There are several other claims to polysaccharide synthesis which will be briefly mentioned.

Hassid (96) in attempting to synthesise Xylans managed to increase the chain length of primer xylan by one unit on incubating an enzyme preparation from asparagus shoots with UDP-xylose and short chain xylans. The low association of the enzyme for short chain xylan units was thought to be the reason for the rather disappointing results.

Investigations have also been carried out on the synthesis of the more complex heteropolysaccharides, the most outstanding

successes having been obtained with hyaluronate (97, 98) and the capsular polysaccharides of Pneumococcii (99, 100).

In hyaluronic acid synthesis radioactive UDP-N-acetyl-glucosamine and UDP-glucuronic acid, which are the constituent sugars of the polysaccharide, were treated with a particulate extract from Rous sarcoma cells. The polysaccharide fraction obtained was analysed for radioactive sugar and the results confirmed using a specific hyaluronidase.

Studies on the synthesis of the capsular polysaccharides of Pneumococcii were carried out in an analogous fashion to the above, in that radioactive nucleotides and a particulate preparation from the bacterium in question were used as enzyme source. In this work more refined studies of the polysaccharide fractions were possible due to the availability of specific antisera.

New light has been cast on cellulose synthesis by the discovery of Elbein, Barber and Hassid (101) that a particulate extract from mung beans could utilise the glucose from GDP-glucose for the synthesis of cellulose. This transfer would not take place when other glucose donors, including UDP-glucose (the substrate used by Glaser in his cellulose synthesising system) were substituted for GDP-glucose in the digest. Enough cellulose was isolated in these experiments to enable its positive identification by both chemical and enzymic means. The isolation of the particulate enzyme fraction

proved to be rather critical as activity was readily lost on washing and could only be partly recovered on adding yeast concentrates, thus indicating the possible involvement of other factors. No primer was required as the low concentration of cellulose present in the enzyme sample was adequate.

SCOPE OF THE PRESENT WORK.

This investigation is concerned, in part, with attempts to establish a direct connection between the presence of sugar containing and other nucleotides in plants and the polysaccharides in these tissues.

Ideally for this purpose it would be desirable to have available adequate quantities of a wide range of likely nucleotides, thus facilitating the certain identification of any naturally occurring compounds found and permitting attempts to demonstrate direct polysaccharide synthesis from one or other of these compounds as substrate.

In the absence of a direct connection, the alternative possibility of there existing a compound or group of compounds which could act as intermediate carriers of sugar residues at some

stage in the process of polysaccharide synthesis had to be borne in mind.

These various aspects of the problem are described in this Thesis as follows:-

PART I is devoted to an account of the methods available and the techniques finally adopted for the synthesis of thirteen selected nucleoside diphosphate sugar compounds together with several more orthodox nucleotides.

PART II describes some early attempts made to detect plant enzyme systems capable of utilising certain of these compounds as donors in polysaccharide formation.

In PART III an account is given of a definitive attempt to relate the nature of the polysaccharide to the nucleotides with which it is associated. For this purpose, starch grains are almost ideal, in that they are morphologically discrete entities, easily obtainable in quantity and possessing the advantage that a great deal is known of the structure of the constituent polysaccharides. This PART thus contains a description of the discovery and identification of substantial quantities of several nucleotides together with a single nucleoside diphosphate sugar viz. adenosine diphosphate glucose (ADP-glucose).

PART IV discusses the possibility that lipoid entities could conceivably play an important role in the functioning of

polysaccharide synthesising systems. Literature references to the discovery of glycolipids in living tissues are cited and the physical properties which could endow these compounds with advantageous properties in this respect are discussed. Because of the relationship already established between the polysaccharide and nucleotide content of starch grains, lipid extracts of potato starch were similarly obtained and, contrary to previous reports, small but significant amounts of at least two distinct glycolipids were discovered.

The significance of these various observations are discussed.

PART 1.

THE SYNTHESIS OF NUCLEOTIDE-SUBSTRATES.

INTRODUCTION.

At the outset of this work it was felt that much advantage would be gained if it was possible to be independent of natural sources for supplies of nucleotides. As has been indicated (Table 1) a large number of sugar containing nucleotides have now been identified from a great variety of tissues. In general, however, the quantities present are very small and variable. The methods used for their recovery are complicated and time consuming and vary depending on the tissue and upon such factors as the lability of the compounds. Frequently the amounts isolated are barely sufficient to permit their certain identification and only rarely, as instanced in the case of UDP-glucose in yeast, can substantial experimental amounts be obtained in this way.

The work of Todd and his collaborators and of Khorana and others in Canada and the United States on the development of a range of phosphorylating agents had shown that it was entirely feasible to contemplate the chemical synthesis of most of the compounds required for this work. Some of the reagents described by

these workers had already been used successfully in this laboratory for the synthesis of mono and diphosphoro-nucleosides and for the preparation of PAPS (the biological sulphating agent) (102).

Before these methods can be applied successfully some background of experience is required. Some are inapplicable in specific cases for reasons of solubility of reactants or because an essential intermediate is unstable or because inversions of configuration occur or because the product resists hydrogenolysis etc. Hence, as the following account will show, it was necessary to become familiar with many methods and then adopt one to suit the particular circumstances.

Because of these complications each of the five sections into which Part 1 is divided commences with a brief theoretical discussion of the various alternative methods and reagents available and comments are made in the text to indicate why for individual compounds a particular course has been followed.

In brief, the starting material for the synthesis of a member of the nucleotide-sugar group of compounds is generally the corresponding nucleoside. All four nucleosides are commercially available at reasonable cost and are of satisfactory quality.

Bases are attached to the ribose moiety of nucleosides through the hydroxyl on C-1 of the sugar. Hence the possibility exists of phosphorylation on the three remaining hydroxyls viz.

C-2, C-3, and C-5 of the ribose. As all the compounds of interest here were C-5 phosphorylated it was merely necessary to protect C-2 and C-3. Section I is devoted to an account of the procedures adopted to this end in each case.

The second stage requires the formation of the C-5 monophosphate of the blocked nucleoside. Hence, Section II contains a summary of available reagents, an assessment of their relative suitabilities and a discussion of the results obtained.

The next step requires that the nucleotides (Sections I and II) should now be coupled to the appropriate addendum (generally a sugar-1-phosphate) through pyrophosphate formation between the phosphate of the nucleotide and the phosphate of the material to be coupled. For this purpose adequate and reliable samples of the sugar phosphates were needed and an account of general methods for their synthesis and isolation together with a discussion of actual results is given in Section III.

Section IV deals briefly with the theory and more extensively with the results obtained on applying the several methods available for coupling to yield the final product.

Section V describes the techniques used for separating and isolating the various products from reaction mixtures.

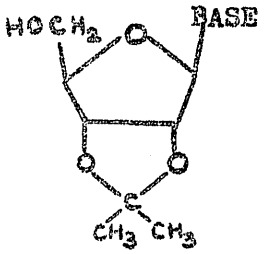
SECTION 1.

PREPARATION OF ISOPROPYLIDENE NUCLEOSIDES.

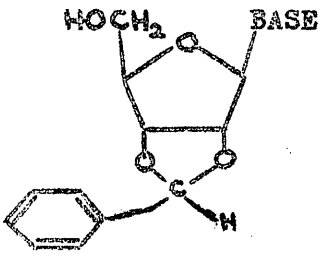
(1) The suitability of isopropylidene derivatives.

Nucleosides may, if required, be prepared by chemical means e.g. by coupling the fully protected 1-bromo ribose derivative with the silver or mercury salt of the appropriate base (103). These procedures are tedious and yields are generally low; hence, no attempt was made to obtain nucleosides by these means. Nucleosides are more readily prepared by mild alkaline hydrolysis of yeast ribonucleic acid, the yields of uridine and guanosine in particular being good. Thus in some of the experiments to be described nucleosides obtained in this way were used; mostly, however, commercially available samples were utilised.

Protection of the C-2 and C-3 positions of the ribose moiety of nucleosides is facilitated by the fact that the hydroxyls on these positions are *cis* relative to each other, hence acetone or alternatively benzaldehyde react to give the corresponding isopropylidene and benzylidene derivative respectively:-



Isopropylidene nucleoside.



Benzylidene nucleoside.

These are the reagents of choice and have been used consistently for similar purposes by many workers. Formation of isopropylidene or benzylidene derivatives is catalysed by the addition of Lewis acids, the ones usually selected being p-toluene-sulphonic acid, sulphuric acid or anhydrous zinc chloride. Some of these catalysts also function as dehydrating agents, thus assisting complete reaction by removing the water eliminated. For this reason, mixtures of anhydrous copper sulphate together with a trace of sulphuric acid may be used and Levene (105), who introduced several of the techniques still used in this field relied largely upon this latter method.

Reaction of benzaldehyde with a sugar structure generally yields a six membered ring derivative because for stereochemical reasons this reagent can "bridge" an intermediate carbon. Influenced possibly by this knowledge, Gulland (106) incorrectly identified the benzaldehyde derivative afforded by cytidine as being the six membered ring product 3-5-O-benzylidene cytidine. Later work by Todd *et al* (107) showed, in fact, that ribose in

nucleoside combination invariably reacts to give a five membered derivative, hence Gulland's compound was shown to be entirely analogous to the usual five membered acetone derivatives and was proved to be 2-3-O-benzylidene cytidine. Khorana (108) has since claimed that benzylidene derivatives are more readily prepared and easier to isolate than the isopropylidene compounds. However, owing to this uncertainty concerning the behaviour of benzaldehyde and because the isopropylidene derivatives are in any case not difficult to obtain, it was decided in this work exclusively to use the acetone derivatives.

(2) Preparation of isopropylidene uridine.

A good commercial sample of uridine (Expt. 1b), dried under vacuum over phosphorus pentoxide for 18 hours (C.H. 11) was stirred continuously at 37° for 68 hours with anhydrous copper sulphate and sulphuric acid according to the method of Levene and Tipson (105) for the preparation of isopropylidene uridine. As the reaction progressed the mixture gradually darkened giving a pale brown solution. This production of colouring matter did not prove troublesome in most cases particularly if good quality uridine was used initially. However, if the uridine sample was impure (Expt. 1a) (prepared by the controlled hydrolysis of yeast

ribonucleic acid (109)) the reaction darkened up excessively, necessitating a decolourising treatment with charcoal, a step which was thought to adsorb some of the nucleoside material present. This opinion was reinforced by the reduced yield of product obtained when a charcoal step was included.

The progress of the blocking reaction was followed by removing samples at intervals and submitting them to an assay by paper electrophoresis at pH 9.6 using a borate buffer (C.M.2).

This buffer system was of considerable value in these experiments because borate, by virtue of its ability to complex with *cis* hydroxyl groups, confers a negative charge on uridine. Thus at pH 9.6 uridine has a significant mobility compared with the corresponding isopropylidene derivative, which remains almost stationary.

When the reaction was complete the excess acid was neutralised by the addition of solid calcium hydroxide and the solution filtered. On removal of solvent under vacuum a pale yellow glass was obtained which could be crystallised from an acetone-pet. ether mixture, a step which incurred a substantial loss in yield. However, experience proved that the glass obtained was of suitable quality to enable its use in the next stage directly; hence, crystallisation as a purification step was frequently omitted in further preparations.

If, as happened occasionally, the preparation at this stage contained an objectionable amount of unblocked material an alternative technique to crystallisation for its removal proved consistently useful and could be applied to all the nucleosides.

The technique, described fully in Expt. 1b, involved preparing a Dowex anion exchange resin in the borate form (C.H. 9-1b). A suitable quantity of this was then added to an aqueous solution of the partially blocked nucleoside and the flask agitated for 1 hour. This treatment resulted in the adsorption of free nucleoside on to the resin which could then be removed by filtration enabling the recovery of the required blocked material without appreciable loss. A column technique was an alternative procedure.

Generally a yield of 65% was obtained by this method (Expt. 1b) compared with a yield of 25% when a charcoal treatment and a crystallisation step were included (Expt. 1a).

(3) Preparation of isopropylidene adenosine.

Baddiley's procedure (110) for the preparation of isopropylidene adenosine was found to be the most suitable of several alternatives tried (111).

This involves the use of zinc chloride as catalyst and dehydrating agent. The adenosine (Expt. 2) was added to a solution of excess zinc chloride in dry acetone and shaken for six hours. After standing for a further ten hours at room temperature the mixture was poured into excess warm barium hydroxide which ensured neutralisation of the acidic zinc chloride and thus avoided hydrolysis of the isopropylidene groups which could occur in the aqueous solution. After precipitation of excess barium with carbon dioxide, followed by filtration and washing of the inorganic precipitate, the aqueous solution was concentrated to crystallisation. The final yield by this method was generally in the region of 75%.

(4) Preparation of isopropylidene guanosine.

Guanosine derivatives in general frequently present additional experimental difficulties because of their insolubility in the usual solvents. Such was the case in the preparation of the isopropylidene derivative and although the procedure for its formation was essentially the same as was used for isopropylidene adenosine in that zinc chloride in dry acetone was shaken with guanosine (Expt. 3a and 3b), the isolation of the product was more difficult. Two methods were used successfully in this work.

In the first (112) after completion of the reaction, the mixture was poured into excess barium hydroxide solution and the excess barium precipitated with carbon dioxide as before (cf. isopropylidene adenosine). At this stage the isopropylidene guanosine may precipitate out of solution owing to its insolubility and be discarded along with the insoluble barium and zinc salts. Repeated extraction of the solid material with hot water and warm cellosolve (2-ethoxyethanol) overcame this difficulty and yields of 60% of crystalline derivative (M.Pt. 297-8°) were obtained.

The alternative isolation procedure (113) required the use of a cation exchange resin to remove zinc ions. To accomplish this it was necessary to select conditions which would not render the guanosine derivative insoluble and furthermore would not cause its destruction. Both of these requirements are satisfied by an alkaline environment, provided experimentally (Expt. 3b) by the addition of concentrated ammonia to the reaction mixture to maintain a clear solution, thereby keeping the guanosine derivative in solution. This alkaline environment is not favourable to the rapid adsorption of zinc ions by the resin but four separate batchwise additions of Dowex 50 cation exchange resin in the ammonium form (C.M. 9-1), with sustained agitation for 15 minutes each time, was ultimately shown to be a satisfactory method. The isopropylidene guanosine could then be crystallised directly from

aqueous solution in yields of ca 70% after concentrating and leaving at 0° for 2 to 3 hours.

(5) Preparation of isopropylidene cytidine.

This compound may be prepared satisfactorily by methods entirely analogous to those already described for the adenosine and guanosine derivatives (114). A method which gives somewhat better yields, however, utilises p-toluenesulphonic acid as dehydrating agent and catalyst (115). In some preliminary trials, yields were disappointing, but it was eventually shown that this was due to the unsatisfactory quality of the p-toluene-sulphonic acid being used. A high quality product gave good results in subsequent preparations. The technique involved the solution of p-toluenesulphonic acid and cytidine in dry acetone (Expt. 4). This was accomplished by continued agitation and by refluxing on a water bath until all the solid material was dissolved. The temptation to proceed before this was satisfactorily effected invariably led to decreased yields.

The gum obtained upon removal of the acetone was treated with excess Dowex anion exchange resin in the bicarbonate form (G.M. 9-1b) suspended in ice water. This ensured the maintenance of alkalinity in the solution and effected the removal of the

p-toluenesulphonic acid. Filtration of the resin and concentration of the aqueous solution afforded a glass of isopropylidene cytidine obtained in yields frequently in excess of 90%.

SECTION 11.

THE 5'-PHOSPHORYLATION OF THE ISOPROPYLIDENE NUCLEOSIDES.

(1) Classification of suitable phosphorylating agents.

The present discussion is confined to dealing with those reagents which are currently considered useful for the phosphorylation of protected nucleosides. Reagents of historical interest only, will not be dealt with unless this illustrates some aspect of the use of modern methods. Similarly an account of phosphorylating agents whose use is largely confined to the special problems presented by stereochemical situations such as are met with in the sugar-1-phosphates will be deferred for separate discussion in the appropriate section.

For convenience, the various methods applicable to

protected nucleosides can be classified into two groups. This division is justifiable from the standpoint of mechanism, at least according to the views most usually accepted.

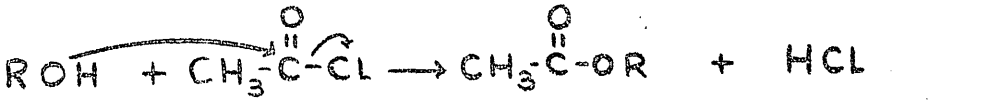
Thus, the first group includes those reagents which are anhydrides of phosphoric acid with some other, usually stronger acid, phosphorylation being accomplished as the result of the elimination of the stronger acid in favour of the entity to be phosphorylated.

The second group comprises those reagents which are empowered to phosphorylate as the result of a condensation brought about by elimination of water in the presence of carbodiimides.

(a) Anhydrides.

Phosphorylations of the first type are similar to acylations in that the reagent is activated as the result of the presence of an anhydride bond. The classical acetylating agents are acetyl chloride and acetic anhydride, both of which are acid anhydrides.

When acetyl chloride is reacted with an alcoholic hydroxyl, the acetyl radical is transferred on to the hydroxyl anion forming an ester bond with the liberation of the stronger acid radical which in this case is the chloride:-

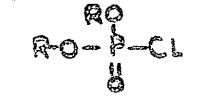


This reaction is an example of the general rule for ester formation whereby exchange favouring the liberation of the acid radical of the stronger acid takes place. This clearly follows from an understanding of the meaning of "strong acid". Hydrochloric acid is a"strong acid" because the chloride ion withdraws electrons so competitively that the proton is readily released. Similarly, in the present case the positive charge on the carbonyl carbon of the acetyl group, which results in attack by the alcohol to be acetylated, has been induced in precisely the same way, by the affinity of chloride for electrons. The positively charged acetyl group thus rejects the chloride in favour of the less electronegative incoming alcohol. It thus follows that the more powerful acetylating agents will be those anhydrides formed from acetic acid and strong acids.

An analogous situation arises in the case of phosphorylating reactions, the phosphate group of the reagent being activated by anhydride formation. However, in this case the fact that phosphates are polyfunctional in that they have three ionisable hydroxyl groups surrounding a comparatively small positively charged phosphorus atom makes the approach of another electronegative group extremely difficult. In effect the positively charged phosphorus atom is protected from attack by the electronegative group of the substance to be phosphorylated, by a shell of negative charges. Thus pyro-

phosphoric acid, which is analogous to acetic anhydride, is a weak phosphorylating agent for this reason.

To reduce the electronegativity surrounding the phosphorus atom and to facilitate the approach of a negatively charged group to be phosphorylated, ester groupings can be introduced into the structure of the phosphorylating agent. Thus a diester derivative of the anhydride formed from orthophosphoric acid and hydrochloric acid:-



di (R) phosphorochloridate would be expected to be a more suitable reagent for use because:-

(a) the ionisable hydroxyls of the phosphoric acid have been masked,

(b) the other component of the anhydride is chloride, the anion of a strong acid, which induces the required positive charge on the phosphorus atom and is itself eliminated preferentially,

(c) such a reagent, unlike pyrophosphoric acid, is monofunctional.

Diesters of phosphoric acid, though fairly easily prepared are generally resistant to acid and alkaline hydrolysis, at least to a degree which limits their use in phosphorylation reactions. The main criterion of any blocking group is that it should be stable during the chemical reaction but easily removable afterwards by methods which do not destroy the desired product. Fortunately, certain aromatic esters are, in fact, more labile to mild acid or alternatively to hydrogenolysis in the presence of catalyst. Hence,

benzyl, phenyl and p-nitrophenyl phosphorochloridates (Fig. 2) are suitable phosphorylating agents. The ineffectiveness of pyrophosphoric acid in this role has already been mentioned. This is caused by the repelling influence of its four anionic groups. If, however, this effect is masked by ester formation then useful reagents are provided. Notably tetra-p-nitrophenyl pyrophosphate (116), which acts in an entirely analogous fashion to the phosphorochloridates, has frequently been used successfully.

A somewhat anomalous, but occasionally effective member of this group is the old established (1864) polyphosphoric acid reagent, obtained by dissolving phosphorus pentoxide in syrupy phosphoric acid (117).

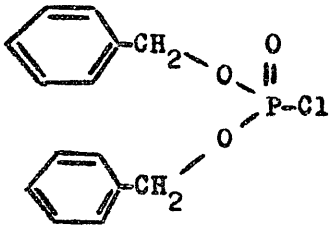
The application of this reagent and the others above mentioned will be deferred to allow consideration of the second group of reagents viz. those dependent upon water elimination by carbodiimides.

(b) Carbodiimides.

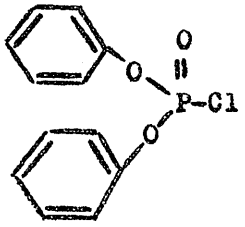
The group of compounds referred to as carbodiimides contain the structure -N=C=N- ; hence a typical member e.g. dicyclohexylcarbodiimide is represented thus :-

F I G. 2.

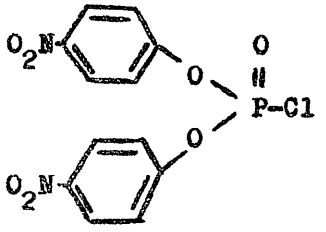
P H O S P H O R Y L A T I N G A G E N T S. (ANHYDRIDES)



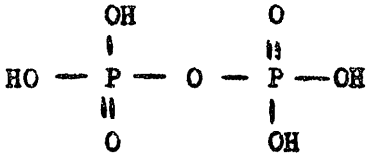
DIBENZYL PHOSPHOROCHLORIDATE.



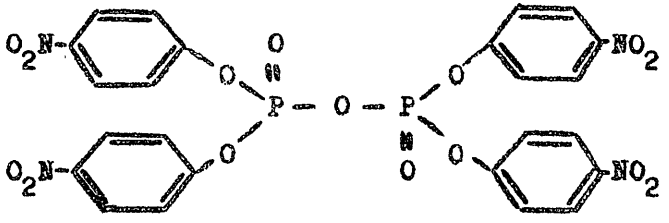
DIPHENYL PHOSPHOROCHLORIDATE.



DI-P-NITROPHENYL PHOSPHOROCHLORIDATE.



PYROPHOSPHORIC ACID.



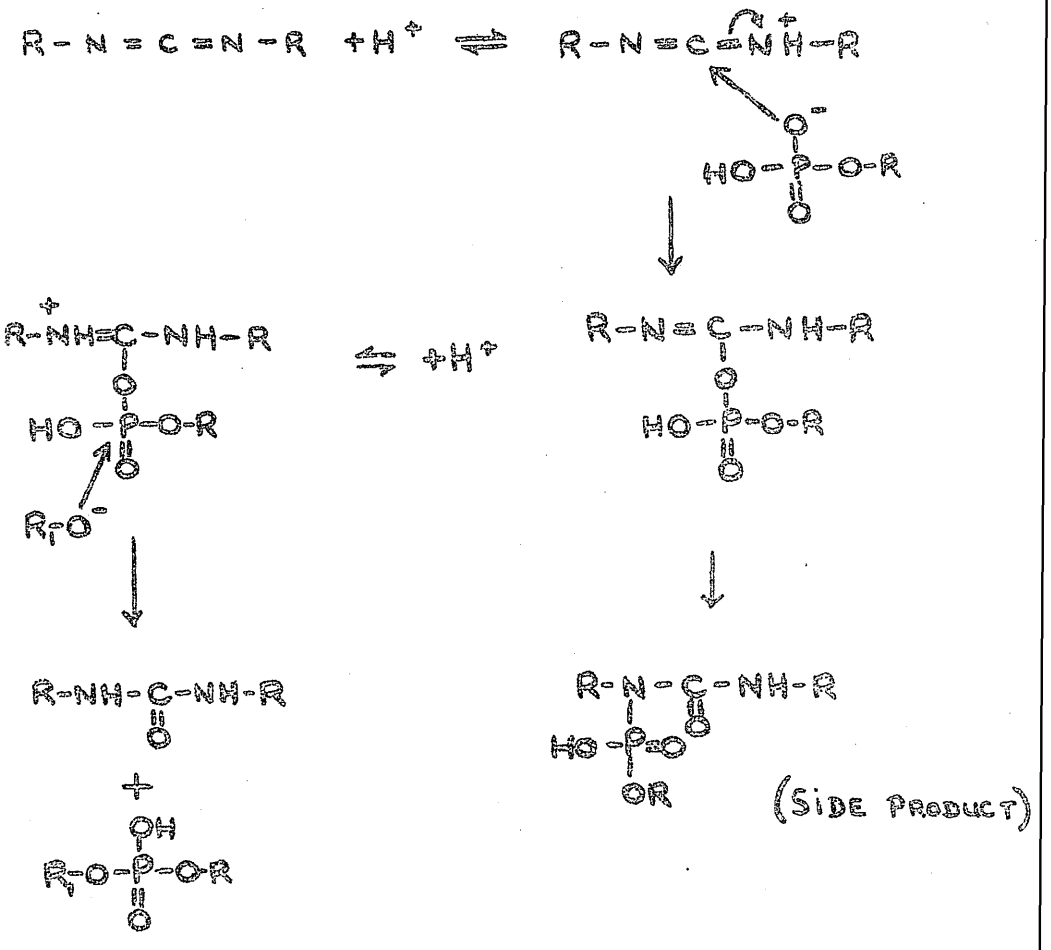
TETRA-P-NITROPHENYL PYROPHOSPHATE.

This particular compound and to a lesser extent certain others, such as p-tolylcarbodiimide have been used in the preparation of peptides, anhydrides of phosphoric and acetic acids, phosphoramidates etc. Their use has been reviewed by Khorana (119).

Their utility depends upon the fact that they readily undergo a two stage addition of the elements of water withdrawn from two reacting molecules, resulting in condensation. This can often be accomplished under partially aqueous conditions, thus the carbodiimides are versatile and powerful reagents in many chemical syntheses. Both symmetrical and unsymmetrical products can be made satisfactorily by judicious choice of carbodiimide and by attention to various experimental factors.

For the purpose of phosphorylation, a suitable reagent, which can now be an inorganic salt of orthophosphoric acid or more usually a blocked reagent e.g. cyanoethyl phosphate or monobenzyl phosphate in a basic medium, is allowed to react with the material to be phosphorylated in the presence of the carbodiimide.

Although alternative mechanisms have been proposed (34) the following scheme due to Khorana appears to be the one most widely accepted to account for the condensation of phosphate to an alcohol grouping by carbodiimides :-



Protonation of a nitrogen induces a positive charge on the carbon which results in attachment of the phosphorylating species - the strongest acid present - to this carbon. The rate of this step is governed by the acid strength, the pH of the solution and the carbodiimide used. Protonation of the other nitrogen leads to a redistribution of charges and the appearance of a positive charge on the phosphorus. Nucleophilic attack by the alcohol to be phos-

phorylated is thus facilitated and the carbodiimide (in hydrated form i.e. the urea derivative) is eliminated and the required product obtained.

Undesirable migration which results in the formation of an N-phosphate bond may occur occasionally but this is inhibited at higher pH values.

The use of cyanoethyl phosphate in conjunction with dicyclohexylcarbodiimide (DCC) proved to be the most generally successful method used in this work; a detailed account is given in a later section.

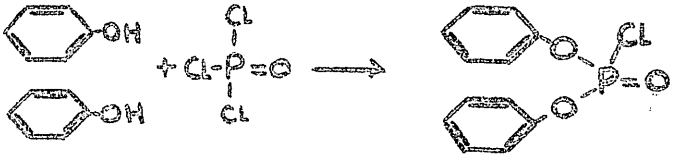
(2) Comparison of the efficacy of three reagents of the first category (anhydride type) for the synthesis of uridine-5'-phosphate as test substance.

Diphenyl phosphorochloridate, dibenzyl phosphorochloridate and polyphosphoric acid were the three reagents of this group which were used in preliminary work on the phosphorylation of blocked nucleosides. At the same time, trials were being carried out on the carbodiimide method; because this latter method proved immediately adaptable and versatile no extensive attempts were made to apply the phosphorochloridate method in all the required syntheses. Hence, what follows is a brief account of one or two of the more

successful applications of this method presented mainly for purposes of comparison with results obtained by the carbodiimide method.

(a) Diphenyl phosphorochloridate.

Diphenyl phosphorochloridate, the first successful monofunctional anhydride type reagent to be introduced (120) was made (Expt. 5) by refluxing phosphorus oxychloride with two equivalents of phenol for 6 hours.



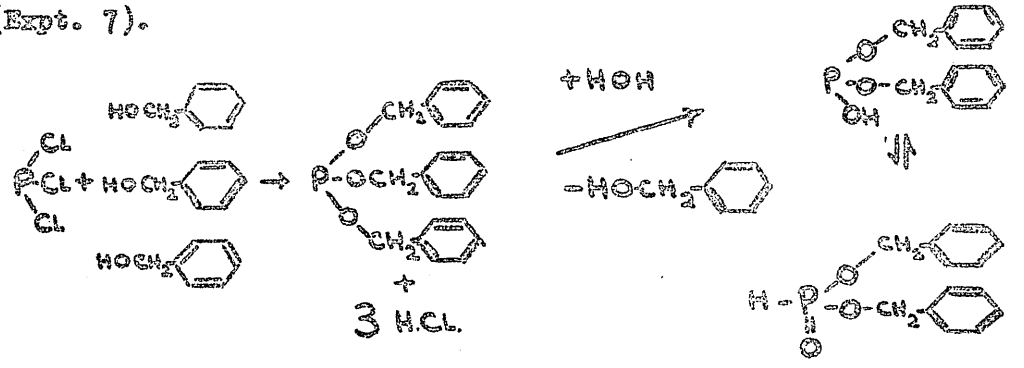
The product was obtained by fractional distillation at a temperature of 178° and 6 mm. Hg in 57% yield.

This was used in attempts to synthesise uridine-5'-phosphate but its use was discontinued in favour of the alternative dibenzyl phosphorochloridate. Thus, although the coupling of the reagent to the blocked nucleoside proceeded satisfactorily (Expt. 6), hydrogenolysis of the phenyl groups to liberate the required 5'-phosphate was difficult and was sometimes not complete even after 8 hours in the presence of excess platinum oxide catalyst.

(b) Dibenzyl phosphorochloridate.

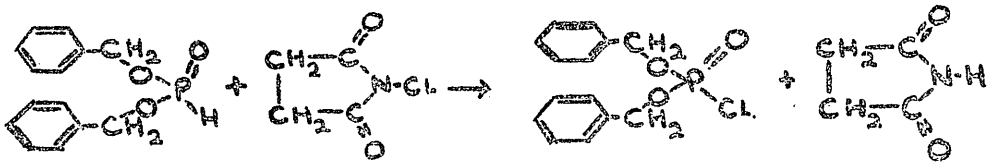
Dibenzyl phosphorochloridate was not so troublesome in this respect in that hydrogen uptake in the presence of palladium-charcoal catalyst was generally rapid. Occasionally, however, debenzylation was incomplete which detracted considerably from the value of the method for the present purposes (121).

The reagent was made by the method of Todd (122) and involved the refluxing of phosphorus trichloride with benzyl alcohol in the presence of dimethylaniline to remove the HCl eliminated (Expt. 7).



The dibenzyl phosphite obtained on thorough stripping of excess reagents under high vacuum could be distilled with some hazard at 170° and 10^{-5} mm. of Hg but experience showed that this was unnecessary. Provided the reactants were pure the dibenzyl phosphite obtained after thorough stripping crystallised satisfactorily at 7° as a pale yellow mass. The product could be stored indefinitely in the cold and suitable quantities removed as

required for conversion into the phosphorochloridate. This was done by dissolving the phosphite in benzene followed by the addition of an equivalent of N-chlorosuccinimide (123) (Expt. 8).



The chlorination proceeds at room temperature and is followed by observing the slow precipitation of succinimide from the benzene solution. After filtration the benzene may either be distilled leaving a pale yellow oil of the dibenzyl phosphorochloridate which must be used immediately or else the benzene solution is used directly.

In the attempted preparation of uridine-5'-phosphate with this reagent, isopropylidene uridine was dissolved in dry pyridine cooled to -40° and excess dibenzyl phosphorochloridate added (Expt. 9). The reaction was allowed to proceed for 3 hours at -40° then left overnight at room temperature. The gum obtained was dissolved in ethanol and subjected to hydrogenolysis using palladium-charcoal as catalyst. After removal of the catalyst and mild acid treatment to hydrolyse the isopropylidene residues, inorganic phosphate was removed by a barium fractionation (G.N. 7-2a). The uridine-5'-phosphate was then isolated after concentration by the addition of four volumes of alcohol in a 20% yield. Using a similar

technique Brown and Todd (124) obtained uridine-5'-phosphate in 44% overall yield. As will be seen later uridine-5'-phosphate was prepared in 70% yield by the simpler carbodiimide method. It was thus evident that no advantage would ensue in attempts to obtain fractional improvements in yield by the phosphorochloridate method and its use was discontinued for this purpose.

In addition, the experience of Moffat and Khorana (116) had indicated the unsuitability of dibenzyl phosphorochloridate for the synthesis of guanosine-5'-phosphate. No such restriction applied to the carbodiimide route, this method being apparently powerful enough to enable the phosphorylation of all the nucleosides.

(c) Polyphosphoric acid.

The polyphosphoric acid method was also briefly investigated. This reagent first described in 1864 is made by dissolving phosphorus pentoxide in syrupy phosphoric acid (Expt. 10). The phosphorylating species (125) is believed to be a three to five unit phosphoric anhydride chain and the anhydrous conditions which obtain, reduce the acidity of the reagent to an acceptably mild level. Thus it can be used for the phosphorylation of acid stable pyrimidine nucleosides, but the purine nucleosides are too labile and are cleaved to free ribose and base.

For purposes of comparison with the other available reagents

it was used for the synthesis of uridine-5'-phosphate.

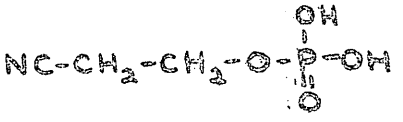
Excess reagent was added to dry isopropylidene uridine and the reaction allowed to proceed for two hours at 60° during which time some charring occurred (Expt. 11). At the end of this period water was added resulting in the liberation of orthophosphoric acid by hydrolysis of the reagent which, in turn, removed the isopropylidene groups from the nucleoside phosphate. This excess acid was neutralised with caustic soda solution, the inorganic phosphate removed with barium acetate and the uridine-5'-phosphate isolated by adding four volumes of ethanol to the clear mother liquor (G.M. 7-2a). The yield by this method was a satisfactory 45%, but as stated the reagent has only limited application.

(3) <u>The use of reagents of the second category (carbodiimides) in conjunction with suitable phosphate donors; the particular advantages of cyanoethyl phosphate in this role.</u>

The generally accepted mechanism for condensations effected by use of carbodiimides has been mentioned earlier in this Section (p47 It will be recalled that the net result is the abstraction of the elements of water from the phosphorylating agent and the hydroxyl or other group to be phosphorylated resulting in condensation with conversion of the carbodiimide to the corresponding urea. Thus the

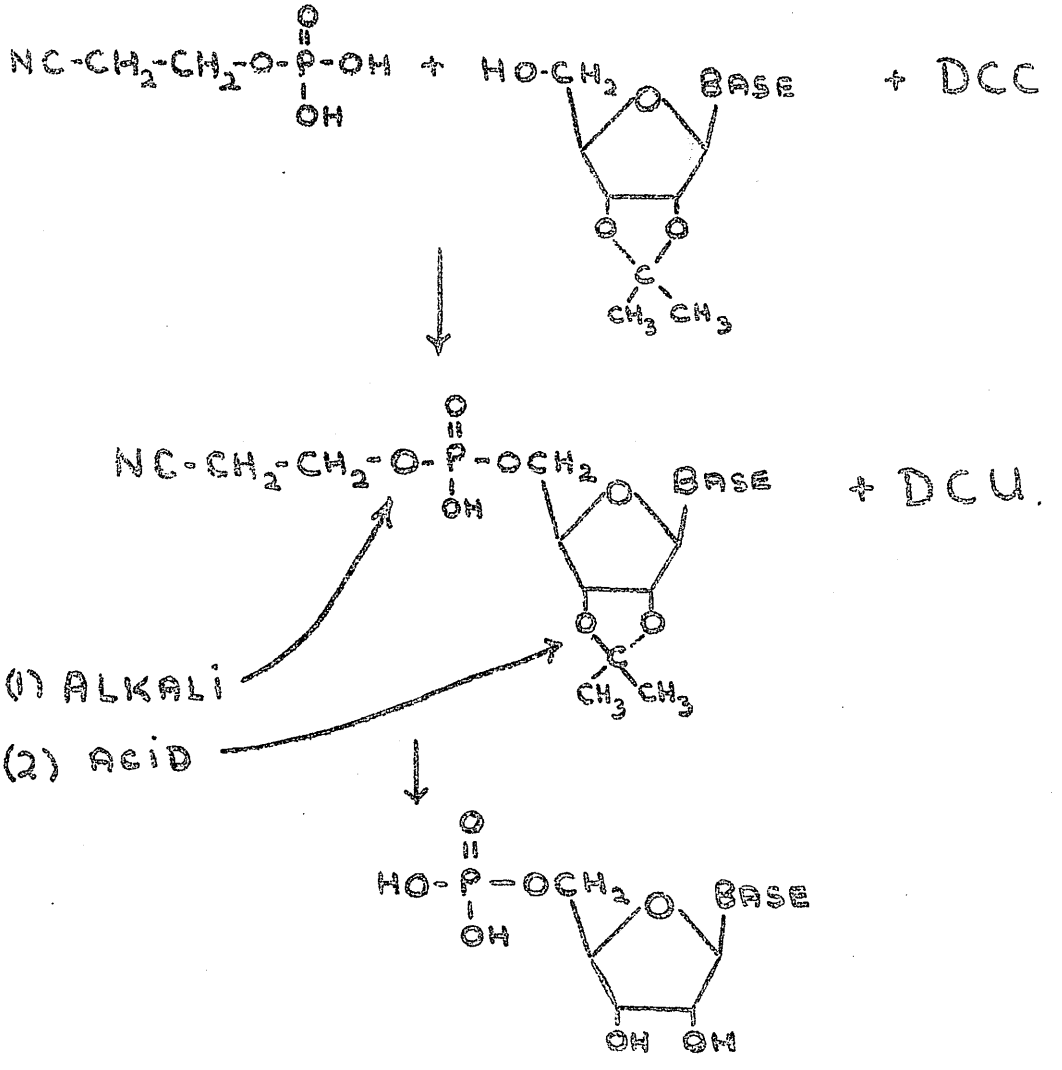
phosphate group of the reagent must have a free hydroxyl function and, indeed, the method even allows the use of inorganic phosphate in this role. In such a case, however, excess of the hydroxylic component is required to prevent formation of diesters and it is more customary to use a blocked phosphate to circumvent this difficulty.

In common with all phosphorylating agents the blocking group must be easily removed without destruction of the desired product. Hence, benzyl groups which can be hydrogenolysed are suitable. Tener, whose work contributed extensively to popularising the carbodiimide method introduced monobenzyl phosphate as a reagent (126). This was soon superseded by cyanoethyl phosphate (127) :-



This was even more suitable because the cyanoethyl group was removable by mild alkaline hydrolysis. Thus the occasionally troublesome hydrogenolysis step was circumvented by use of this reagent. The alkali lability of the cyanoethyl group is consistent with the behaviour of phosphate esters which have an electron withdrawing group in β relation to the phosphorus (34). Thus glyceraldehyde-3-phosphate is similarly labile.

In summary, conversion of a blocked nucleoside to a nucleotid by means of cyanoethyl phosphate in presence of DCC occurs as follows:-



(a) Preparation of cyanoethyl phosphate. (CEP)

Two methods for the preparation of cyanoethyl phosphate have been described by Tener (127). The first procedure, adopted without modification in this work, involves the slow addition of ice

cold cyanoethanol in pyridine to an ether solution of phosphorus oxychloride, the reaction being cooled meantime (Expt. 12). After removal of inorganic phosphate with barium acetate, the barium salt of cyanoethyl phosphate was obtained in 70% yield by precipitation of crystalline platelets with ethanol. This product could be stored indefinitely at room temperature without deterioration.

The alternative method requires the use of polyphosphoric acid for the phosphorylation of the cyanoethanol and appears to have no advantages over the above.

In the phosphorylations to be described the solvent of choice is pyridine, hence, it is advisable to use the cyanoethyl phosphate also in pyridine solution i.e. as its pyridinium salt.

The barium salt was, therefore, converted into the free acid form by treatment with cation exchange resin in the hydrogen form (G.M. 9-1). Addition of pyridine followed by removal of water by vacuum distillation gave a pyridine solution of the pyridinium salt, the concentration of which was adjusted to a convenient 1m. mole/ml (Expt. 13).

The dicyclohexylcarbodiimide (DCC) used in the condensations to be described was a good quality commercial product.

The plan adopted in the following discussion of the phosphorylations of the isopropylidene nucleosides is to discuss a typical example (uridine-5'-phosphate) in full and to deal with the remainder

more briefly. Many details are common to each account. Repetition is thus avoided and scope is provided for drawing attention to procedural variations which are unique to a particular preparation. Full experimental details are, however, provided in each case in the Section devoted to this.

(b) Preparation of uridine-5'-phosphate. (using DCC and CEP)

When isopropylidene uridine is phosphorylated with cyanoethyl phosphate in pyridine in the presence of DCC, phosphorylation of the pyrimidine ring hydroxyl substituent may also occur as a side reaction. In practice this does not result in any serious contamination of the required end product because during an acid hydrolysis step in the isolation procedure the pyrimidine-OH phosphate is hydrolysed. However, this side reaction reduces the effective amount of cyanoethyl phosphate available for the phosphorylation of the C-5 position of ribose and it is necessary to use a substantial excess of reagent for this reason.

Hence, to isopropylidene uridine (Expt. 14) dissolved in dry pyridine, a three molar excess of the standard cyanoethyl phosphate (CEP) solution in pyridine was added. The water content of the mixture was reduced to a minimum by repeated azeotropic distillation with pyridine under vacuum. A five molar excess of DCC in dry pyridine was then added and the reaction allowed to proceed for about

6 hours at 26°. During this time the progress of the phosphorylation was followed by paper electrophoresis of samples withdrawn from the reaction vessel in M/50 phosphate buffer pH 7.5 (G.M. 2). For this purpose the samples for electrophoresis were treated with alkali to remove the cyanoethyl blocking group. This served to increase the mobility at pH 7.5 of any isopropylidene uridine-5'-phosphate formed, since at this pH both of the phosphate hydroxyls are fully dissociated. Thus electrophoresis for 1 hour at 600 volts (30v/cm) provided adequate differentiation between the mobile phosphate derivative and the immobile non-phosphorylated blocked nucleoside.

In practice, for the alkaline hydrolysis of the blocking group it was sufficient merely to add an equal volume of concentrated ammonia and to hydrolyse at 60° for 30 minutes. This procedure differed from the technique eventually used for the final isolation of the main product when it was usual to use 0.5N lithium hydroxide. Both procedures, however, are satisfactory and can be used interchangeably.

When, after 6 hours, phosphorylation was judged to be complete, water was added to convert excess DCC to dicyclohexylurea (DCU), the pyridine being distilled off under vacuum and the gum obtained triturated with excess 0.5N lithium hydroxide. Heating this alkaline suspension for 2 minutes at 100° is sufficient to effect hydrolysis of the cyanoethyl group from the isopropylidene

uridine-5'-derivative of cyanoethyl phosphate (or any other diester). The heating was nevertheless continued for an hour in order to decompose unused cyanoethyl phosphate to cyanoethanol and inorganic phosphate. After filtration to remove insoluble material (mainly DCU), the clear filtrate was passed through a column of cation exchange resin in the hydrogen form (C.H. 9-1) giving a solution of pH ca 2.5. This acidity was sufficient to effect hydrolysis of the isopropylidene group upon refluxing the solution for 90 minutes.

Adjustment of the pH of the cooled solution to 7.5 followed by precipitation of the inorganic phosphate at this pH by addition of barium acetate gave a solution from which barium uridine-5'-phosphate could be precipitated by the addition of four volumes of ethanol (C.H. 7-2a).

At this stage and following the practice adopted for all four nucleoside-5'-phosphates and the nucleoside diphosphate sugar compounds, a further purification step was included. As this is a procedure of wide applicability it is described in detail in C.H. 7-1b (128).

Briefly, its success depends upon the fact that lithium salts of nucleotides are soluble in dry methanol but insoluble in dry acetone, whereas lithium phosphate and lithium salts of sugar phosphates are insoluble in methanol.

Thus, conversion of the barium salt of uridine-5'-phosphate

to its free acid form by passing a water solution of the salt through a cation exchange resin in the hydrogen form (G.M. 9-1) was followed by neutralisation of the acid solution to pH 7.0 with lithium hydroxide using a pH meter. After careful removal of water followed by a final thorough drying of the residue by azeotropic distillation with dry methanol, the residue was triturated thoroughly and repeatedly with dry methanol. The insoluble residue, consisting mainly of inorganic phosphate, was discarded. The lithium salt of uridine-5'-phosphate was recovered from its methanol solution in 85% yield based on the amount of crude barium salt used, by the addition of 10 volumes of dry acetone.

(c) Preparation of cytidine-5'-phosphate. (using DCC and CEP)

Attention was drawn previously (p. 57) to the possibilities that exist of side reaction due to phosphorylation of heterocyclic ring substituents. As explained in the case of uridine-5'-phosphate such products are destroyed by the hydrolytic conditions involved in subsequent isolation steps. Unfortunately, such is not the case with the side product formed when the ring amino substituent of isopropylidene cytidine becomes phosphorylated. Several unsuccessful attempts to prepare cytidine-5'-phosphate were made before it was realised that this side product survived the conditions hitherto regarded as severe enough for its destruction.

Phosphorylation of isopropylidene cytidine, electrophoresis of samples, removal of cyanoethyl groups with lithium hydroxide, detailed in Expt. 15, were essentially similar to the procedures used for uridine-5'-phosphate. In the latter case the removal of isopropylidene groups was effected by heating a mildly acid (pH 2.5) solution of crude isopropylidene uridine-5'-phosphate for 90 minutes. It was expected that these conditions would be adequate for the dephosphorylation of pyrimidine amino groups. Unsatisfactory yields of final product and aberrant analytical results for phosphate led to the realisation that this was not so. Thus, the yield of supposed barium cytidine-5'-phosphate was anomalously high, whereas when attempts were made to isolate the product as its lithium salt the yields of the latter were much lower than expected. In addition, much UV absorbing nucleotide material at this stage remained insoluble in the methanol, contrary to the usual behaviour of lithium salts of nucleotides (p.59). However, treatment of this methanol insoluble material with 0.2N hydrochloric acid for 30 minutes at 100°, followed by neutralisation of the acid to pH 7.0 with lithium hydroxide and freeze drying, gave a product largely soluble in dry methanol. The insoluble portion was mainly lithium phosphate and the soluble UV absorbing component precipitable with excess acetone, was characterised as the lithium salt of cytidine-5'-phosphate obtained in an overall yield of 61%.

(d) Preparation of adenosine-5'-phosphate. (using DCC and CEP)

The procedure for the phosphorylation of isopropylidene adenosine (Expt. 16) did not differ substantially from that used in the preparation of uridine-5'-phosphate. Two details of modification are worthy of mention.

In the first place, isopropylidene adenosine is not as soluble in pyridine as the uridine analogue and somewhat larger amounts of solvent had to be used. It was also advisable to aid solution by gentle warming. Reaction was also slower as evidenced by the paper electrophoresis assay technique (C.H. 2) but was generally complete in 15 hours at room temperature. Details of isolation provided in the Experimental Section, are almost identical with those given for uridine-5'-phosphate. The lithium salt of adenosine-5'-phosphate could consistently be obtained in overall yield of 60%.

(e) Preparation of guanosine-5'-phosphate. (using DCC and CEP)

The solubility of isopropylidene guanosine in pyridine is even lower than is that of the adenosine derivative alluded to above. Various alternatives have been tried to overcome this difficulty. The use of excess pyridine is inconvenient and unsatisfactory. The addition of dimethyl formamide or other polar solvent can assist in solubilising the starting material, but

the most satisfactory technique used in this work was based on one suggested by Toner (127).

If the isopropylidene guanosine was gently warmed with a pyridine-water mixture containing the pre-dissolved pyridinium salt of cyanoethyl phosphate (CEP), solution of the former was slowly accomplished. Clearly, in the absence of DCC, phosphorylation cannot occur and the solubilising effect is not caused by reaction of a heterogeneous mixture but by a genuine contribution to the solution of the isopropylidene derivative by the CEP and the water. When solution was complete the water was carefully but thoroughly removed by repeated azeotropic distillation at a bath temperature of 30° with dry pyridine, which did not normally result in the deposition of isopropylidene guanosine from solution.

Subsequent operations upon this homogeneous reaction mixture, detailed in Expt. 17, did not differ materially from those previously discussed until the stage involving isolation of the crude product as its barium salt was reached.

It will be recalled that the barium salt of uridine-5'-phosphate was separable from barium phosphate by virtue of the solubility of the former in water as opposed to its insolubility in 80% ethanol (G.M. 7-2a). Application of this procedure to the present case resulted in unacceptably high losses of nucleotide and it became apparent that the barium salt of guanosine-5'-phosphate was

being co-precipitated with barium phosphate from aqueous solution at pH 7.5. The difficulty was eventually overcome by precipitation of inorganic phosphate with barium bromide from hot aqueous solution at pH 7.5. Under these conditions the guanosine-5'-phosphate remained soluble, enabling filtration of the barium phosphate. The subsequent addition of 4 volumes of ethanol to the filtrate resulted in the precipitation of the barium salt of guanosine-5'-phosphate in yields of 65 to 70%, uncontaminated with any significant amounts of inorganic phosphate.

Further purification of the barium salt by its conversion to the lithium form (G.M. 7-1b) gave an overall yield of guanosine-5'-phosphate of 65%.

SECTION III.

THE PREPARATION OF ANOMERIC SUGAR PHOSPHATES.
(ALDOSE-1-PHOSPHATES AND KETOSE-2-PHOSPHATES)

(1) Assessment of directive influences operating during acetylation, halogenation, and methylation at the anomeric carbon of sugars.

The chemical reactivity of the C-1 hydroxyl of aldoses and the C-2 hydroxyl of ketoses as compared with that of other hydroxyl groups of sugars is thought to be due to the electromeric effect of the adjacent ring oxygen. The existence of a large and varied series of derivatives including polymers in nature all of which arise as a consequence of reaction at C-1, however accomplished, underlines the special properties and importance of this functional group.

The C-1 hydroxyl is, of course, capable of assuming an α or β configuration with respect to the two major ring forms of sugars viz. pyranose and furanose and derivatives of all these possible alternatives are frequently found.

A matter of great interest to organic chemists and of prime importance in the present work is to be able to predict with reasonable certainty, whether, in attempts to synthesise a C-1 derivative of an aldose sugar (C-2 of ketoses) the ultimate product

will be in the α or β form. Frequently this requires a knowledge of whether some intermediate step involving reaction at the anomeric carbon has resulted in inversion, or whether some subsequent step will, in effect, reverse this inversion.

Although it is now customary to invoke mechanistic arguments to interprete and to predict the course of organic reactions it is nevertheless noteworthy that these same arguments cannot be applied to substitutions into heterocyclic rings (of sugars) with equal precision.

The reactions of sugars at the anomeric carbon which have been most widely studied from the standpoint of suitability of reagents and experimental conditions in the past have been :-

(a) the formation of acyl derivatives e.g. α and β penta-O-acetyl glucose,

(b) the conversion of these into their respective C-1 bromo-derivatives and

(c) the formation of α and β glycosides.

The aim of the discussion for the next 16 pages will be to examine what principles of mechanism have been, or can be deduced from a knowledge of the conditions under which the above listed compounds are formed and to see whether these principles can be extrapolated to deal with the complexities introduced by using various reagents to phosphorylate this anomeric position.

Returning to the derivatives (a), (b) and (c) above mentioned, the complexity of the situation can be best illustrated by commenting upon examples of each in turn:-

(a) If the experimental conditions for the formation of penta-O-acetyl glucose are alkaline (pyridine-acetic anhydride) or at most only faintly acid (acetic anhydride) the product is almost exclusively the β form.

If acid conditions are utilised however, (acetic acid-zinc chloride or acetic anhydride-sulphuric acid mixtures) the product obtained is the α form. Furthermore, if β penta-O-acetyl glucose itself is submitted to these self-same acid conditions it is converted into the α form (129). Strongly basic conditions (solid sodium hydroxide in an inert solvent) are claimed by Wolfrom (130) to produce the same effect.

Similar effects are produced with other sugars of the D-series.

(b) C-1 bromo-derivatives of sugars, important as intermediates in synthetic reactions, are usually made by conversion of the penta-O-acetyl sugar into the corresponding 1-bromo-tetra-O-acetyl derivative using a reagent such as HBr in glacial acetic acid. Sometimes the alternative procedure of starting with the free sugar and treating with a reagent which effects acetylation in situ, followed by bromination of the anomeric carbon, is used (131).

For the common D-aldohexoses for which information is available, unless exceptional precautions are taken, the bromo-derivative produced under these acid conditions irrespective of the sugar or of the anomeric form of its penta-O-acetyl derivative used as starting material is always the α form.

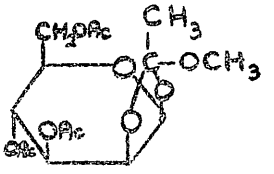
(c) Methyl glycosides, as examples of C-1 ethers, can be made by one of two usual methods. More commonly the Fischer procedure (132) is used, which involves the passage of dry HCl gas into the sugar dissolved in anhydrous methanol. The situation is, of course, complicated by virtue of the fact that both furanose and pyranose products are possible, but with regard to the pyranose derivatives the predominant product is the α-methyl derivative. This is a further example of the α product being produced under acid conditions.

The other common procedure for glycoside synthesis is via the appropriate 1-halogeno derivative utilising the Koenigs-Knorr reaction (133). Thus, for example, the 1-bromo-O-acetylated sugar in the obligatory α form is reacted with the alcohol in the presence of silver oxide or carbonate which removes the HBr liberated and maintains a mildly alkaline environment for the reaction.

When the parent sugar is glucose, galactose or xylose, the product is the β-glycoside, which means that an inversion has taken place.

Mannose is notable in behaving exceptionally, and

depending upon the precise conditions, two products may be formed. One is the ortho-ester:-



which is produced in response to a stereochemical situation which will be discussed later. The other product is the expected glycoside, mainly in the α form, which indicates a mechanism favouring retention of configuration.

Several attempts have been made to accommodate such facts as are exemplified above into adequate schemes of mechanism (134, 131, 129). Such arguments have a relevance to an understanding of phosphorylations at the anomeric carbon of sugars. Hence, there follows a summarised account of the main items of evidence and theory which can be marshalled to accommodate the above facts. This is followed by an attempt to apply similar reasoning to the expected results obtainable by the use of various reagents and conditions for phosphorylations at C-1.

Haworth ring structures for pyranose sugars do not provide an accurate picture of the positions of substituents in relation to each other and to the ring. Reeves (135) and others have recognised that the pyranose ring of sugars is conformationally analogous to

cyclohexane and based upon arguments previously used by Barton and co-workers (136) there is now the realisation that many features of the behaviour of sugars towards esterifying reagents etc. can be more easily understood by applying similar reasoning.

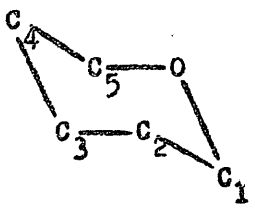
As is the case with cyclohexane a six membered pyranose ring cannot be planar. It can in theory assume any one of eight puckered conformations. Of these, six are boat shaped and two are in the chair form. As can be seen from constructing models, the boat conformations require that groups in these, assume a high degree of crowding, do not permit of maximal staggered arrangements (Fig. 3) and are therefore unstable.

Of the two chair forms as exemplified by β-glucopyranose (Fig. 4), one conformation maintains the bulkier hydroxyl substituents in an axial relationship to one another, whereas in the other chair form an equatorial arrangement is obtained. The former, despite the absence of eclipsing of groups, is unfavoured because the axial arrangement brings the bulky substituents into closer proximity than does the equatorial; hence the latter is favoured. This particular case serves to illustrate the complexity of the situation. It might be argued that α-glucopyranose, being that the anomeric hydroxyl is axial, should be considerably less stable than the β form. That this is not so is explained (135) by taking into account the contribution made by the massive ring oxygen in repelling the anomeric

F I G. 3.

THE EIGHT POSSIBLE STRAINLESS RING FORMS OF THE PYRANOSE RING.

(2 CHAIRS AND 6 BOATS)



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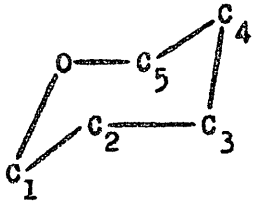
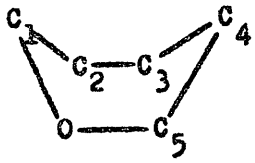


1 C



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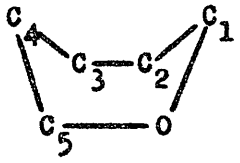

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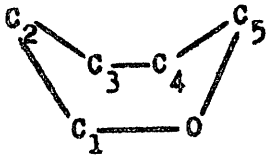

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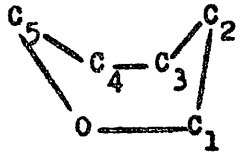

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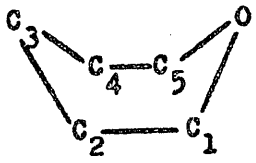

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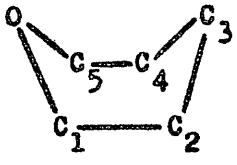
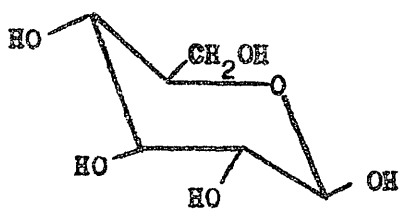


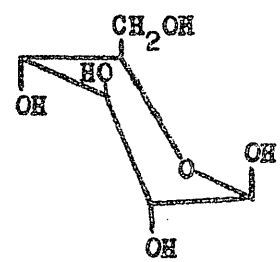
3 B

F I G. 4.

β - G L U C O P Y R A N O S E. (CHAIR FORMS)

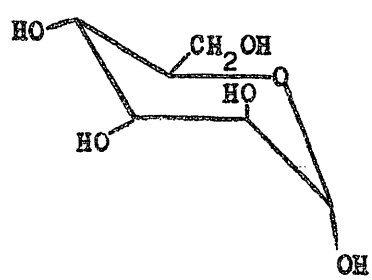


EQUATORIAL
(STABLE)



AXIAL
(UNSTABLE)

α - M A N N O P Y R A N O S E. (STABLE CHAIR FORM)



hydroxyl into the otherwise unfavourable axial position. Hence, both α and β forms exhibit approximately equivalent stabilities.

The general validity of this argument is substantiated by noting that β substituted mannopyranose derivatives either do not exist or are prepared only with the greatest difficulty. Fig. 4 illustrates the mannopyranose ring in its favoured form. The bulky substituents on C-5, C-4 and C-3 respectively, which are $-CH_2OH$, -OH and -OH assume the staggered equatorial position, thus forcing the hydroxyl on C-2 to assume an axial relation to the ring. The configuration of substituents on the anomeric carbon are thus under the strong directing influence of two large groups on either side of and above the general plane of the ring viz. the ring oxygen on one side and the axially orientated hydroxyl of C-2 on the other (Fig. 5). Consequently α derivatives of mannose predominate irrespective of the nature of the anomeric addendum or of the conditions.

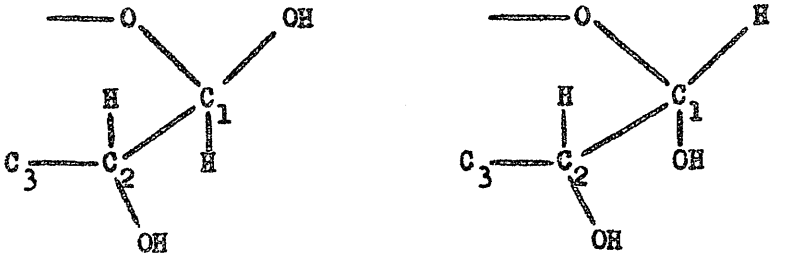
If mannose is acetylated the O-acetyl on C-2 plays a direct role which reinforces the group effects described (see p. 81). Hence, owing to this further effect the likelihood of obtaining a β derivative of mannose via the Koenigs-Knorr reaction using the O-acetylated sugar is even further reduced.

Thus these two powerful effects may operate to ensure that mannose derivatives almost invariably are of the α configuration and more minor influences which may become manifest in the case of

F I G. 5.

ARRANGEMENT OF GROUPINGS AT C-1 AND C-2 OF PYRANOSE SUGARS.

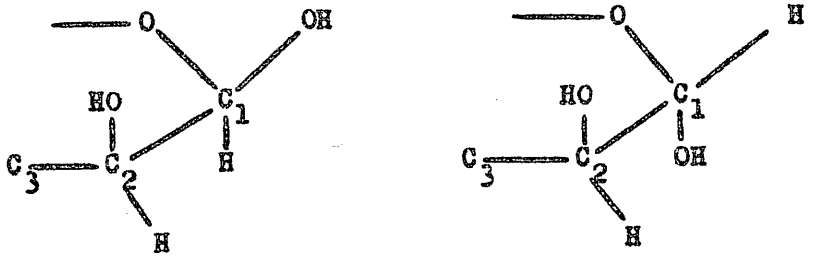
GLUCOPYRANOSE.



(β) (α)

Approximately equal stabilities.

MANNOPYRANOSE.



(β) (α)

Unstable. Stable.

other sugars cannot effect the issue.

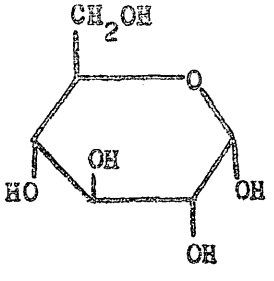
In an attempt to evaluate these other minor influences whose effects can be all-important in the absence of the fortuitous juxtaposition of groups as with mannose, Hassel and Ottar (137) attempted to relate the stability of the α and β forms of glycosides, O-acetyl and halogeno derivatives of sugars with the configuration of the respective sugars. Thus the purpose of their study was to attribute to the various substituents on C-2, C-3, C-4 and C-5 of the pyranose sugar a degree of influence upon the configuration of substituents on C-1.

In brief, they concluded that under conditions which permit anomerisation the group which exerted the greatest influence was that positioned on C-5. In other words in the case of D-glucopyranose the C-5 substituent being $-CH_2OH$ and orientated above the general plane of the ring, influences the C-1 substituent to assume the α or trans configuration. In L-glucopyranose the trans configuration is the β form and is thus favoured (Fig. 6).

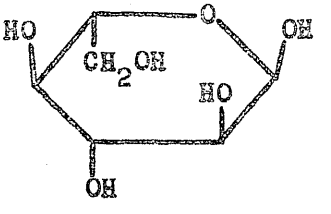
They also noted an analogous subsidiary effect exerted by the C-3 substituent. This effect becomes dominant in sugars where the C-5 substituent is only hydrogen. Thus D-xylopyranose derivatives lacking a bulky group on C-5 assume an α configuration in that the C-3 hydroxyl above the ring directs the substituent into the position trans to itself. The C-3 hydroxyl of D-ribopyranose

F I G. 6.

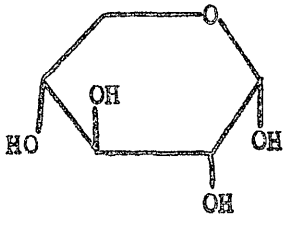
FAVOURED FORMS OF PYRANOSE SUGARS UNDER CONDITIONS WHICH PERMIT ANOMERISATION. (HAWORTH FORMULAE)



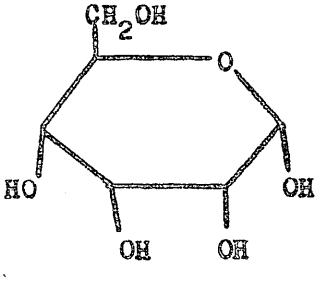
α-D-GLUCOPYRANOSE
(TRANS C-1,C-5)



β-L-GLUCOPYRANOSE
(TRANS C-1,C-5)



α-D-XYLOPYRANOSE
(TRANS C-1,C-3)



α-D-ALLOPYRANOSE
(TRANS C-1,C-5)

is below the ring, hence the favoured derivatives are β in this case.

If a sugar has both C-5 and C-3 substituents on the same side of the ring their directive effects reinforce one another (D-glucopyranose→α); if, on opposing sides, the influence of the C-5 substituent predominates (D-allopyranose→α).

These Hassel-Ottar rules appear to be obeyed without exception, but as emphasised they are applicable only under conditions which permit anomerisation.

Lemieux (134) has reviewed four possible mechanisms whereby the phenomenon of anomerisation can conceivably be brought about. The classical explanation for alpha-beta interconversions of simple sugars, first used to explain the phenomenon of mutarotation, invokes the formation of the intermediate open chain form.

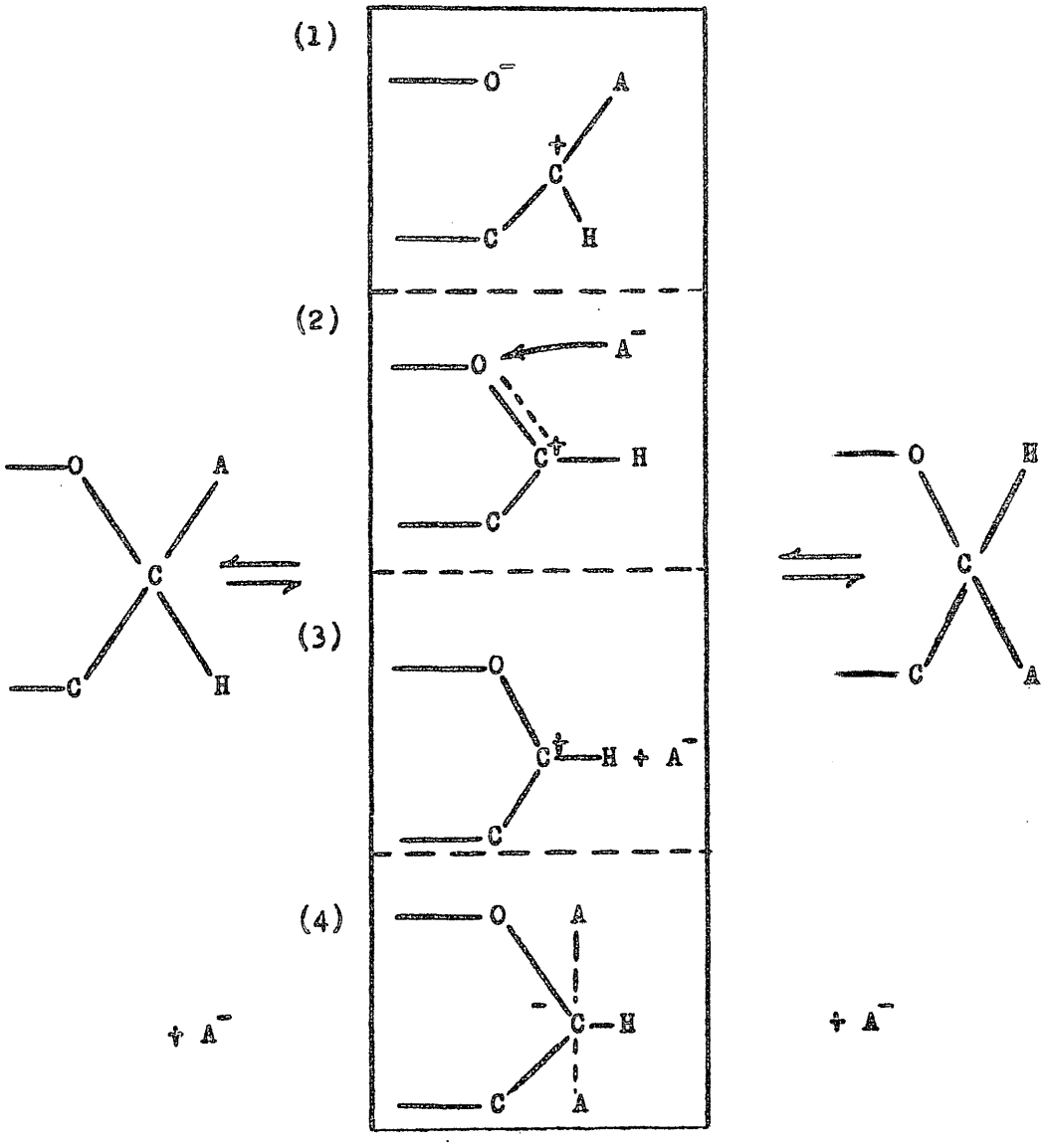
With substituted sugars this mechanism is not wholly debarred but evidence in its favour over alternative proposals, which envisage the formation of carbonium intermediates, is lacking (Fig. 7).

However, the experimental facts concerning anomerisation and considering O-acetylated D-glucopyranose derivatives as examples are as follows:-

In inert neutral solvents both α and β methyl tetra-O-acetyl glucopyranosides are stable and do not anomerise. The conversion of the β to the favoured α form occurs in the presence of

F I G. 7.

POSSIBLE MECHANISMS FOR ANOMERISATION OF SUGARS.



a strong acid catalyst (e.g. sulphuric acid in methanol).

On the other hand the analogous α and β penta-O-acetyl glucopyranosides which can both be isolated, demonstrate the anomerisation effect much more readily. The more weakly acid conditions provided, for example, by stannic chloride in chloroform, are now sufficient to convert the β into the α form.

The corresponding α and β bromo tetra-O-acetyl glucopyranosides do not normally co-exist, spontaneous anomerisation to the α form occurring.

Thus for the three instances cited and for other examples also, the rule appears to be that if the aglycon is derived from a neutral substance, strong acid catalysis induces anomerisation; if derived from a weakly acidic substance, less strong conditions are required and if from a strong acid the process is spontaneous. This generalisation is consistent with the observations that α and β methyl glycosides are readily isolated, β-O-acetyl sugars are less readily obtained (the α readily) and β bromo derivatives are virtually unknown.

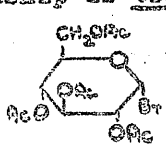
However, in all cases the change is from β to α in the case of glucose. It is inferred that under conditions where this spontaneous change occurs the preferred configuration is formed under the influence of the Hassel-Ottar effects described earlier. Similar considerations can be applied to other sugars.

We have thus far seen how (a) as exemplified by D-manno-pyranose, α derivatives are favoured as the result of C-2-ring oxygen repulsion effects and (b) how, under conditions which permit of free anomerisation the configuration adopted by a sugar C-1 derivative is governed by the rules deduced by Hassel and Ottar.

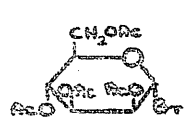
One further important situation remains to be examined viz. the effect of groups attached to C-2 of pyranose sugars upon the reactivity and configuration of C-1 substituents. Clearly, such influences cannot be assessed under conditions which permit anomerisation. Hence what follows describes what takes place on the assumption that conditions are controlled so that the Hassel-Ottar effects are at a minimum.

The importance of the C-2 group is well illustrated by considering the use of the bromo derivatives of the O-acetylated derivatives of glucose and mannose respectively as starting materials for the synthesis of their C-1 methyl glycosides.

The bromo derivatives of both these sugars, for reasons already presented, are in the α configuration. Thus the C-2 O-acetyl of glucose is in *cis* relation to the bromo, whereas in mannose the relationship is *trans*.



Bromo tetra-O-acetyl α-gluco-pyranoside. - *cis* -



Bromo tetra-O-acetyl α-manno-pyranoside. - *trans* -

Under the usual conditions of the Koenigs-Knorr reaction the bromo O-acetylated sugar is dissolved in chloroform or benzene. The methanol is added and the reaction allowed to proceed in the presence of silver oxide to remove the HBr produced. Under these conditions anomerisation of the product cannot take place, hence the configuration of the product will reflect the mechanism of formation and not the eventual configuration as dictated by the directing influences of the Hassel-Ottar effects.

The C-1 methyl glycosides actually formed are in fact methyl tetra-O-acetyl β-glucopyranoside (Fig. 8) and methyl tetra-O-acetyl α-mannopyranoside (Fig. 9). Hence, while configuration is retained in the latter case there has been an inversion in the reaction with the bromo derivative of glucose.

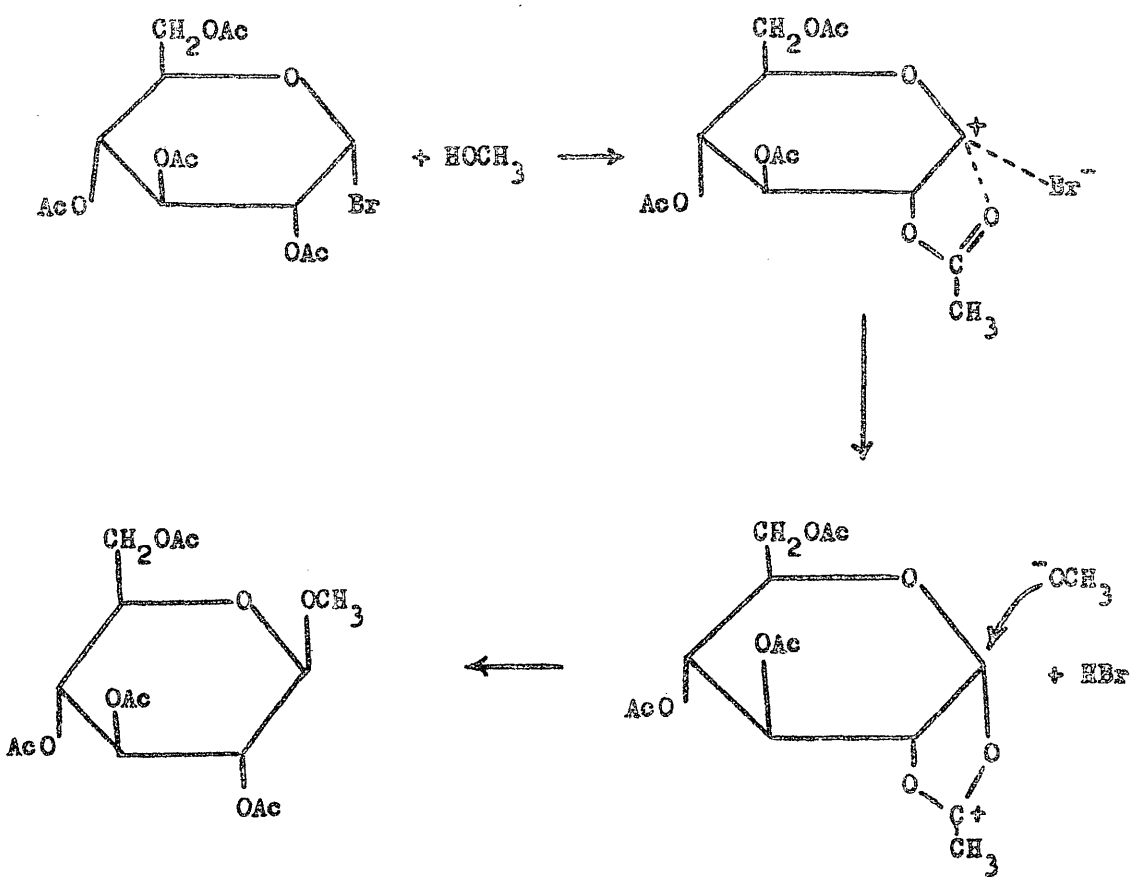
This anomaly has been explained by postulating that the course of the reaction is governed by the initial dissociation of the bromo group which induces a positive charge on C-1 and results in attack by the carbonyl oxygen of the C-2 O-acetyl group upon this positive site (Figs. 8 and 9). There is thus produced a transient cationic intermediate of the "ortho-ester" type which bridges C-1 and C-2. This ortho-ester bridge will be formed on the same side of the ring as the C-2 hydroxyl was found originally.

Because of the trans relationship of the C-1 bromo and the C-2 O-acetyl groups in mannose the ortho-ester derivative forms above

F I G. 8.

PREPARATION OF METHYL TETRA-O-ACETYL GLUCOPYRANOSIDE.

(KOENIGS-KNORR REACTION)



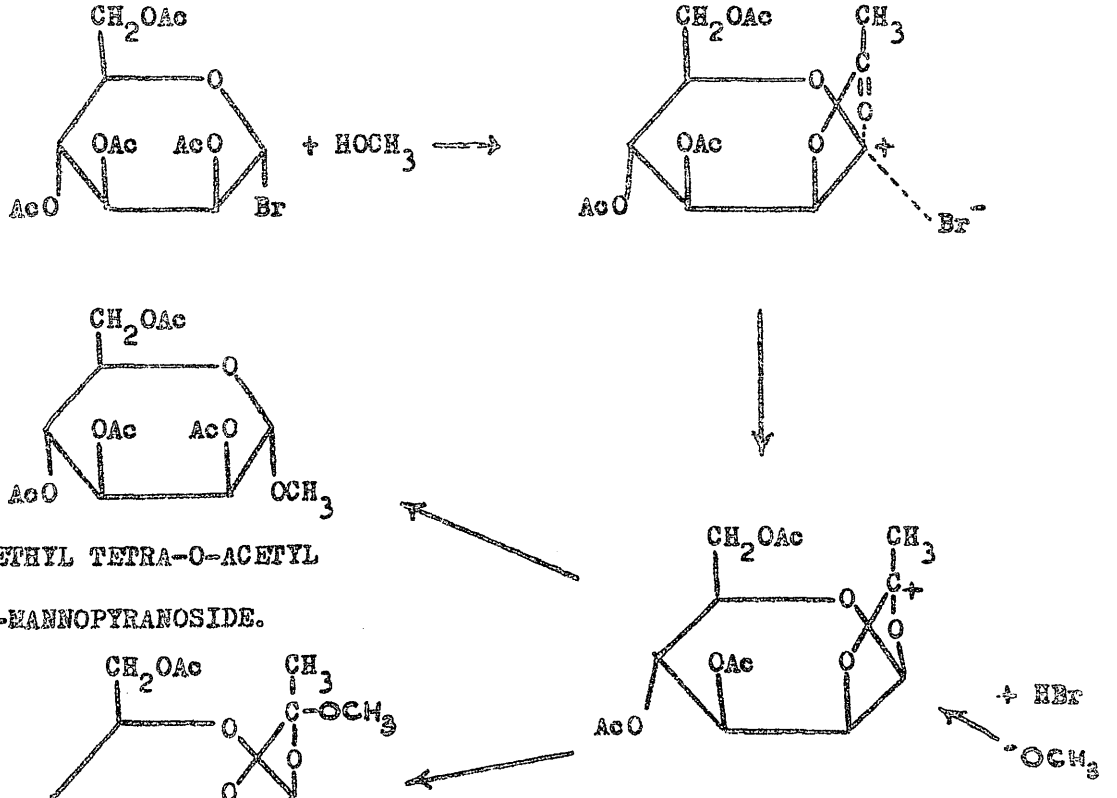
METHYL TETRA-O-ACETYL

β-GLUCOPYRANOSIDE.

F I G. 9.

PREPARATION OF METHYL TETRA-O-ACETYL MANNOPYRANOSIDE.

(KOENIGS-KNORR REACTION)



the pyranose ring and expulsion of the bromine appears to be assisted by nucleophilic attack by the carbonyl oxygen from the opposite side. This would be in agreement with the rapid rate of formation of methyl tetra-O-acetyl α-mannopyranoside found experimentally. With glucose where a cis relationship holds, reaction is considerably slower and this may be due to an interference with the expulsion of the bromine by the C-2 O-acetyl group (131).

The formation of the ortho-ester bridge has a second important effect. The methylation is assumed to occur as the result of nucleophilic attack by methoxyl on C-1, which displaces the ortho-ester oxygen and this whole group then reverts to its original form of an O-acetyl ester on C-2. Clearly, if the ortho-ester bridge is formed above the ring (mannose) methoxyl attack is from the opposite side and the product is the α-methyl derivative. The reverse is thus true in the case of glucose, the β-methyl derivative being formed.

It will be seen that for mannose this neighbouring group effect augments the various other effects already alluded to, all of which combine to ensure the formation of the α derivative.

In the case of glucose it is stressed that the conditions of the Koenigs-Knorr reaction enable the neighbouring group effect to become predominant in directing β-methyl formation. If conditions were adjusted to favour anomerisation (increased acidity of the

medium) the product formed would be the α derivative, although mechanistically this would have been produced via the β derivative.

Similar arguments can be applied to other sugars. The C-2 neighbouring group mechanism is not without some experimental support. Mention has already been made of the relative rates of formation of the methyl derivatives of the tetra-O-acetyl pyranosides of mannose and glucose. In addition, in some cases a stable ortho-ester derivative can actually be isolated. This is formed as the result of preferred methoxyl attack upon the carbonyl carbon after formation of the "ortho-ester cation" bridge (Fig. 9). If such an intermediate is sufficiently stable it may be formed in preference to the methyl O-acetylated sugar derivative (138).

Other C-2 substituents are also not without effect on reactivity at C-1. Thus it is known that glycosides of C-2 deoxy sugars are much more acid labile than the corresponding glycosides of the parent sugars (139). In contrast the glycosides of C-2 amino-C-2 deoxy sugars are only acid hydrolysed with great difficulty. If the amino group is acetylated the corresponding glycoside may then be hydrolysed with approximately the same ease as the parent (C-2 hydroxyl) sugar. This latter effect is attributed to the positive charge on the amino group in acid solution, providing an electrostatic shield for C-1 thus hindering the approach of a proton to effect hydrolysis at C-1 (140).

(2) Assessment of the relevance of these influences upon phosphorylations at the anomeric carbon of sugars.

With these principles in mind some attempt can now be made to ascertain whether, with the reagents customarily used, analogous or explicable results are obtained when the C-1 position of sugars is phosphorylated.

The starting material for such phosphorylations is almost invariably the corresponding halogeno O-acetyl or more rarely the halogeno O-benzoyl sugar derivative (131). Thus the conditions for preparing the C-1 phosphate derivatives of aldoses by this route are those applicable for the Koenigs-Knorr reaction generally.

The phosphorylating agents most widely used for this purpose have been:-

(a) Trisilver phosphate (Ag_3PO_4).

(b) "Monosilver" phosphate (AgH_2PO_4).

(c) Silver diphenyl phosphate ($(C_6H_5O)_2P(O)OAg$).

(d) Silver dibenzyl phosphate ($(C_6H_5CH_2O)_2P(O)OAg$).

Each of the reagents can be reacted with the halogeno O-acetylated sugar derivatives dissolved in a suitable solvent (dry benzene), when during the course of the reaction silver halide is eliminated. Without commenting upon yields it is instructive to compare the products obtained from a typical halogeno sugar derivative

e.g. bromo tetra-O-acetyl α-glucopyranoside with each of the above reagents in turn. These are as follows:-

(a) Trisilver phosphate $\longrightarrow$ α-glucose-1-phosphate (141).

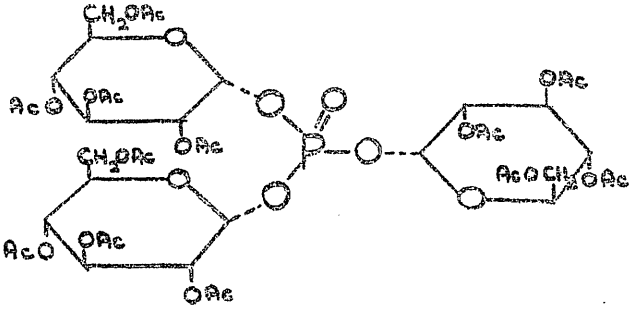
(b) "Monosilver" phosphate $\longrightarrow$ β-glucose-1-phosphate (142).

(c) Silver diphenyl phosphate $\longrightarrow$ α-glucose-1-phosphate (143).

(d) Silver dibenzyl phosphate $\longrightarrow$ β-glucose-1-phosphate (144).

If the arguments used previously for the C-1 O-methyl derivatives are applicable the predicted product should, in each case, be the β-phosphate. The trisilver phosphate and the silver diphenyl phosphate thus appear to have behaved anomalously.

The explanation for these results may be connected with the conditions under which the product is eventually isolated. Thus when trisilver phosphate is the reagent the initial crude product is largely the tri-ester:-



In order to convert this into the monophosphate it is customary to subject it to a mild acid treatment (0.2N HCl in methanol) for some hours (141). This serves to produce the mono-ester, the treatment being interrupted before any substantial

hydrolysis of the latter takes place. Under these conditions anomerisation can be expected to occur, resulting in the formation of the α-phosphate.

The procedure with silver diphenyl phosphate (143) is that after coupling with the bromo O-acetylated sugar, silver bromide and solvent are removed, the residue is taken up in dry alcohol and submitted to hydrogenolysis in the presence of platinum oxide catalyst. The difficulty of removal of phenyl groups requires that hydrogenolysis be continued for several hours and under these conditions anomerisation can also occur. Two factors contribute to produce this result. In the first place mono-dephenylation releases an acidic group at an early stage. The slower removal of the second phenyl group releases glucose-1-phosphate, itself in free acid form. Thus, despite the largely anhydrous conditions the medium is sufficiently acidic and the time sufficiently long to induce conversion of the β into the α form. This is consistent with the observation that the yield of α-glucose-1-phosphate by this method is frequently low, caused by the breakdown of the required product into glucose and free phosphate as the result of the prolonged exposure to these conditions.

The second factor which may contribute is the nature of the aglycon. In the discussion presented earlier on the conditions necessary to induce anomerisation in a variety of C-1 substituted

products (p. 77), it was pointed out that if the aglycon was derived from a substance of sufficiently strong acidic character, strongly acidic conditions in the medium are not required to induce conversion into the preferred α configuration.

It is likely that an entirely comparable situation exists here. Diphenyl phosphoric acid is a much stronger acid than dibenzyl phosphoric acid, which, in turn is stronger than orthophosphoric acid. Thus when the aglycon is diphenyl phosphoric acid, for similar reasons, inversion to the preferred α form may occur prior to hydrogenolysis.

The weaker dibenzyl phosphate as aglycon (144) is much more readily hydrogenolysed thus reducing the time required and hence the hazards of hydrolysis. Additionally the likelihood of this more weakly acidic aglycon anomerising prior to hydrogenolysis is lessened. Hence, when dibenzyl phosphate is the phosphorylating agent the product is β-glucose-1-phosphate.

The remaining procedure (142) which utilises "monosilver" phosphate uses at all stages conditions which are unlikely to lead to anomerisation in that neutral solvents or weakly basic conditions are involved. Hence the product as expected is the β anomer.

The results are comparable when these various reagents are used to phosphorylate D-galactose (145, 143, 142), D-xylose (146, 147) and L-arabinose (147, 148). With this latter sugar no

results are available for the silver diphenyl phosphate method but it would be expected that the product would be the β anomer.

It will be recalled that for D-mannose, because of the trans relationship between the C-2 O-acetyl and the C-1 halogeno substituents in bromo tetra-O-acetyl α-mannopyranoside the various directive influences operative in the formation of C-1 derivatives each augmented the others to produce the α derivative of the sugar.

Three of the four phosphorylating agents discussed above, have been applied to the phosphorylation of bromo tetra-O-acetyl α-mannopyranoside. In each case, viz. with trisilver phosphate (149), silver diphenyl phosphate (150) and silver dibenzyl phosphate (150), α-mannose-1-phosphate was obtained. Hence, the cumulative effects of the influences, discussed previously, in this special case again appear to operate predictably and no preparation of β-mannose-1-phosphate has thus far been described.

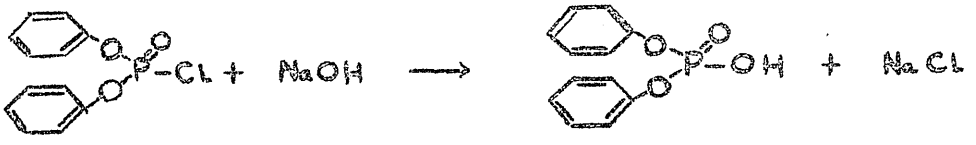
In the various preparations of anomeric sugar phosphates to be described attention has been confined to the use of the diphenyl and dibenzyl phosphate reagents. In terms of yield and convenience of solubility the mono and trisilver phosphate reagents are not as satisfactory; in addition the trisilver salt, being multi-functional, yields unwanted di and tri-esters.

It will also be noted that in some cases use has been made of the triethyl ammonium salt of dibenzyl phosphate in preference

to the silver salt. This variation, introduced by Wright and Khorana (148), avoids the light sensitive effects associated with the use of silver compounds which are very pronounced with this particular reagent. There is the further advantage of convenient solubility in the solvent of choice, which is dry benzene, thus resulting in rapid reaction at room temperature. This can be followed by observing the concomitant precipitation of triethyl ammonium bromide which is insoluble in benzene.

(3) Preparation of silver diphenyl phosphate.

This reagent was conveniently prepared by the method of Pasternak (151) and involved the alkaline hydrolysis (Expt. 18) of diphenyl phosphorochloridate (Expt. 5).at 100°.



The reaction was judged complete when a one phase mixture was obtained. Neutralisation of excess alkali with nitric acid was followed by the addition of silver nitrate. Insoluble silver chloride was separated from the silver salt of diphenyl phosphate by warming the solution, which served to dissolve the latter. The yields of just over 40% obtained were low compared with the value of 75% claimed by Pasternak, but the preparations obtained were satis-

factory white friable powders.

(4) Preparation of dibenzyl phosphate reagents.

Dibenzyl phosphate and its various salt forms may be made in a variety of ways. A method occasionally used (152) is to reflux benzyl chloride with trisilver phosphate to form tribenzyl phosphate. A cautious alkaline treatment results in mono-debenzylation and thus the eventual isolation of dibenzyl phosphate.

A second method used here (Expt. 19) utilised the convenient dibenzyl phosphite (Expt. 7) which could be converted into dibenzyl phosphorobromidate by treatment with bromine in carbon tetrachloride (153). This unstable intermediate was readily hydrolysed to dibenzyl phosphate which could either be isolated in acid form or as its silver salt.

The method of preference, however, was to reflux dibenzyl phosphite directly with sodium hydroxide (Expt. 20) (154). This treatment resulted in the immediate precipitation of sodium dibenzyl phosphate but refluxing was continued for a prolonged period to decompose pyrophosphates. The sodium dibenzyl phosphate was filtered, washed, and conveniently stored in free acid form, obtained by acidifying a hot water solution and extracting it into chloroform. This free acid crystallised readily from ether in good yield.

The dibenzyl phosphate is customarily used for phosphorylation either as its silver or its triethyl ammonium salt.

To prepare the former (Expt. 21) the water insoluble free acid form was brought into solution with dilute sodium hydroxide and the silver salt precipitated by the addition of silver nitrate to the neutral solution.

The triethyl ammonium form of the reagent was prepared as required (Expt. 22) by mixing equimolar quantities of triethylamine and dibenzyl phosphate, each dissolved separately in dry benzene (148).

(5) Preparation of bromo O-acetyl sugars.

The Experimental Section contains details of the preparation of the bromo tetra-O-acetyl α-pyranose derivatives of D-glucose, D-mannose, and D-galactose, and the corresponding bromo tri-O-acetyl α-pyranose derivative of D-xylose (Expts. 25 to 30).

In the earlier preparations of these compounds it was customary to prepare the penta-O-acetyl derivatives from the free hexose sugars (tetra-O-acetyl derivatives from free pentose sugars) (155) and after purification, treatment with a saturated solution of HBr in glacial acetic acid afforded the required bromo derivatives.

In later preparations the procedure was simplified in that

acetylation and bromination were carried out simultaneously. The technique described by Smith (156) and the reagent he describes (Expt. 24) gave very satisfactory results in all cases tried, and furthermore led to a considerable saving of time.

(6) Preparation of α-D-glucose-1-phosphate.

The enzymic method of Hanes (13) using a crude potato phosphorylase preparation, is a quick and reliable method of obtaining substantial quantities of this phosphate ester. Hence, no attempt was made to synthesise α-glucose-1-phosphate chemically. Details of this well-known preparation are provided (Expt. 31).

(7) Preparation of β-D-glucose-1-phosphate.

(a) Using silver dibenzyl phosphate (Expt. 32a).

Bromo tetra-O-acetyl α-glucopyranoside (Expt. 27) dissolved in dry benzene was treated with a slight excess (molar basis) of dry phosphorylating agent (Expt. 21), with warming and agitation for some hours. Despite precautions taken to exclude light the mixture darkened considerably. The gum obtained after filtration of the insoluble material and evaporation of the solvent was submitted to hydrogenolysis in methanol with palladium-charcoal

as catalyst. The uptake of hydrogen was rapid. Deacetylation was followed by a barium fractionation (G.M. 7-2a) carried out in the largely methanolic solution at pH 7.5. To this, methanol soluble barium bromide was added which resulted in the co-precipitation of inorganic phosphate with the barium salt of the ester. The latter was recovered from the mixed precipitate by prolonged water extraction. The method was generally unsatisfactory because yields of barium β-glucose-1-phosphate were seldom in excess of 10% of theory.

(b) Using triethyl ammonium dibenzyl phosphate (Expt. 32b).

Equimolar amounts of phosphorylating agent in benzene (Expt. 22) and bromo tetra-O-acetyl α-glucopyranoside (Expt. 27) in benzene were mixed and because of the sluggish rate of reaction (p. 84) it was allowed to proceed at a slightly elevated temperature for several hours. When precipitation of the benzene insoluble triethyl ammonium bromide appeared complete the mother liquor was concentrated to a gum which was dissolved in methanol and hydrogenolysed as before. Isolation of the barium salt of the β-glucose-1-phosphate was performed as for the previous preparation (G.M. 7-2a), the product being obtained in substantially improved yields which varied from 40 to 50% of theory.

The hexose content of the repeatedly reprecipitated

product equated satisfactorily with acid labile phosphate content (G.M. 8). There was no evidence of phosphate release when samples, converted to their sodium salt form, were submitted to the action of potato phosphorylase in the presence of primer.

(8) Preparation of α-D-mannose-1-phosphate.

(a) Using silver diphenyl phosphate (Expt. 33a).

Silver diphenyl phosphate (Expt. 18) does not darken excessively, hence, it was permissible to reflux a benzene solution of this reagent with a molar quantity of bromo tetra-O-acetyl α-manno-pyranoside (Expt. 28) in benzene without the need to exclude light. After a brief (30 minutes) reaction period further reagent was added and refluxing continued. Insoluble material was filtered off and the benzene solvent completely removed by vacuum distillation. Hydrogenolysis of the phenyl groups was accomplished slowly (8 hours) in ethanol solution over platinum oxide. Complete removal of benzene was thus essential to avoid a spuriously high value for hydrogen uptake caused by the reduction of benzene to cyclohexane. De-acetylation followed by the usual barium fractionation (G.M. 7-2a) gave α-mannose-1-phosphate in yields of ca 30%.

(b) Using triethyl ammonium dibenzyl phosphate (Expt. 33b).

As previously explained (p.89) with sugars other than mannose this reagent would give rise to the β-phosphate, but because of the combined influences discussed earlier the α-mannose derivative will be obtained irrespective of the reagent. This circumstance can be turned to advantage in that it permits the use of the more convenient triethyl ammonium dibenzyl phosphate reagent in this synthesis.

The preparation of α-mannose-1-phosphate thus did not differ essentially from the method used for the preparation of β-glucose-1-phosphate using this reagent. As expected the reaction was much faster and precipitation of the eliminated triethyl ammonium bromide was complete in 2 hours. The yield of barium salt varied between 45 and 50%.

The authenticity of samples of α-mannose-1-phosphate, obtained by this and the previous method, was established by conversion of the reprecipitated barium salt into the brucine salt (G.M. 7-2c). The melting point of this derivative (179°) agreed with the literature value (180°-181°) and it was possible to recover the α-mannose-1-phosphate in barium salt form of high purity by reconversion of this brucine derivative in a yield of ca 75%.

(9) Preparation of α-D-galactose-1-phosphate (Expt. 34).

This ester was prepared from bromo tetra-O-acetyl α-galactopyranoside (Expt. 29) and silver diphenyl phosphate (Expt. 18) by a method entirely analogous to that used in the preparation of α-mannose-1-phosphate. The yield was somewhat smaller (ca 25%) but the product analysed satisfactorily in terms of hexose and phosphate content and acid lability (G.M. 8).

(10) Preparation of α-D-xylose-1-phosphate (Expt. 35).

The phosphorylating agent was again silver diphenyl phosphate (Expt. 18) and the reaction with bromo tri-O-acetyl α-xylopyranoside (Expt. 30) proceeded typically. The yield of barium salt (G.M. 7-2a) was some 25% overall, but in some earlier preparations losses were exaggerated because it was not appreciated that the water solubility of the barium salt was unusually low. The product analysed satisfactorily in terms of pentose and phosphate content and acid lability (G.M. 8).

(11) The special case of the fructose phosphates.

If one accepts the supposition that fructosan synthesis

or degradation or mobilisation may involve a nucleoside diphosphate fructose intermediate, then even in its simplest terms the possible structural variations of such an intermediate are considerable.

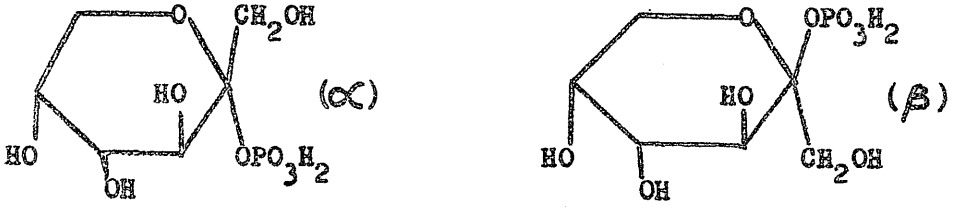
In the first place, even in the simplest analysis there are four series of nucleosides to which the intermediates may be related. Attachment of the fructose moiety to the second phosphate of the appropriate nucleotide may, however, occur in one of several ways. These are illustrated in Fig. 10 and it can be seen that the α and β anomers of both furanose and pyranose forms are separate possibilities. In addition, the existence of 1-2 linked polymers of fructose (inulin) is a reminder that the attachment of fructose via its C-1 primary alcohol grouping to a nucleoside diphosphate could lead to transfer reactions of the kind envisaged.

Hence, for any nucleoside series there are five possible fructose derivatives and to cover only the more likely cases twenty nucleoside diphosphate fructose compounds are required. Clearly, even if reliable synthetic routes to each were available the task would be formidable.

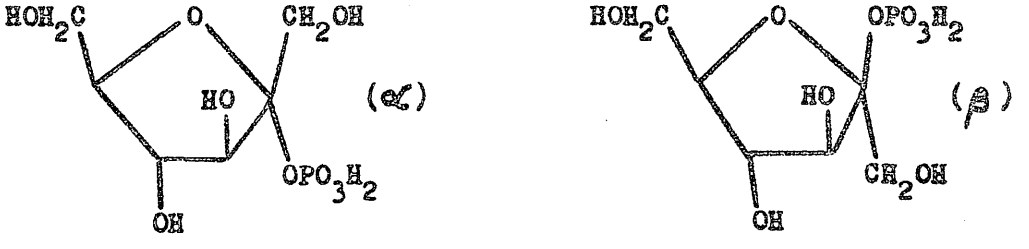
However, some guidance towards a simplification is found in the identification by Baddiley et al (57) of a guanosine diphosphate fructose in extracts of Streptomyces griseus. More recently, Gonzales and Pontis (47) have shown the presence of a uridine diphosphate fructose in dahlia tubers.

FIG. 10.

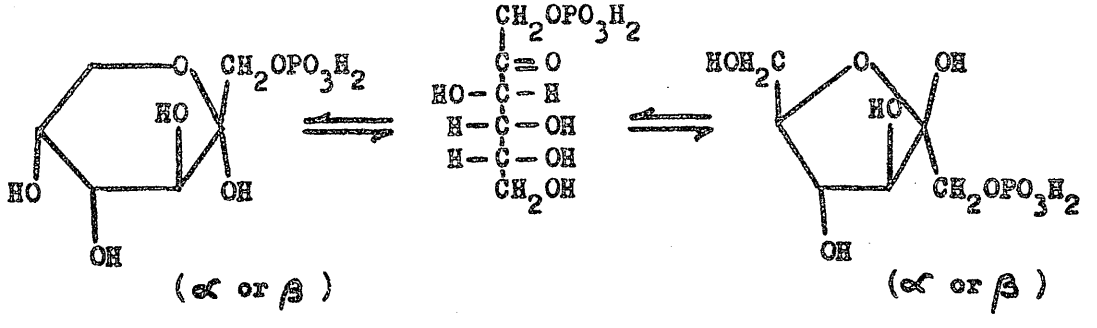
FRUCTOPYRANOSE-2-PHOSPHATE.



FRUCTOFURANOSE-2-PHOSPHATE.



FRUCTOSE-1-PHOSPHATE.



It is a comparatively simple matter to synthesise nucleoside diphosphate fructose compounds in which attachment of the sugar is via the C-1 hydroxyl and the above mentioned workers were able, in consequence, to compare the properties of the naturally occurring compounds with the corresponding synthesised derivatives containing the fructose thus attached (157, 158). Each group concluded that on the basis of acid lability their compounds did not correspond, the naturally occurring ones in each case being more acid labile.

It would thus appear likely that as is the case with nucleoside diphosphate aldose sugar derivatives, linkage of fructose to the nucleoside diphosphate moiety also occurs through the anomeric carbon viz. C-2.

The only account of the synthesis of fructose-2-phosphate (furanose and pyranose) so far available in the literature is that provided by Pontis and Fischer (159). Their method involves the formation of the cyclic 1-2 phosphate derivative from unblocked fructose-1-phosphate in the presence of DCC. Controlled alkaline hydrolysis of this cyclic diester leads to a non-random scission which results in regeneration of the fructose-1-phosphate to the extent of about 80% together with the formation of some 6% fructofuranose-2-phosphate and about 12% fructopyranose-2-phosphate. Observations on the optical rotations of the two esters suggest that

they are in the β configuration.

Despite their success in obtaining quantities of these two esters no report has yet been made by these authors of a successful attachment of either to a nucleoside monophosphate to provide a novel fructose containing nucleotide.

With the same end in view, their synthesis, prior to its publication, had been attempted here (Expt. 38). No success had attended these efforts however, and in the light of what Pontis and Fischer ultimately stated it is now clear that these failures were largely caused by a lack of appreciation of the extreme acid lability of these two phosphate esters. From the data now available it appears that the fructofuranose-2-phosphate is 50% hydrolysed at pH 4 in 5 minutes. The other form although somewhat more stable is also very readily destroyed.

In addition to its acid sensitivity the furanose ester does not survive the conventional operations involved in a barium fractionation. Pontis and Fischer discarded this technique in favour of a borate anion exchange method to circumvent this difficulty and this also allowed them to isolate their product under more safe alkaline conditions. As will be seen from Expt. 38, in the cyclisation trials of fructose-1-phosphate with carbodiimide, attempted in this work prior to the Pontis-Fischer publication, a barium fractionation procedure was applied on the assumption that

such destruction (160) would not be excessive. Clearly, this was an underestimate of the magnitude of this destructive barium effect.

In any case an alternative approach, which seemed more unambiguous, was being attempted at this time. An account of this alternative and higher yielding route to D-fructopyranose and D-fructofuranose-2-phosphate syntheses has been submitted for publication.

There were now available three phosphate esters of fructose of interest to the present purpose. In addition to the two above mentioned, fructose-1-phosphate was prepared by the phosphorylation of a fructose derivative with dibenzyl phosphorochloridate. A discussion of these three syntheses follows.

(a) Preparation of D-fructose-1-phosphate (Expt. 37).

Several methods, both chemical (161, 162) and enzymic (163, 164) are available for the synthesis of this ester. The one applied here differed from others previously published only in that the usual fructose derivative, 2-3, 4-5 di-O-isopropylidene fructose (Expt. 36) was treated with dibenzyl phosphorochloridate (Expt. 8) in preference to phosphorus pentoxide (161) or diphenyl phosphorochloridate (162).

The sugar derivative in dry benzene was admixed with a molar equivalent of the reagent in the same solvent and as reaction progressed at room temperature, triethylamine was added dropwise to

remove the eliminated hydrogen chloride. Centrifugation of the triethyl ammonium chloride was followed by vacuum distillation of solvent which allowed the gum obtained to be dissolved in methanol for hydrogenolysis which was both rapid and quantitative in the presence of the palladium-charcoal catalyst. The isopropylidene groups were removed by heating an aqueous solution of the debenzylated product to 100° for 30 minutes. Undoubtedly at this stage there was some dephosphorylation, because Tanko and Robison (163) state that fructose-1-phosphate in 1N HCl at 100 for 5 minutes is 50% hydrolysed. Yields of the product isolated as its barium salt (G.M. 7-2a) were somewhat variable but were in the region of 30%.

(b) Preparation of D-fructopyranose-2-phosphate (Expt. 41).

When it is required to induce fructose to react in the pyranose ring form a convenient derivative is the 1,3,4,5-tetra-O-acetyl compound (155). Ring closure in this is via C-2 and C-6; closing of the ring followed by "activation" of C-2 by formation of a halogeno derivative would furnish a potentially promising starting material. It is probable that attempts to obtain fructopyranose-2-phosphate by this route previously may have been unsuccessful because certain minor but important improvements of technique have only recently become available.

In the first place satisfactory crystalline and authentic

preparations of 1,3,4,5-tetra-O-acetyl fructose (Expt. 39) are not by any means the easiest of sugar derivatives to obtain, and in this work repeated attempts were necessary before this was achieved.

Furthermore, the C-2 bromo derivative (Expt. 40) is unstable and can only be prepared if scrupulous care is taken to exclude water. Literature references (165) refer to it as being "transiently produced" as an intermediate for various purposes. The customary procedure for the isolation of a sugar bromo O-acetyl derivative is to pour the mixture upon completion of reaction into excess of ice cold sodium bicarbonate solution (cf. Expt. 27). Clearly, this aqueous environment would result in hydrolysis of the bromo tetra-O-acetyl fructopyranoside. This difficulty has been circumvented both here and by others (166) by removal of the brominating agent (HBr in glacial acetic acid) by repeated co-distillation with Analar toluene (G.H. 11) *in vacuo* at a low temperature. It was discovered that much improved yields could be obtained by including a further procedure at this stage designed to ensure complete removal of acid. This consisted of the careful dropwise addition of triethylamine to a benzene solution of the bromo O-acetylated compound to a point where the solution was "neutral" as judged by the pH 7 colour towards an indicator, of small samples withdrawn and shaken with water.

To the neutral dry benzene solution of the β (C-4 directed) bromo derivative (Expt. 40) was added an equimolar quantity of

triethyl ammonium dibenzyl phosphate in dry benzene. The reaction, judged by the rate of precipitation of triethyl ammonium bromide was rapid at room temperature. Hydrogenolysis, de-acetylation and fractionation by conventional methods led to the isolation of a barium salt (G.M. 7-2a) in yields in excess of 20%. It is likely that the barium salt form is not the most suitable in terms of stability of product but this point has not been examined closely. There was some evidence of loss of product when the preparations were submitted to reprecipitation with barium.

(c) Preparation of D-fructofuranose-2-phosphate (Expt. 47).

A readily prepared furanose derivative of fructose is the 1,3,4,6-tetra-O-benzoyl compound (Expt. 42) (167) and it has usually been accepted that this is as suitable a derivative for use in synthetic work as are analogous O-acetyl compounds in terms of reactivity and ease of removal of the blocking groups. Because of the need to select methods of minimal violence to remove blocking groups after phosphorylation, a preliminary experiment to test alkali lability was carried out on the above tetra-O-benzoyl fructofuranose with the equivalent 1,3,4,6-tetra-O-acetyl fructofuranose (Expt. 44) as control. In each case (Expt. 45) equimolar amounts were suspended in a convenient volume of dilute standard lithium hydroxide and after a similar heating period unneutralised lithium hydroxide was estimated.

This experiment clearly indicated that by far the more alkali labile and hence preferred blocking group was the O-acetyl.

The contemplated use of the O-benzoyl derivative was therefore abandoned and a quantity of 1,3,4,6-tetra-O-acetyl fructofuranoside was prepared as an alternative. This was obtained by the acetylation of a commercial inulin sample (British Drug Houses) (Expt. 43) which on subsequent acetolysis with an acetyl bromide reagent gave a clear thin syrup of 1,3,4,6-tetra-O-acetyl fructofuranose (168).

The bromo derivative of this intermediate is at least as unstable as its pyranose equivalent (Expt. 46) but provided the rigid precautions described previously are adhered to it can be prepared successfully.

Phosphorylation was accomplished also in a similar fashion, using as before triethyl ammonium dibenzyl phosphate. The product after hydrogenolysis and de-acetylation was contaminated because of its labile nature with substantial quantities of fructose and inorganic phosphate.

As explained earlier, purification by means of a barium fractionation was prohibited because of the instability of the ester in the presence of barium ions (160). Furthermore, its quantitative estimation by phosphate analysis was rendered wholly unreliable because the acidity of the reagents (C.M. 6-2) was sufficient to

cause the immediate hydrolysis of the fructose-2-phosphate; hence reliable values for inorganic as opposed to ester phosphate content could not be obtained.

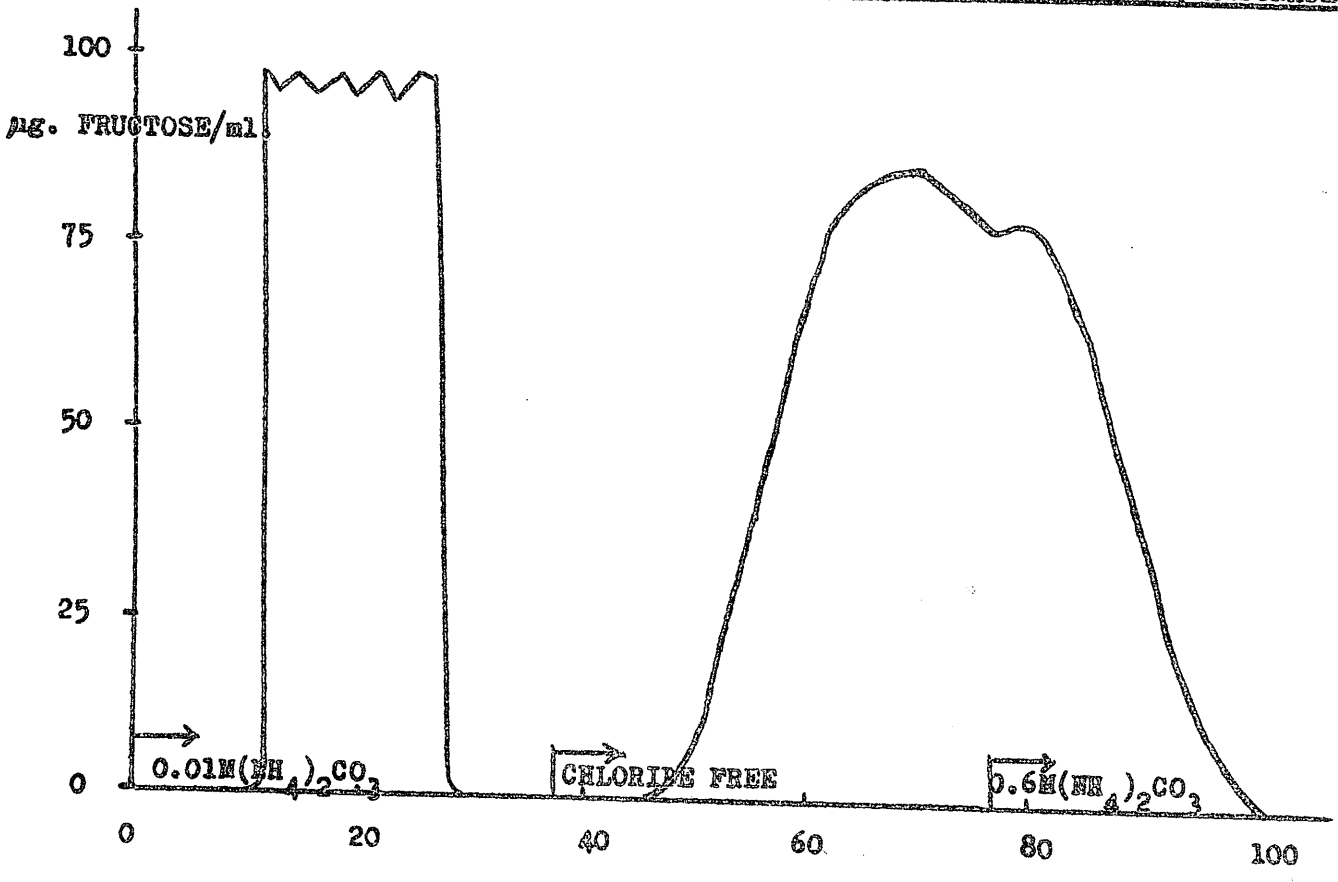
These difficulties were largely circumvented by removal of inorganic phosphate with magnesia mixture (C.M. 7-2b). This alkaline reagent precipitates inorganic phosphate as the insoluble magnesium ammonium phosphate without bringing about hydrolysis of the fructose-2-phosphate. Estimation of reducing sugar by the Somogyi method prior and subsequent to mild acid hydrolysis (C.M.8), despite a rather high blank gave values for esterified sugar which corresponded fairly closely with the phosphate obtained after removal of inorganic phosphate.

The preparation at this stage was heavily contaminated with magnesium and ammonium chlorides from the magnesia mixture used to remove the inorganic phosphate. Because the instability of the ester prohibited the use of nearly all the usual methods for their separation recourse was made to the use of a diethylaminoethyl (DEAE) cellulose column (C.M. 9-2).

It was reported by Rushizky and Sober (169) that mono-nucleotides could be desalted by the carbonate form of this exchanger if the mixture to be desalted was applied to the column in highly dilute ammonium carbonate solution at pH 8.6. Rushizky and Sober had observed that chlorides and sulphates present in their nucleotide

F I G. 11.

ELUTION OF FRUCTOFURANOSE-2-PHOSPHATE FROM DEAE-CELLULOSE (CARBONATE FORM).



solutions emerged from the column immediately under these conditions but that phosphates were delayed somewhat and that nucleotides required a change of eluting concentration.

Thus it was hoped that the slight retentive behaviour of the exchanger towards phosphate could be turned to advantage in the present instance. The magnesium ion was initially removed (Expt. 47) as its insoluble 8-hydroxyquinoline complex (C.M. 7-2b), the solution then being adjusted to pH 8.6 and 0.01M with respect to ammonium carbonate. Free fructose and inorganic salts were eluted immediately (Fig. 11), the fructose phosphate ester being delayed. The contents of appropriate tubes were then bulked and the solution carefully concentrated under vacuum in a rotary evaporator at a low bath temperature. The ammonium carbonate volatilised slowly under these conditions and the ammonium salt of fructofuranose-2-phosphate was obtained as a somewhat deliquescent white powder. As the result of these various purification procedures the overall yield was in the region of 15% as compared with about 25% at the earlier stage of inorganic phosphate removal.

If the theories earlier discussed concerning the influences which affect the configuration assumed by anomeric addends are applicable the esters synthesised here should be of the α configuration i.e. there should be an inversion of the configuration of the original β bromo derivatives.

Thus by an entirely analogous argument as was applied on p. 81, elimination of the bromo group with attack on the positive C-2 site by the acetyl group of C-3 from above the ring should induce attack by the incoming group from below the ring giving rise to an α derivative. The one complicating factor here, is the effect of the bulky $-CH_2OAc$ group which is already positioned in α relation to C-2. This group could reverse the effect of the influences which favour formation of an α derivative. Since the magnitude of this effect cannot be assessed it is not justifiable to claim categorically that the fructose phosphate esters here synthesised are of the α-configuration.

SECTION 1V.

PYROPHOSPHATE BOND FORMATION AND THE SYNTHESIS OF NUCLEOSIDE DIPHOSPHATE SUGARS.

(1) Survey of the available methods for pyrophosphate bond formation.

Methods for the coupling of nucleoside monophosphates (Section 11) to sugar-1-phosphates (Section 111) leading to nucleoside diphosphate sugar synthesis have gradually been developed by a variety of workers (34, 119, 170).

Essentially the problem consists of inducing the nucleus-seeking oxygen of the hydroxyl of one of the phosphate groups to attack the other phosphate, thus resulting in pyrophosphate formation.

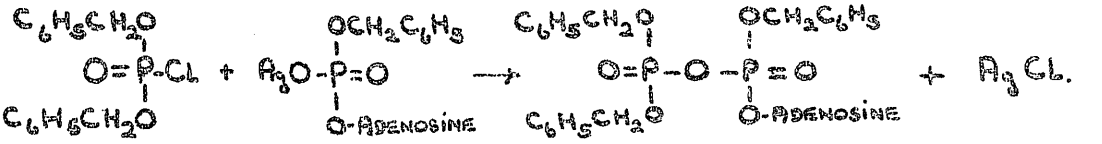
Under the conditions of an actual experiment one of the main factors which can lead to decreased yields will be the presence of sites which may compete for the phosphorylating species. On occasion this can be the phosphorylating agent itself, in which case the main product will be a symmetrical pyrophosphate. Alternatively,

if one of the two reactants carries an alcohol grouping, preferential phosphorylation of this may occur at the expense of pyrophosphate formation. Other undesirable possibilities which will be referred to can also occur. Thus many of the newer improved procedures for pyrophosphate synthesis have been attempts to minimise such side reactions or to encourage the main reaction.

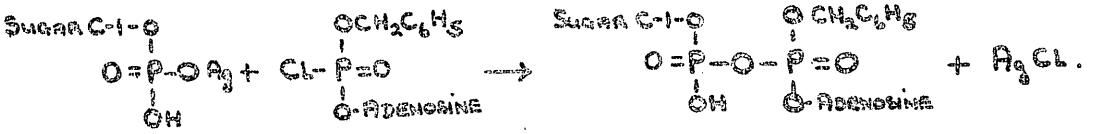
The general mechanism for the phosphorylation of a hydroxyl group has already been discussed (Section 11 p. 41). The analogy was drawn between phosphorylation and acylation reactions in general. It was pointed out that phosphorylations were favoured if a positive charge could be induced upon the phosphorus atom, thus encouraging nucleophilic attack by the alcohol upon the phosphorus. In addition, if the phosphorylating agent was a mixed anhydride the reaction occurred in the direction consistent with the expulsion of the stronger acid.

The same arguments hold for pyrophosphate bond formation. What follows is intended to illustrate this point and to indicate the measures progressively adopted to minimise the side reactions alluded to.

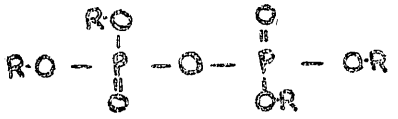
In one of their early methods for nucleoside diphosphate synthesis Todd and his colleagues (171) reacted the silver salt of monobenzyl adenosine-5'-phosphate with dibenzyl phosphorochloridate.



Hydrogenolysis of the tribenzyl derivative gave a low yield of adenosine diphosphate. By an extension of the same method adenosine triphosphate was synthesised. Clearly, however, this method was not suited to the synthesis of nucleotide-sugar derivatives. By adopting the reverse stratagem of introducing a chloro group into the nucleoside monophosphate and coupling this to the silver salt of a sugar phosphate, these workers (123) were able to extend what was essentially the same reaction to this purpose.



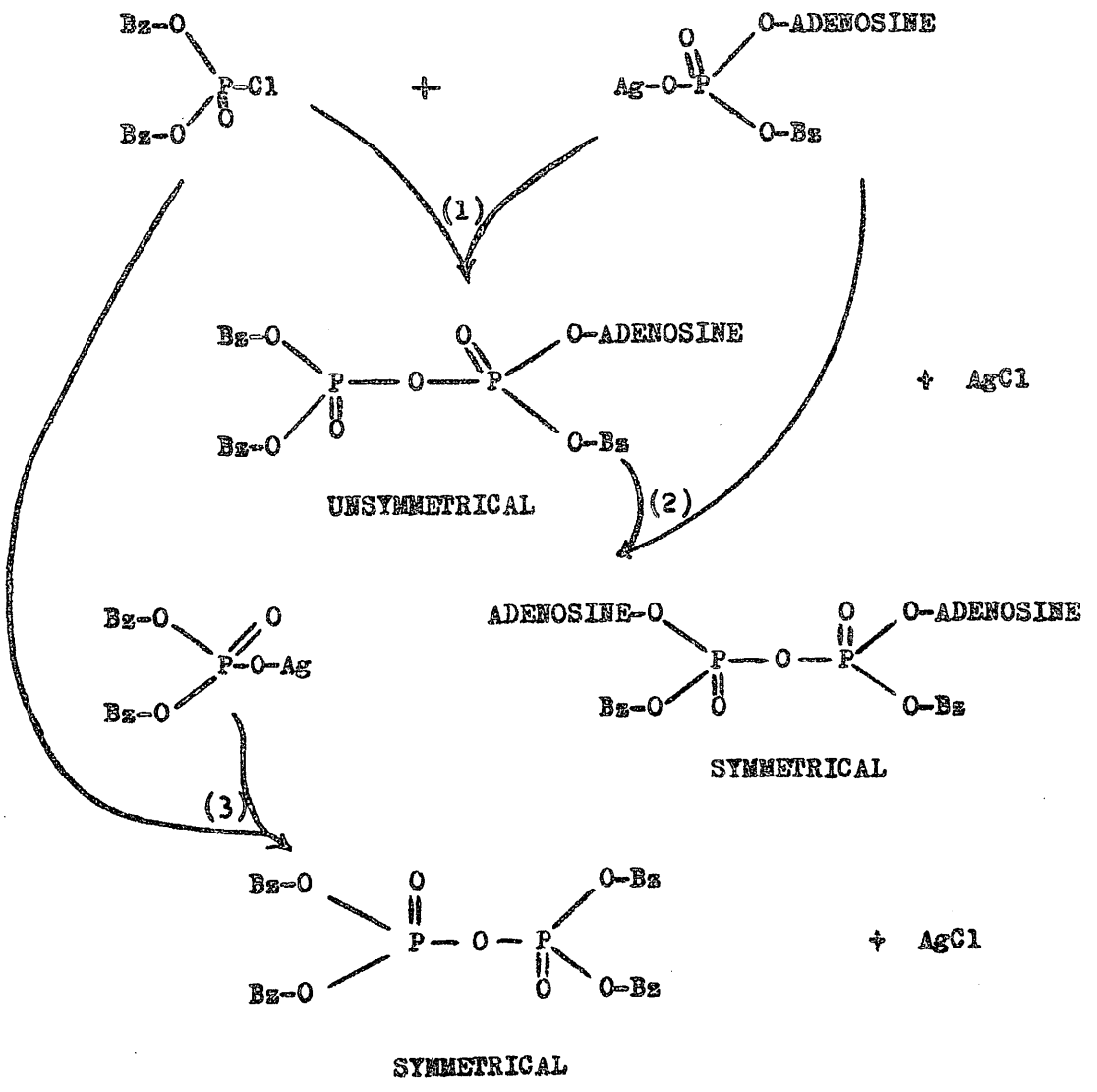
Yields by these methods were low for a number of reasons. Fully protected intermediates of the type:-



are very unstable and are prone to undergo spontaneous disproportionation reactions which lead to the formation of symmetrical side products. This may be envisaged as occurring as follows:-

F I G. 12.

DISPROPORTIONATIONS OF COMPLETELY SUBSTITUTED PYROPHOSPHATES.



Assuming for the sake of the present argument that the stronger acid moiety is the P_2 or dibenzylated phosphorus (Fig. 12) there will be a residual positive charge on P_1 which will encourage attack by the adenosine monobenzyl phosphate (silver salt). This will result in the expulsion of dibenzyl phosphate and the simultaneous formation of symmetrical diadenosine dibenzyl pyrophosphate. The dibenzyl phosphate in turn may react as the silver salt with dibenzyl phosphorochloridate to yield unwanted tetrabenzyl pyrophosphate.

It was realised that these undesirable side reactions could be inhibited if the reacting phosphorus-containing species were permitted to react in partially unprotected form.

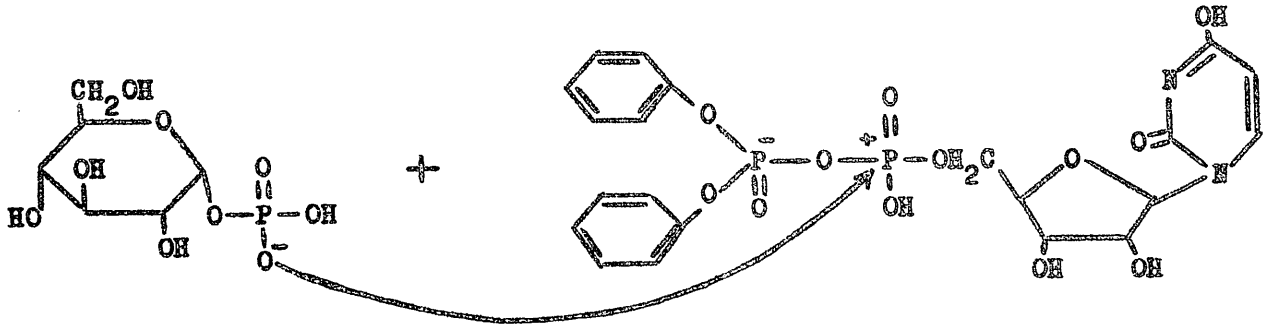
For example, Michelson (172) devised an attractive route to the synthesis of uridine diphosphate glucose by allowing P_1 uridine P_2 diphenyl pyrophosphate to react in pyridine with α-glucose-1-phosphate (Fig. 13).

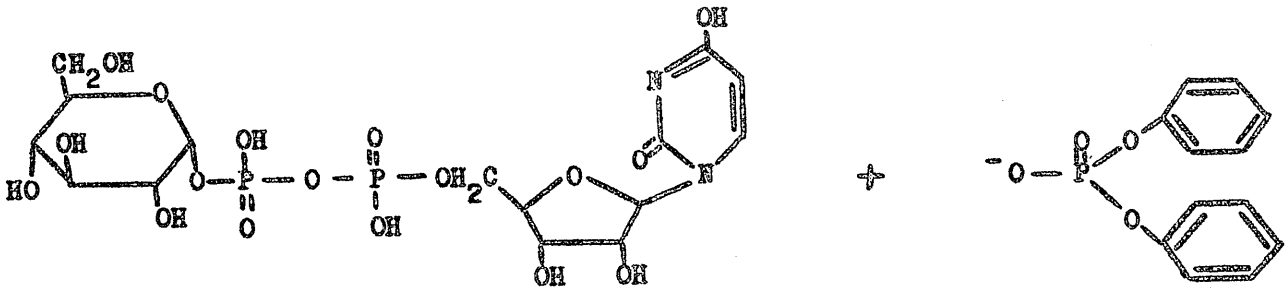
The former compound when dissolved in benzene containing an excess of triethylamine is stable and does not undergo the spontaneous disproportionation reactions characteristic of the fully protected tetra-substituted pyrophosphates (p.113). This stability is attributed to the presence of an ionisable group on P_1 which repels attack at this phosphorus.

Transfer of this compound into pyridine solution, with

FIG. 13.

FORMATION OF UDP-GLUCOSE. (MICHELSON)





UDP-α-GLUCOSE.

DIPHENYL PHOSPHATE.

removal of the strong tertiary base triethylamine, and admixture with α-glucose-1-phosphate in pyridine, now permits the usual course of events to occur viz. the expulsion of the stronger anion by the weaker. Thus the diphenyl phosphate moiety of the original pyrophosphate, being the anion of a strong acid induces a positive charge on P_1, which encourages its displacement by the anion of the weaker acid α-glucose-1-phosphate.

The product of the reaction is the doubly charged anion uridine diphosphate glucose which under the conditions of the experiment and due to its charge is not susceptible to any further degradative exchange reactions.

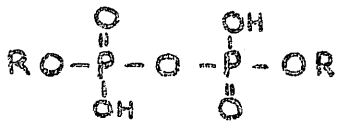
More recently, Michelson (173) has published details of this method and has shown that it is capable of exploitation in the synthesis of a wider range of analogous unsymmetrical pyrophosphates.

It will be recalled that one of the most successful methods for the synthesis of nucleoside-5'-phosphates from isopropylidene nucleosides involved the use of dicyclohexylcarbodiimide (DCC, Section 11 p. 44). This reagent operated by abstracting the elements of water from the group to be phosphorylated and the phosphorylating agent. Formation of a pyrophosphate bond can be brought about in a similar fashion and DCC has been used in a variety of instances for this purpose (174, 175). In certain cases, however, yields of product are exceptionally good, whereas in others

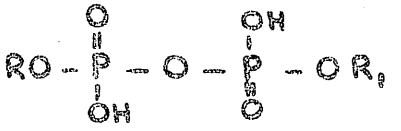
they are poor. It is profitable to consider why this should be so.

If DCC is used to couple two monophosphates:-

via their phosphate groups one could expect three possible products:-

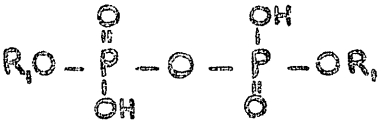


(1) SYMMETRICAL



(2) UNSYMMETRICAL

and

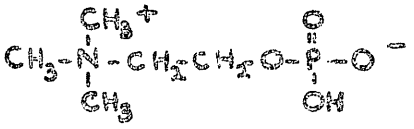


(3) SYMMETRICAL

Reference to the mechanism for the action of DCC (p. 47) makes it apparent that if the affinity of the protonated DCC for either acid is identical, then the three possible pyrophosphates will be formed in equimolar amounts. If, however, one of the monophosphates is a stronger acid than the other then this will form the preferred adduct and the formation of the unsymmetrical product will predominate.

This circumstance was turned to great advantage by Kennedy

in his synthesis of cytidine diphosphate choline (176). The two monophosphates involved were cytidine-5'-phosphate and choline phosphate respectively. Because of its dipolar nature:-



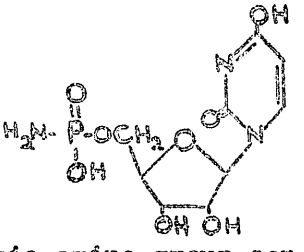
choline phosphate is an appreciably stronger acid than is cytidine-5'-phosphate so that formation of the unwanted symmetrical dicytidine pyrophosphate and dicholine pyrophosphate was minimal.

It cannot be assumed, however, that at worst a product will be obtained in 33% of theoretical yield. Other complications can diminish the amounts of product formed. Thus when Kenner, Todd and Webb (177) attempted the synthesis of UDP-glucose by coupling uridine-5'-phosphate to α-glucose-1-phosphate in the presence of DCC the yield was only 3½%. This was brought about as the result of the readiness with which DCC could induce the α-glucose-1-phosphate to undergo an internal cyclisation to form glucose 1-2 cyclic phosphate.

Thus the DCC coupling reaction will have merit over other alternatives only if (a) the intention is to synthesise symmetrical pyrophosphates or (b) if as was the case in the synthesis of CDP-choline one of the monophosphate reactants is a substantially stronger acid than the other.

An entirely different procedure possessing the merit of

wide applicability was introduced by Khorana in 1958 (178). In an attempt to increase the positive charge upon the phosphorus atom of one of the reacting moieties in the synthesis of UDP-glucose he prepared the amidate of uridine-5'-phosphate.



This basic amino group can become protonated even in pyridine as solvent giving rise to the withdrawal of electrons from the phosphorus atom. This positive site is thus more prone to attack by the other phosphate moiety (α-glucose-1-phosphate), the result being the formation of the required pyrophosphate bond with elimination of ammonia. Yields of UDP-glucose were about 59% of theory but the same technique applied to the synthesis of GDP-mannose (178) and other derivatives proved less successful. These results were partly attributable to the low solubility of certain of the nucleoside phosphoramidates in dry pyridine which was the reaction solvent. In an attempt to overcome this difficulty Khorana (179) investigated the possibility of utilising organic base analogues of phosphoramidates and he was able to make observations upon the suitability of a variety of such compounds for this purpose.

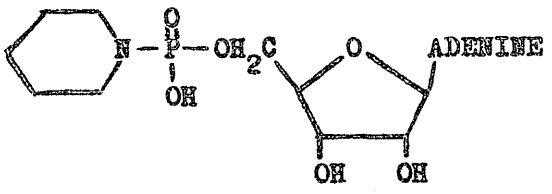
For example, he prepared piperidine, morpholine and

p-anisidine analogues of the 5'-phosphoramidates of adenosine (Fig.14). As with the original phosphoramidates these compounds were prepared by refluxing an aqueous tertiary butanol solution of the free acid form of the nucleoside monophosphate with the appropriate base in the presence of DCC. Water elimination between the phosphate and the base resulted in the production of the various phosphoramidates; the tertiary butanol component of the solvent was utilised in preference to the more obvious methanol or ethanol because these alcohols would readily esterify the nucleotide whereas a "hindered" alcohol such as tertiary butanol would not result in this side reaction.

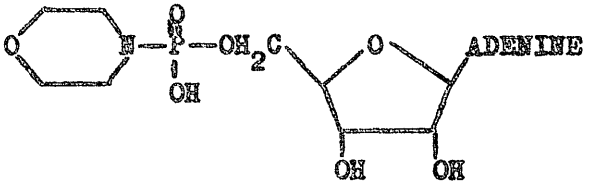
Khorana found that the ease with which these products were formed was inversely related to the base strength of the various amines. Thus the p-anisidine derivative was prepared in good yield whereas the derivative of the strong base piperidine was never produced in amounts in excess of 20% of theory. It transpired that the reason for this was the greater ease with which the stronger base reacted directly and undesirably with the DCC to form a guanidine derivative (Fig. 14). This side reaction thus prohibited the ready formation of the most basic and therefore most highly activated derivative of the nucleoside-5'-phosphate in suitably high yield. The p-anisidine derivative in turn, although readily prepared was insufficiently activated to serve as a suitable

F I G. 14.

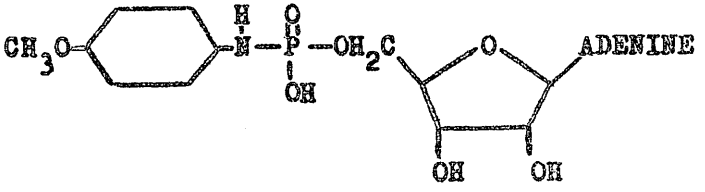
ADENOSINE PHOSPHORAMIDATE AND ORGANIC BASE ANALOGUES.



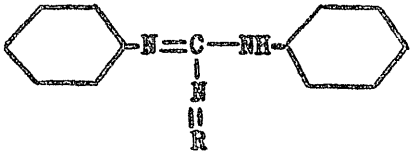
ADENOSINE-5'-PHOSPHOROPIPERIDATE.



ADENOSINE-5'-PHOSPHOROMORPHOLIDATE.



ADENOSINE-5'-PHOSPHORO-P-ANISIDATE.



GUANIDINE DERIVATIVE.
(UNDESIRABLE SIDE PRODUCT)

intermediate.

Hence the compromise was struck of utilising the morpholine derivatives. The nucleoside-5'-phosphoromorpholidates could be prepared in reasonable yields, especially if further precautions regarding technique were taken. They were suitably soluble and satisfactorily reactive in undergoing pyrophosphate formation with sugar-1-phosphates.

This latter part of the process could be carried out at room temperature by dissolving the guanidine salt of the nucleoside-5'-phosphoromorpholidate in pyridine and adding to it a pyridine solution of the tri-n-octylamine salt of the sugar-1-phosphate. Formation of the nucleoside diphosphate sugar was generally complete within three days and the formation of the product could be followed by paper chromatography.

Yields by this method are consistently good and generally in excess of 60%. Khorana and others have utilised the morpholidate method for the synthesis of a wide variety of pyrophosphates. As described in the next sub-section this and two other methods for obtaining UDP-glucose were compared experimentally.

(2) A comparison of the amidate, the Michelson anion exchange and the morpholidate methods for the synthesis of UDP-glucose.

UDP-glucose was preferable to alternative similar compounds for assessing the merits of a variety of routes for its production because literature references to analytical data, stability etc. were much more plentiful than for these other compounds. In addition authentic samples isolated from yeast (180) were available in this laboratory for comparative purposes.

(a) The amidate route.

Phosphoramidates, which are stable in their unprotonated form (favoured by alkali) but susceptible to anionic attack when protonated (acid) are satisfactory intermediates for pyrophosphate bond formation (p. 120). They were first introduced by Khorana (178) who synthesised UDP-glucose by this pathway in yields of 59%.

Uridine-5'-phosphoramidate was prepared in a one step process (Expt. 54) by treating the free acid of uridine-5'-phosphate with ammoniacal aqueous formamide in the presence of excess DCC in tertiary butanol at a temperature of 80° for approximately 8 hours. The mixture which was two phased initially became homogeneous after two hours at this temperature. The carbodiimide by an analogous mechanism to that discussed on p. 53 for the synthesis of nucleoside-

5'-phosphates extracted the elements of water from the uridine-5'-phosphate and ammonia producing the required amidate in quantitative yield.

The progress of the reaction was followed by removing aliquots from the reaction flask and assaying by paper electrophoresis (G.M.2) with a phosphate buffer pH 7.5 (20 volts/cm.). These conditions were selected to differentiate between the amidate and the parent nucleotide. At this pH the singly charged amidate has significantly less mobility than the corresponding doubly charged nucleotide component. This mildly alkaline environment also ensures that the phosphoramidate remains unprotonated and is therefore stable during the assay.

On completion of the reaction the product 1,3-dicyclohexylguanidinium uridine-5'-phosphoramidate was isolated as a white powder in quantitative yield and was stored at 0° over phosphorus pentoxide until required.

In order to effect the coupling of the phosphoramidate with α-glucose-1-phosphate it might appear necessary to use acidic conditions to encourage the formation of the active protonated form of the uridine-5'-phosphoramidate. Fortunately, even in solvents such as pyridine and in the presence of trialkylamines protonation of phosphoramidates still occurs but at a significantly slower rate. These experimental conditions are necessary in order to aid the

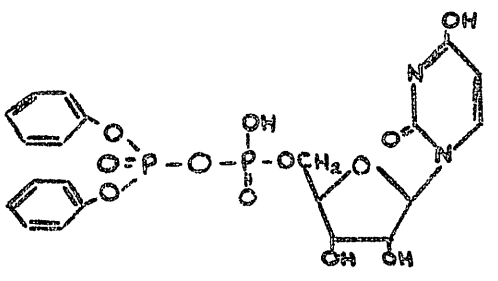
solubility of the reactants and to minimise breakdown of the pyrophosphate bonds formed.

To dissolve the glucose-1-phosphate in pyridine which was the most suitable solvent, it was converted into its tri-n-octylamine salt form (Expt. 48). This was obtained as an anhydrous glass by repeated azeotropic distillations with dry absolute alcohol.

1,3-dicyclohexylguanidinium uridine-5'-phosphoramidate (Expt. 60) which dissolved with difficulty in dry pyridine was added to a three molar excess of tri-n-octylamine glucose-1-phosphate dissolved in the same solvent. After four days at room temperature with exclusion of moisture the reaction was complete. Pyridine was removed under vacuum and the mixture submitted to ion exchange chromatography (Section V), UDP-glucose being ultimately isolated as its lithium salt in 25% overall yield.

(b) The Michelson anion exchange route.

The anion exchange method utilised by Michelson for the synthesis of UDP-glucose (173) depends on the formation of an intermediate of the type (Fig. 13):-



which was readily prepared (Expt. 55) by treating the tri-n-octylamine salt of uridine-5'-phosphate dissolved in dioxan-benzene with a molar quantity of diphenyl phosphorochloridate in the presence of excess tributylamine. After 3 hours at room temperature when the reaction was complete, the product was isolated as a gum by removing the solvents under vacuum. In the presence of an excess of a strong tertiary base the product was quite stable. However, removal of excess base by trituration with ether-pet.ether followed by dissolution of the gum obtained in pyridine and the addition in the same solvent of a three molar excess of tri-n-octylamine glucose-1-phosphate (Expt. 48) resulted in a controlled exchange taking place (Expt. 61). The mechanism of this exchange, which resulted in the production of UDP-glucose with the concomitant release of diphenyl phosphate, has already been discussed (p. 115).

This method gave an overall yield of UDP-glucose as its lithium salt of 17% of theory.

(c) <u>The morpholidate route.</u>

Uridine-5'-phosphoromorpholidate was prepared in quantitative yield (Expt. 56) by the dropwise addition of an aqueous tertiary butanol solution of DCC to the refluxing solution of the nucleotide and morpholine in the same solvent. In this way contact between the morpholine and the DCC was minimal which encouraged the

formation of the desired product at the expense of the synthesis of the competitive guanidine base (p. 121).

The progress of the reaction was followed in a similar manner to that used for assaying the uridine-5'-phosphoramidate (G.M. 2).

On completion of the reaction the product was ultimately isolated as a white powder in quantitative yield which could be stored over phosphorus pentoxide until required.

A three molar excess of tri-n-octylamine glucose-1-phosphate (Expt. 48) dissolved in pyridine was added to a readily prepared solution of 1,3-dicyclohexylmorpholinoguanidinium uridine-5'-phosphoromorpholidate in the same solvent and left for four days at room temperature under anhydrous conditions (Expt. 62). The ultimate yield of UDP-glucose isolated as its lithium salt was 56% of theory.

TABLE 111.

%age yield of UDP-glucose (lithium salt) based on U5'P used initially	Amidate	Michelson anion exchange	Morpholidate
	25	17	56

Table 111 allows a comparison to be made of the yields

here obtained by the three methods under test. The morpholidate route is clearly superior to the others and after some further confirmatory work use of the amidate and the Michelson routes was discontinued. Thus, with the amidate route, the most pronounced limitation was the sparing solubility in pyridine of the uridine-5'-phosphoramidate. This objection became unacceptable when attempts were made to use guanosine-5'-phosphoramidate for the attempted synthesis of GDP-glucose and the method was consequently abandoned.

On the other hand there is no doubt that the yields by the Michelson route could have been improved with perseverance. However, at this stage of the work, Michelson had not published any experimental details and indeed, the trial above described was devised without benefit of such details.

As the main purpose of this phase of the work was to obtain authentic and substantial quantities of the sugar containing nucleotides and since the morpholidate method was clearly capable of fulfilling this requirement it was decided to utilise this method for all subsequent preparations.

(3) Preparation of adenosine-5'-phosphoromorpholidate, guanosine-5'-phosphoromorpholidate and cytidine-5'-phosphoromorpholidate.

Adenosine-5'-phosphoromorpholidate (Expt. 57), guanosine-

5'-phosphoromorpholidate (Expt. 58) and cytidine-5'-phosphoromorpholidate (Expt. 59) were each prepared in satisfactory yield by an analogous process to that used for the synthesis of uridine-5'-phosphoromorpholidate. The products, obtained as white powders were stored over phosphorus pentoxide at 0° until required.

(4) Preparation of nucleoside diphosphate sugars.

For the preparation of a particular nucleoside diphosphate sugar it is recommended (179) that between 0.5 and 2 m.moles of the nucleoside phosphoromorpholidate be treated with a three molar excess of the tri-n-octylamine salt of the sugar-1-phosphate. In all the experiments carried out here, unless otherwise stated, amounts of morpholidate equivalent to 1 m.mole were used consistently, this being treated as recommended with the three molar excess of the sugar-1-phosphate. When this ratio of sugar-1-phosphate to morpholidate was reduced, due to a shortage of the former, then the yield of product was substantially lessened (UDP-galactose).

Thus, in practice, the coupling of the two phosphorylated entities to provide the required pyrophosphate was a reaction that proceeded spontaneously at room temperature for some days. The two reactants, separately dissolved in dry pyridine were mixed and the flask tightly stoppered. The morpholidates were suitable amounts

of the dry powders obtained as previously described (p. 127, p. 129, Expts. 56 - 59). The sugar-1-phosphates were conveniently used as their tri-n-octylamine salts obtained from their barium salts by methods analogous to that used for glucose-1-phosphate (p. 126, Expts. 48 - 53).

Experience showed that reaction was not usually complete for some three or four days but reaction progress could be ascertained by paper chromatography of aliquots withdrawn from the flask (Section V).

The nucleoside diphosphate sugars listed below were each prepared by the morpholidate method. Procedures for their isolation are described in the next Section.

UDP-α-glucose	(Expt. 62).	ADP-α-glucose	(Expt. 68).
UDP-β-glucose	(Expt. 63).	ADP-α-mannose	(Expt. 69).
UDP-α-mannose	(Expt. 64).	ADP-α-xylose	(Expt. 70).
UDP-α-xylose	(Expt. 65).	ADP-l-fructose	(Expt. 71).
UDP-l-fructose	(Expt. 66).	GDP-α-glucose	(Expt. 72).
UDP-α-galactose*	(Expt. 67).	GDP-α-mannose	(Expt. 73).
		CDP-α-glucose	(Expt. 74).

* 1½m.moles of galactose-1-phosphate used.

SECTION V.

THE IDENTIFICATION, ISOLATION AND PURIFICATION OF NUCLEOSIDE DIPHOSPHATE SUGARS.

(1) Methods for assessing the concentrations of nucleotide components in reaction mixtures.

Before attempting the full-scale isolation of the various nucleoside diphosphate sugars prepared by coupling together nucleoside phosphoromorpholidates and sugar-1-phosphates in pyridine, trials were carried out in an attempt to find a suitable micro technique for assessing the proportions of nucleotides and sugar phosphates present in the reaction mixtures. For this purpose paper chromatography, paper electrophoresis and thin layer chromatography were considered.

(a) Paper electrophoresis.

Mixtures containing the phosphoromorpholidate, monophosphate, diphosphate and diphosphate sugar derivatives of a particular nucleoside are, on the whole, rather too complex for paper electrophoresis, it being very difficult to separate all four components into discrete spots. This problem becomes accentuated in the presence of inorganic

salts. In addition, the lability of the nucleoside diphosphate sugars to both acid and alkali exerts a further restrictive effect, as a pH not far removed from 7 is essential if breakdown of the products is to be avoided.

(b) Thin layer chromatography.

Thin layer chromatography mainly using cellulose coated plates has proved successful for the analysis of sugar phosphates (181) and nucleotides (182) and has several advantages over paper. Thus, less material is required, the technique is quicker and allows the use of drastic reagents for the development of the spots. In preliminary experiments carried out on sugar phosphate mixtures, satisfactory separations were obtained by this method (G.M. 3). On developing the spots with acid molybdate (G.M. 4-2a) the medium did not char and disintegrate as sometimes occurred when paper was used.

(c) Paper chromatography.

For nucleotide mixtures paper chromatography proved most suitable despite the disadvantage of being rather time consuming. Satisfactory results were obtained using, in the main, Leloir's two solvents (183) which were composed of alcohol-ammonium acetate mixtures at pH 3.8 in one case and 7.5 in the other.

An important advantage these solvents possessed over many

others was their comparative insensitivity to inorganic salts, an ingredient which produces aberrant results with most solvents. Of even greater utility, however, was the reliability which could be placed upon a particular class of component appearing always in the same general region of the chromatogram. Experience showed that this characteristic could be relied upon almost to a diagnostic extent and when both solvents were used in conjunction inclusion of this step was of very substantial advantage (Fig. 15).

(2) Separation of nucleotide components by ion exchange chromatography.

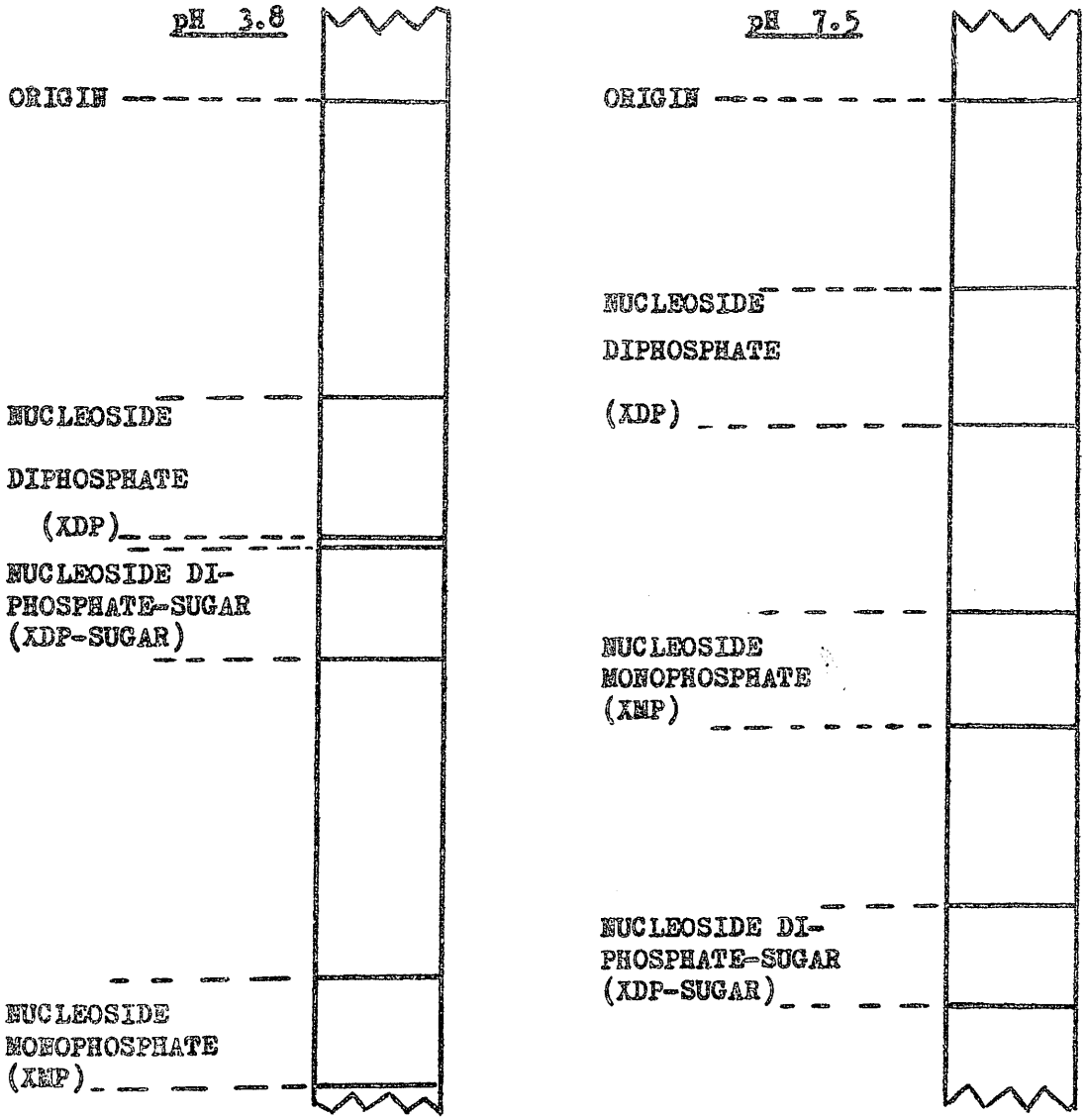
(a) Theory of the process.

The requirement at this stage of the work was for a method which could differentiate between closely related substances and yet be versatile enough to handle both large and small amounts of material. Precipitation techniques were of little use due to the difficulties of co-precipitation and mutual solubility. Adsorption methods, although satisfactory for purifying families of compounds would not readily permit the individual separations of closely related compounds.

Ion exchange procedures would appear to be the most promising approach and have proved generally satisfactory. Several

F I G. 15.

PAPER CHROMATOGRAPHIC SEPARATIONS OF XMP, XDP and XDP-SUGAR USING LELOIR'S ETHANOL-1M AMMONIUM ACETATE (7.5/3, v/v) SOLVENTS.



workers have used this technique to effect a separation of nucleotide mixtures into their individual components and Cohn (184) has provided a theoretical basis which makes possible the forecasting of the order of elution of the components.

To separate members of a family of ions by ion exchange it is necessary to find a set of conditions under which each exhibits a different degree of affinity (distribution coefficient) for the exchanger. While the affinity is governed by a number of variables it is reasonable to assume that in a group as closely related as nucleotides the net charge per ion will be the most important. Since nucleotides possess both acidic (phosphate) and basic (amino) groups the pH of the medium will determine the net charge on each molecule.

TABLE IV.

pKb (185) of nucleoside monophosphates.

Nucleoside monophosphate	Amino	Primary phosphate	Secondary phosphate
Cytidine monophosphate	4.3	0.80	6.0
Adenosine monophosphate	3.7	0.90	6.0
Guanosine monophosphate	2.3	0.70	5.9
Uridine monophosphate	0.0	1.0	5.9

From Table IV it can be seen that above pH 6 all four nucleoside monophosphates are strongly negative and would be held on an anion exchange resin. On gradually lowering the pH the secondary dissociation of each of the phosphates is suppressed. Additionally, as the pH is lowered, the influence of the amino groups may become manifest. If, as in the case of cytidine monophosphate the tendency of the amino group to take up a proton is considerable, the resulting positive charge due to the $-NH_3^+$ nullifies the remaining negative charge on the phosphate. Hence at best, at pH's below 6 the nucleotides have only one negative charge per ion.

At pH 3.5 the net charge on each of the four mononucleotides is as follows:-

TABLE V.

Nucleotide	Charge
Cytidine monophosphate	-0.16
Adenosine monophosphate	-0.46
Guanosine monophosphate	-0.95
Uridine monophosphate	-1.0

Thus the ease of elution at pH 3.5 is in the order C5'P, A5'P, G5'P, U5'P. From the available data on the pK's of the

various ionisable groups (Table IV), the most suitable pH for the elution of the nucleotides is in the range 2.5 to 3.5. Separation of nucleotides at high (>6) pH's is virtually impossible since, under these conditions each possesses at least two negative charges and the amino groups are uncharged.

If, on the other hand, the pH for ion exchange separations is below 2.5 then acid hydrolysis of the components becomes a hazard. Nucleoside diphosphate sugars are particularly labile at these low pH values.

An alternative to lowering the pH is to increase the anion concentration of the eluting solution and in practise a compromise is struck to utilise these two effects to their greatest advantage.

(b) Choice of eluting agent.

Although several anions have been used for eluting nucleotides from anion exchangers, in the main formate and chloride have been the ones of choice. Formate owed its popularity to its high buffering capacity which enabled strict pH control, to its suitability as eluting agent over the pH range 3.5 to 5, and to its ease of removal from the nucleotide components by volatilisation. Chloride, on the other hand, does not buffer so effectively, which has the advantage of allowing elution at pH 2.7 to 3. Due to the

higher competing ability of the chloride anion (stronger acid) compared with formate lower concentrations of chloride are required. Thus, the advantages displayed by the chloride system viz. elution with a low salt level at pH 2.7, the most suitable pH for separating the four nucleotide components, in most cases outweighed the advantages of the formate system particularly with the introduction of suitable methods for the removal of chloride ions from the resolved nucleotide samples.

(c) Removal of inorganic salts.

In the initial experiments using chloride as eluant the sodium salt was used, its removal being accomplished with activated charcoal (186).

This method (G.M. 7-1a) involved the application of the nucleotide sample at pH 6.8 to a charcoal-celite column, the charcoal having been previously activated by treatment with 6N HCl for 6 to 8 hours with subsequent extensive washing, followed by a strictly controlled heat treatment (110-115° for 15 hours). The nucleotide material held on the column was removed with 50% aqueous alcohol, the pH of which was adjusted to 7.5 with ammonia. Satisfactory results were obtained, but the extraction of UV absorbing contaminants from the charcoal, somewhat devalued the method. The inclusion of an EDTA wash before removing the nucleotides from the charcoal, substantially

reduced this contamination.

Pontis and Blumson (187) found that by substituting calcium chloride for sodium chloride in the eluting solvent they could make use of the differential solubility of calcium chloride compared with the calcium salts of nucleotides in dry ether-ethanol mixtures, the former being soluble and the latter insoluble under these conditions. The fact that this method did not give identical elution patterns to those obtained with sodium chloride, together with the difficulty of removing other calcium compounds such as hydroxide and carbonate which are insoluble in ether-ethanol mixtures, minimised the usefulness of the technique. The lithium chloride technique introduced by Khorana (128) was found preferable.

The success of this alternative technique depends upon the fact that lithium nucleotides, obtained by elution of a column with lithium chloride are readily soluble in dry methanol as is the lithium chloride itself. But, whereas, the addition of excess acetone will result in precipitation of the lithium nucleotides, lithium chloride remains soluble. This step was used regularly for preparing pure samples of nucleoside-5'-phosphates at an earlier stage of the work (p. 59) and brought about a very effective purification. As well as eliminating lithium chloride and other salts soluble in acetone, other components, such as lithium phosphate were removed as they were insoluble in dry methanol. This method gave satisfactory

results in all cases, the only difficulty being the practical one of obtaining a dry residue in the presence of high concentrations of deliquescent lithium chloride.

(d) Choice of eluting gradient.

Regarding the actual process of elution several possibilities exist. One solvent which can separate two components with different affinities for an ion exchange resin cannot be used to separate a range of compounds with widely differing affinities without either telescoping the earlier compounds together or widely spacing the latter ones, which are thus obtained in high dilution. Therefore, it is usual to change the solvent periodically, preferably in a region where no compound is being eluted. However, in spite of this improvement, a stepwise increase in concentration of the solvent tends to produce a discontinuous pattern which may be reflected in overlap of compounds in some instances. This effect has been overcome by the introduction of gradient elution systems which allow an even and continuous change in concentration. The actual gradient used can be one of three basic types, the choice of which is governed by the mixture to be separated. When the constituents are likely to be eluted towards the end of a chromatographic run (with high concentrations of salts) then more control over this part of the system can be obtained by using a convex gradient i.e. one in which the

concentration builds up quickly to begin with and then gradually tapers off. If, on the other hand, the most interesting components are likely to be displaced at low salt concentrations then a concave gradient is advisable i.e. one in which the concentration increases slowly to begin with and then more rapidly. The third alternative, sometimes the most useful, is to use a linear gradient, in which the salt concentration increases uniformly throughout the elution.

The actual gradient chosen can be varied by using systems of vessels of different shapes and sizes either by keeping the levels of liquid constant or by varying the rates of flow into and from a mixing vessel.

The technique favoured in this laboratory for producing the three main variants discussed above involved the use of two parallel-walled flasks of differing diameters connected by a siphon which allows the levels in both to drop simultaneously.

A convex gradient is produced by using a smaller diameter flask as the mixing unit, with a large flask as the reservoir, a reversal of these flasks producing a concave gradient. Where both flasks are identical a linear gradient is obtained.

The concentration at any one point using this system of two parallel-walled flasks, with the liquid levels dropping simultaneously, can be readily worked out using a formula derived by Bock and Man-Sing Ling (188):-

$$\text{Concentration} = C_2-(C_2-C_1)(1-\alpha)^{A_2/A_1}$$

where C_1 and C_2 are the initial concentrations in the vessels of cross sectional area A_1 and A_2 respectively, $\alpha = v/V$ where v is the volume of eluant passed through the system and V is the total initial volume present.

(e) Choice of resin.

Factors other than charge on the ions involved have to be considered in selecting a resin for ion exchange separations. For the best separations, ideally, equilibrium conditions should obtain at each stage throughout the column length. For practical purposes, a suitable resin will be one in which equilibration occurs as rapidly as is consistent with a reasonable flow rate. Although this is favoured by a low degree of cross linking (1-2%) such resins are prone to unacceptably excessive swelling and contracting. Hence resins of a higher degree of cross linking are generally used. Ease of access to the charged sites also has a bearing on equilibrium rates and modern resins in fine bead forms are designed to provide good flow rates with maximal diffusion within the beads.

A resin satisfying the above criteria which proved to be satisfactory for most of the present purposes was a 200-400 mesh Dowex 1 quaternary ammonium anion exchanger(4% cross linked).

(f) Choice of column dimensions, flow rate and fraction size.

For practical purposes, increasing the length of a column, up to a point, results in improved separations of components. On the other hand, if the requirement is for a column capable of handling larger amounts of the same mixture, this can be achieved merely by increasing the diameter, always assuming that the length was adequate in the first place for the smaller amount.

For the reaction mixtures described in Section IV , involving generally 1m.mole amounts of nucleotide reactant, column dimensions of 12 cm by 1.6 cm diameter were found suitable.

Columns of the same resin, of similar dimensions, were used by Cohn (184) who commented that symmetrical peaks, indicative of good equilibrating conditions, were obtained with flow rates of 1ml per minute. The front-sharpening conditions obtained with any gradient system however, permit the use of higher flow rates; in practice it was found possible to use flow rates of 2-3ml per minute without loss of peak definition with all gradients described in this work.

It was customary to run columns in conjunction with a fraction collector. Large fractions, up to 50ml in volume were permissible in preparative experiments where the number of nucleotide components was small. Recovery of nucleotide material applied was assessed by UV spectrophotometry of each fraction at 260 mμ.

(3) The actual ion exchange procedures followed for isolating the nucleoside diphosphate sugars prepared in Section IV.

In each case the nucleotide mixtures, after removal of pyridine by vacuum distillation below 30° and extraction with ether, to remove tri-n-octylamine, were applied in dilute solution, at a neutral pH, to a column of Dowex 1 by 4 chloride form resin (200-400 mesh), 12 cm by 1.8 cm diameter in the cold room (G.M. 9-1). The solution applied was diluted to below 0.02M to ensure complete adsorption of components. Copious washing with distilled water to a zero UV value confirms removal of contaminants such as pyridine. The column was then washed with a litre of 0.003N HCl of pH 2.7, to bring the column in its entirety to this optimal acidity for nucleotide separations (p. 138). In no case did this step remove any UV absorbing material. If this pre-treatment was not included, the components being eluted were telescoped together when the gradient elution was commenced. A linear gradient of lithium chloride at a pH of 2.7 was used to separate the nucleotide components in all the experiments attempted.

The steepness of the gradient applied and the total volume of salt mixture in the two chambers of the eluting system was determined by such factors as the column dimensions and the nature of the entities to be separated.

As explained earlier, the standard preparation of nucleoside diphosphate sugar involved reaction of 1m.mole of monophosphate for the ultimate coupling and a resin column of 12 cm length by 1.8 cm diameter was suitable for the subsequent separation. An eluting system containing a total volume of 3 litres of solution was usually used in conjunction with this column (Fig. 16). Thus for producing the linear gradients used here, the two identical flasks which comprised the reservoir and mixing chamber respectively each contained 1.5 litres of solution initially. For all the cytidine and adenosine diphosphate substrate compounds described here, a reservoir concentration of 0.05M lithium chloride (pH 2.7) was suitable, the mixing chamber containing 0.003M HCl (pH 2.7).

The uridine and guanosine series required a stronger salt solution in the reservoir and it was customary to commence elution with a 0.12M lithium chloride solution in this vessel. If, as occasionally happened, displacement of compounds was not complete with the 3 litres of solution, this could be accomplished by application of further salt solution of strength equal to that originally in the reservoir.

The actual elution patterns obtained with the glucose derivatives of each of the four nucleoside diphosphates (analogues of UDP-α-glucose) are shown in Figs. 17 and 18. It will be observed that, as shown, the expected order of elution (under the

F I G. 16.

THE ELUTING SYSTEM EMPLOYED FOR SEPARATING NUCLEOTIDE MIXTURES ON DOWEX 1 x 4 RESIN.

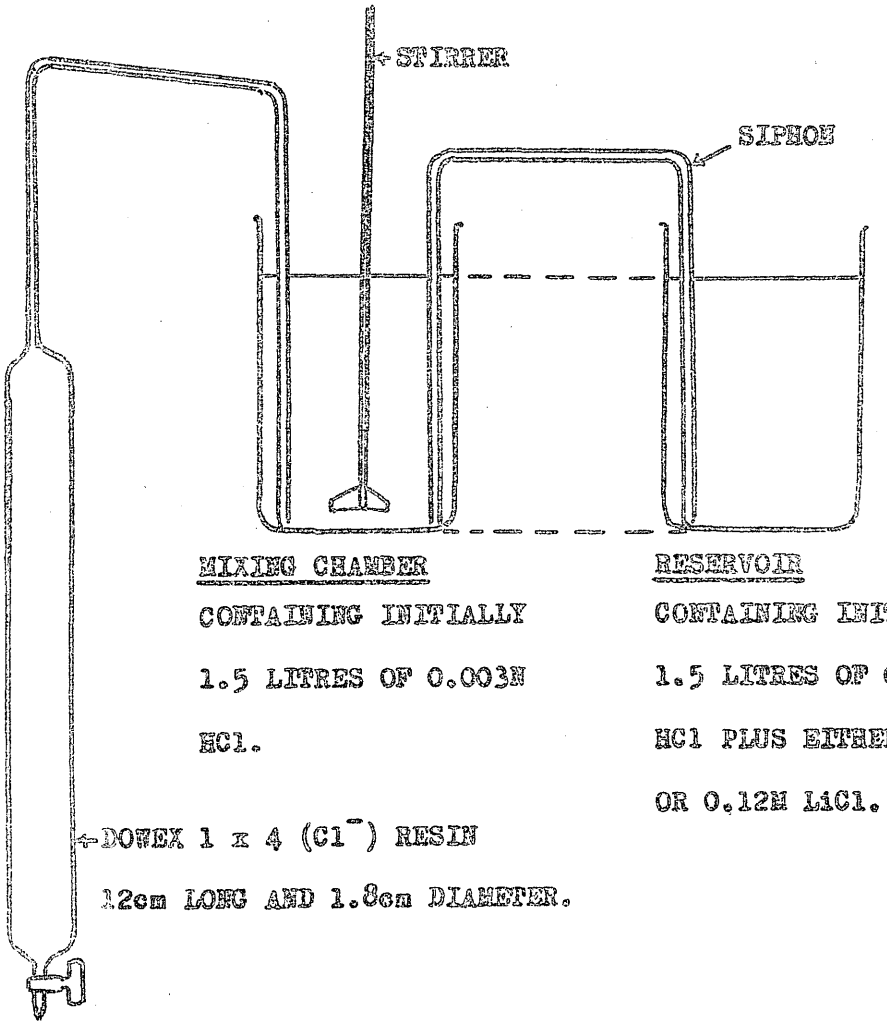
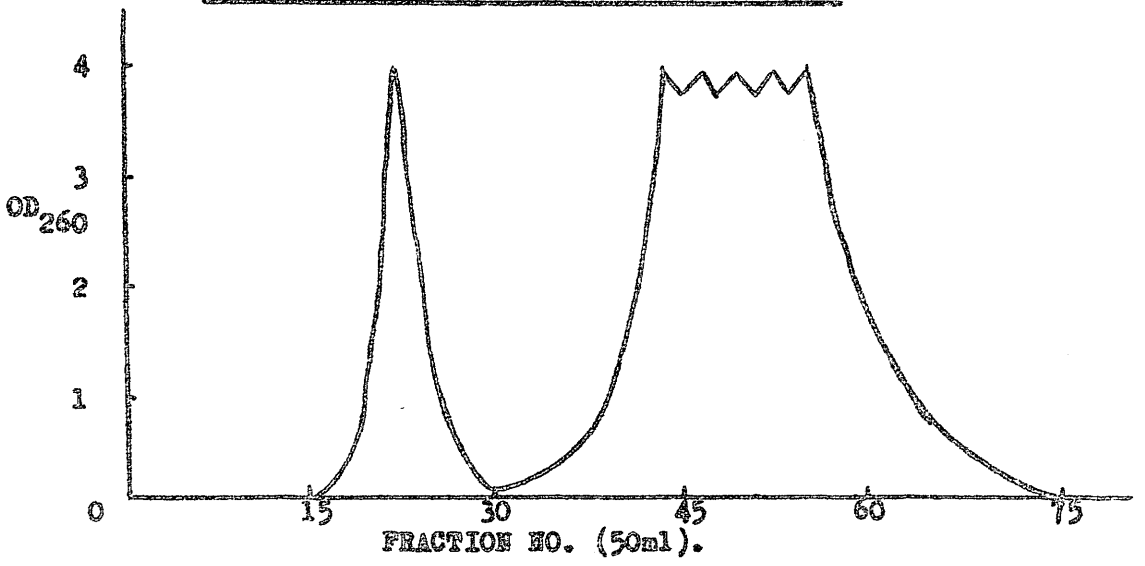
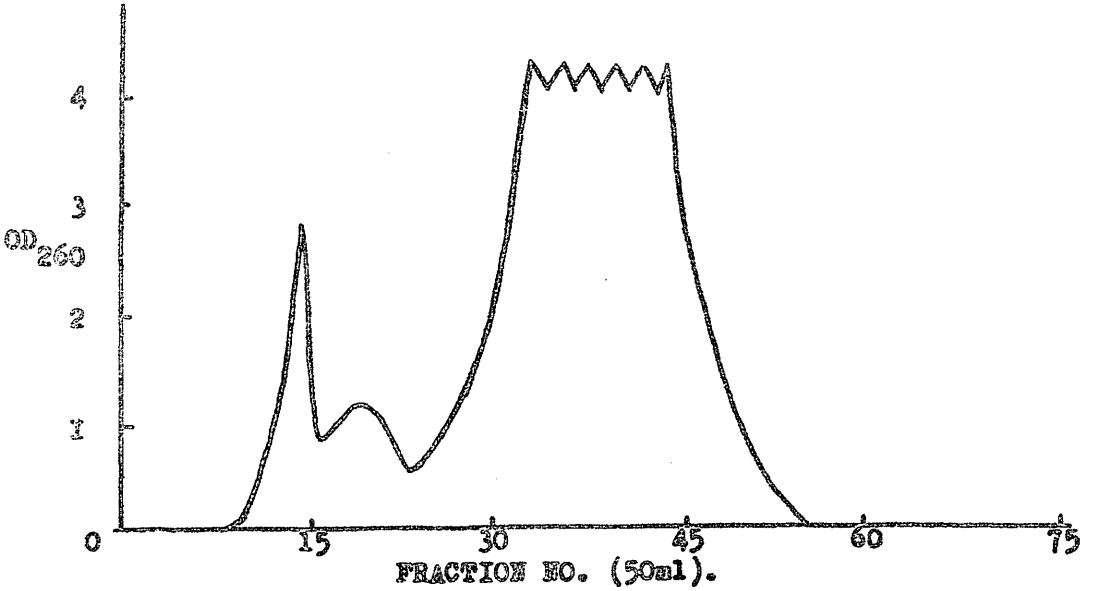


FIG. 17.

ELUTION OF ADP-GLUCOSE FROM DOWEX 1 x 4.

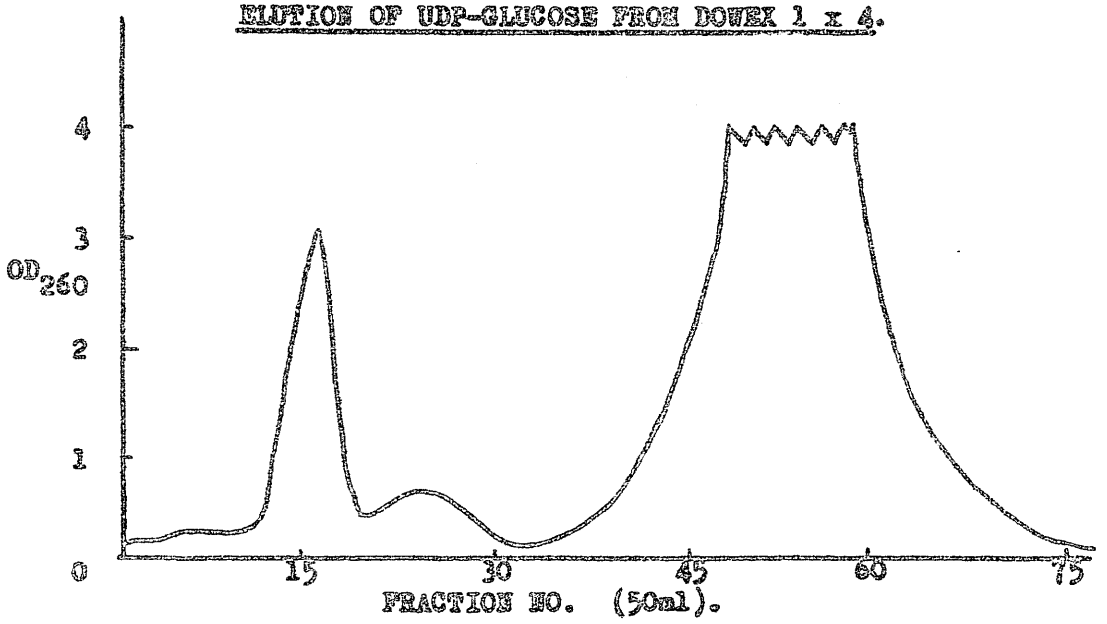


ELUTION OF CDP-GLUCOSE FROM DOWEX 1 x 4.

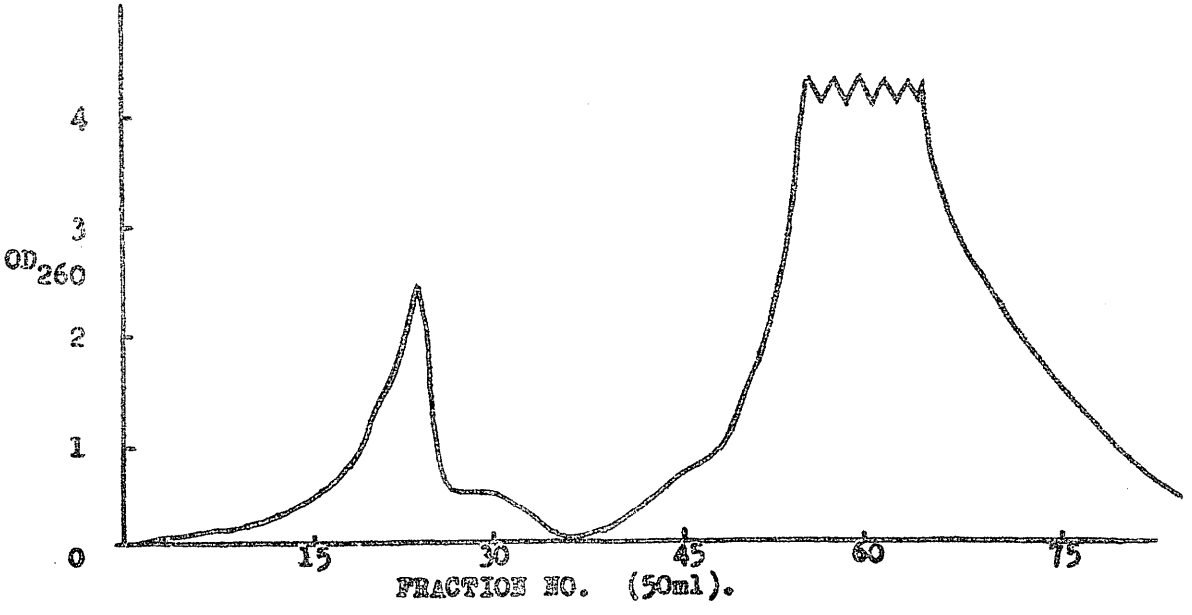


F I G. 18.

ELUTION OF UDP-GLUCOSE FROM DOWEX 1 x 4.



ELUTION OF GDP-GLUCOSE FROM DOWEX 1 x 4.



standard and therefore comparable conditions employed) was in the expected theoretically predicted order C,A,G,U (p.137). It is worthy of note however, that occasionally the order of elution of various phosphorylated derivatives of guanosine and uridine at pH 2.7 would be reversed. This occurred on several occasions in this work. The phenomenon, which has not been satisfactorily explained, has been noted by other workers (187, 192). The affinity of these derivatives for the resin does not appear to be influenced by the nature of the sugar moiety, provided the latter is uncharged.

In every case by far the largest peak, (judging by UV measurement) was that of the nucleoside diphosphate sugar compound which was collected and purified by the lithium technique (G.M. 7-1b). The yields of the nucleoside diphosphate sugar compounds isolated as their dilithium salts averaged just over 50% (range 75-43%, Table VI) based on the amounts of nucleotide used originally. The quantities of unreacted phosphoromorpholidates and monophosphates were usually very low. The exception was the attempted synthesis of UDP-galactose, when both of these entities were present in substantial amounts, the UDP-galactose being isolated in the disappointing yield of 22%. The reason for this was almost certainly the sub-optimal amounts of galactose-1-phosphate used in the reaction mixture (Expt. 67).

The yields of nucleoside diphosphate sugar compounds which

were obtained on treating 1m.mole of nucleotide converted into its morpholidate (or other intermediate where appropriate) with 3m.moles of sugar-1-phosphate, are given in Table V1.

The yields obtained are expressed in OD units, weight, and in m.moles assuming the molecular weight of each to be 600.

TABLE V1.

Expt.	Products	OD units	Wt.(mgs)	m.moles	%age yield
60	UDP-α-glucose (amidate)	2,650	150	0.25	25
61	UDP-α-glucose (Michelson)	1,900	102	0.17	17
62	UDP-α-glucose (morpholidate)	6,000	340	0.56	56
63	UDP-β-glucose	6,800	350	0.58	58
64	UDP-α-mannose	5,100	290	0.48	48
65	UDP-α-xylose	4,950	280	0.47	47
66	UDP-l-fructose	4,650	260	0.43	43
67	UDP-α-galactose	2,400	130	0.22	22 *
68	ADP-α-glucose	11,700	450	0.75	75
69	ADP-α-mannose	8,900	360	0.60	60
70	ADP-α-xylose	8,500	325	0.54	54
71	ADP-l-fructose	7,800	300	0.50	50
72	GDP-α-glucose	6,400	270	0.45	45
73	GDP-α-mannose	7,000	300	0.50	50
74	CDP-α-glucose	6,350	360	0.60	60

* 1½m.moles of galactose-1-phosphate used.

(4) The characterisation of the nucleoside diphosphate sugars.

Standard solutions (G.M. 8) of the nucleoside diphosphate sugar samples were made up and their purity checked by the extensive use of paper chromatography (G.M. 1) and paper electrophoresis (G.M. 2) After mild acid hydrolysis of the standard solutions, inorganic and total phosphorus determinations by the method of King (189, G.M. 6-2) and reducing power determinations by the method of Somogyi (190, G.M. 6-3a) were carried out on suitably diluted aliquots (G.M. 8). The results obtained were expressed in μ.moles/ml of primary standard, comparisons being drawn both on a weight and UV absorption basis.

Table VII represents in tabulated form the results obtained on submitting the nucleoside diphosphate sugar samples to the above determinations.

The fructose derivatives, as explained (p. 98) differ fundamentally in their structure from the others, in that the sugar moiety is not glycosidically linked. This feature is reflected in their properties. They possess an inherent reducing power because their glycosidic carbons are free. But, in common with most other sugar phosphates with free reducing groups (e.g. ribose-5-phosphate), this cannot be used as a reliable criterion for their estimation. Furthermore, the mild acid hydrolysis which serves to cleave the orthodox derivatives, with release of free sugar, does not achieve

TABLE VII.

SUBSTANCE.	CONCENTRATION BASED ON O Ds.	PHOSPHATE. ACID LABILE.	PHOSPHATE. TOTAL.	SUGAR.	RATIO. OD_{260}/ACID LABILE/TOTAL/SUGAR.
UDP-α-glucose	1.43	1.32	2.84	1.39	1 / 0.92 / 1.9 / 0.97
UDP-β-glucose	1.45	1.35	3.01	1.41	1 / 0.94 / 2.1 / 0.97
UDP-α-mannose	1.42	1.36	2.95	1.37	1 / 0.95 / 2.1 / 0.96
UDP-α-xylose	1.46	1.42	3.11	1.44	1 / 0.97 / 2.1 / 1.0
UDP-l-fructose	1.44	0.00	3.08	1.41*	1 / ---- / 2.1 / 0.98
UDP-α-galactose	1.40	1.22	2.50	1.38	1 / 0.87 / 1.8 / 1.0
ADP-α-glucose	1.28	1.21	2.49	1.26	1 / 0.94 / 1.9 / 1.0
ADP-α-mannose	1.25	1.16	2.38	1.23	1 / 0.92 / 1.9 / 1.0
ADP-α-xylose	1.28	1.19	2.43	1.25	1 / 0.93 / 1.9 / 0.98
ADP-l-fructose	1.27	0.00	2.51	1.23*	1 / ---- / 1.9 / 0.97
GDP-α-glucose	1.26	1.20	2.46	1.25	1 / 0.95 / 1.9 / 1.0
GDP-α-mannose	1.24	1.22	2.45	1.20	1 / 0.98 / 1.9 / 1.0
CDP-α-glucose	1.41	1.34	2.65	1.39	1 / 0.93 / 1.8 / 0.98

All concentrations are expressed in μ.moles/ml.

* Fructose concentration.

this with these fructose derivatives. However, fructose content, as estimated by the method of Cole as recorded by Bacon and Bell (191) correlated in both cases with phosphorus and OD values.

As mentioned, the yields of nucleoside diphosphate sugars calculated from the data in Table VI were based on the assumption of a molecular weight of 600 for the products. This figure takes no account of the likelihood of the lithium salts being comparatively heavily hydrated. Solutions of some preparations obtained by direct weighing (Table VII) gave OD 260 values which indicated a hydrate content of some 6-8 molecules of water. Not one sample contained any substantial quantity of inorganic phosphate, nor was there any evidence of serious contamination with other inorganic salts such as lithium chloride.

Hence it is believed that the preparations of nucleoside diphosphate sugars obtained as described here were in fact substantially free of contamination other than with the somewhat indeterminate quantity of water of hydration.

PART II.

A SEARCH FOR ENZYMES UTILISING NUCLEOTIDE-SUBSTRATES FOR POLYSACCHARIDE FORMATION.

INTRODUCTION.

It will be recalled that in the General Introduction evidence was reviewed which pointed to the correlation between the sugar patterns present in the nucleotide and polysaccharide fractions of individual tissues, the discrepancies which did exist being largely accounted for by known sugar interconversions.

Attention was also drawn to the direct evidence provided by the work of Leloir, Hassid, Oliver and others who examined enzyme systems apparently capable of utilising certain nucleotide-sugar compounds as donors in polysaccharide synthesis.

These systems are referred to again in Table VIII which indicates the nucleotide-substrate involved, the source of the enzyme, the need for activator and whether the preparation is soluble or particulate.

As is apparent from the Table, particle-bound activity for the syntheses listed appears to be the rule rather than the exception. These particles can be complex entities containing

TABLE VIII.

POLYSACCHARIDE SYNTHESISED	NUCLEOTIDE SUBSTRATE	ENZYME	
		SOURCE	PROPERTIES
Callose	UDP-glucose	Mung beans (85)	Particulate. Solubilised with digitonin with modified properties. Activated by glucose.
Chitin	UDP-N-acetyl glucosamine	Mould (86)	Particulate. Solubilised with butanol with modified properties. Chitodextrin required as primer and activated by N-acetylglucosamine (2 enzs.?).
Glycogen	UDP-glucose	Yeast (203) Insects (204) Animals (89)	Closely associated with glycogen. Solubilised with difficulty or loss of stability. Maltodextrins compete with glycogen. Activated by G-6-P.
Starch	UDP-glucose ADP-glucose	Plants (92, 93, 94)	(a) Particulate, attached to starch grain. Cannot be solubilised (activity lost on damaging). Maltodextrins compete with starch. Activated by G-6-P?. (b) Soluble enzyme. Starch grain is acceptor.
Xylan	UDP-xylose	Asparagus (96)	Particulate. Cannot be solubilised. 2-5 unit xylan required as primer (1 unit transfers only).
Cellulose	(a) UDP-glucose (b) GDP-glucose	Mould (85) Mung beans (101)	Particulate. Cannot be solubilised. Cellodextrins required as primer. Particulate (4% cellulose). Loss of activity on washing. Activated by yeast concentrates.
Hyaluronic acid	UDP-glucuronic acid UDP-N-acetyl glucosamine	Animal (97) Bacteria (98)	Particulate. Cannot be solubilised. Attached to protoplast membrane. 8-12 unit oligosaccharide required as primer.

polysaccharide material which may have "priming" properties and co-factors in varying amounts depending on the mode of preparation. But of even greater importance from the standpoint of interpretation of mechanism is the likelihood of the particles being multi-enzyme systems. Attempts to solubilise the enzyme(s) or alternatively to remove interfering polysaccharide frequently lead to loss of activity or to modifications in the properties of the system.

The reality of some of these complications is illustrated by the following observations:-

The particulate preparation from mung beans, capable of bringing about the formation of callose (a β-1-3 glucan, 87), shows increased activity upon addition of glucose but nevertheless functions in its absence. The enzyme can be solubilised by treatment with digitonin but is now almost totally dependent upon the presence of glucose before activity becomes manifest.

In an account of their studies on glycogen synthetase (89) Leloir et al describe a method for dissociating the enzyme from the glycogen particles. The protein is stable only if it is immediately adsorbed on to amylose as carrier. In the absence of amylose it rapidly inactivates. The amylose-protein complex is itself not as stable as the original association.

In the same system addition of glucose-6-phosphate results in an enhanced rate of glycogen synthesis without apparent incorp-

oration of the activator or of its sugar moiety into the product. Thus it appears that here the glucose-6-phosphate plays a somewhat analogous role to that of glucose in the callose synthesising system.

Presence of a competing acceptor e.g. a maltodextrin, can result in elongation of the maltodextrin chain by one unit at the expense of further glycogen synthesis.

Leloir's studies and those of others on the analogous starch synthetase have given rise to some conflicting reports. It was initially thought by Leloir (92) and generally confirmed (94) that the synthetase was held irreversibly on the surface of, or within the grain. Thus enzyme studies were customarily conducted with freshly obtained whole grain suspensions as enzyme source.

Such preparations used by Pettinger and Oliver showed a requirement for glucose-6-phosphate, Leloir's preparations, on the contrary, were active in the absence of this co-factor.

In close analogy with glycogen synthetase, dextrin acceptors competed successfully for the donated glucose with formation of a dextrin one unit greater in length at the expense of starch grain build up. UDP-glucose was inferior to ADP-glucose as donor in Leloir's system.

More recently, Frydman and Cardini (95) appear to have modified earlier views concerning the close association of enzyme with grain and have described a soluble enzyme in potato juice which

is capable of carrying out starch grain synthesis from ADP-glucose as donor. The role of the grain here would be better envisaged as being that of a specific acceptor.

Thus there now exists the possibility of there being two systems for grain formation; alternatively the earlier systems may be artifacts which have arisen as the result of a high affinity between substrate and the protein responsible for its formation.

These points illustrate the complexities inherent in the problems posed by the use of particulate enzyme preparations in in vitro studies on the synthesis of macromolecules which are themselves particulate or even, as exemplified by cellulose, virtually insoluble.

But despite the conflict of agreement on many important details the emergence of some pattern common to several of these systems seems to be appearing.

In the first place, as with phosphorylase, most seem to require a priming molecule. Examples are the glycogen and starch synthetases mentioned overleaf.

Secondly, there seems to be a very close association between enzyme and acceptor substrate to the extent that the whole system may be particulate. This association may be necessary for the functioning of the enzyme(s) since dissociation of the enzyme-polysaccharide complex leads to a loss of activity which can sometimes

be regenerated by supplementation with smaller sugars or their derivatives (e.g. glucose or glucose-6-phosphate).

A more puzzling aspect of the behaviour of several systems seems to be that the transfer of a single glycosyl residue to acceptor results in a molecule which does not then serve immediately as an acceptor for further residues. For example, Hassid (96), investigating xylan synthesis, found that particulate preparations from asparagus were capable of catalysing the transfer of radioactively labelled xylose units from UDP-xylose as donor, to primer molecules composed of three to five unit xylodextrins. From his analysis of the ultimate product Hassid concluded that the chain length of primer molecules had been increased by only one unit each.

Similar conclusions have been reached by others in investigations on starch synthetase (92, 93), capsular polysaccharide synthesis (194), the formation of Salmonella lipo-polysaccharide (56) etc. The review by Ginsburg (193) comments on this phenomenon.

From such details as are available it seems that the conversion (for example) of a three unit acceptor to a four unit product ceases at that point, the four unit entity, for whatever reason, not then acting as acceptor in a further transfer. Ginsburg and others have sought to explain this by postulating that the enzyme involved in the further transfer is different to the one which effected the three to four unit conversion and that the attachment

of further residues involves a separate and unique enzyme at each stage in the elongation of the polysaccharide chain.

This hypothesis is admirable in accounting for the formation of complex polysaccharides in a non-random fashion, since the specificity of each separate enzyme would require to be satisfied by a particular end group arrangement before transfer could be effected by that enzyme. It is somewhat more difficult however, to see why, in for instance the xylan synthesising system, a preparation which can convert a three to a four unit chain and separately a four to a five cannot convert a three to a four and use this latter product as primer to maintain the synthesis with eventual production of long chains.

At this stage in these investigations it was felt that the alternative of two main stratagems was available. A preliminary success in either would mean that the alternative course would have to be temporarily abandoned because of limitations on time.

The first of these alternatives was to utilise the relatively large quantities of nucleotide-substrates (Part I) in a search for enzyme systems which were capable of polysaccharide production provided substantial additions of solely these compounds as donors were made to the digests.

The remainder of this Part is devoted to a description of such attempts.

The second alternative was to select a particulate system of known potentiality and to attempt a clarification of the role of nucleotides, nucleotide-sugar derivatives and other compounds, such as lipoid materials, in this single system. The results obtained by applying this approach to the starch grain problem are described in Parts III and IV.

(1) The preparation of some convenient enzyme systems.

It was decided to confine this search for a polysaccharide synthesising system to tissues which had already been used in this laboratory for other purposes. Clearly, the number of plant sources and the alternative methods of obtaining enzyme preparations from these would prohibit an exhaustive coverage of all possibilities and some method of selection dictated by experience was essential. Nor was it thought cogent to the present purpose to concentrate attention upon particulate preparations since the main aim of this phase of the work was to attempt to use simplified systems which would serve to highlight the role, if any, of the new plentiful nucleotide-substrates. The studies to be described in Parts III and IV on starch grains would on the other hand give opportunity for investigations into the more complex factors offered by a particulate system.

Soluble protein preparations from wheat or other grain scutella are relatively easy to obtain and can be relied upon to be in a state of high metabolic activity. Keys (195) has demonstrated that acetone powders of scutella upon extraction with a phosphate buffer gave extracts containing the various enzymes which could account for the conversion of glucose into sucrose via UDP-glucose.

His technique for obtaining extracts was in fact used with occasional minor variations in all present trials with scutella (Expt. 75). Briefly, wheat grains after germination and growth for 60 hours in the dark were harvested and the scutella excised. This tissue was ground in 0.1M glucose and the macerate added quickly to ten volumes of cold acetone. The preparation after removal of acetone was stored at 0° under vacuum and used as required. To extract soluble enzymes from this powder suitable quantities were triturated with pH 7.3 phosphate buffer and after centrifugation of solid the supernatant was treated with ammonium sulphate to 80% saturation. A reprecipitation followed by dialysis gave a liquid which could be used directly.

Crude enzyme preparations possessing high glutamine synthetase (196) and UDP-glucose-fructose-6-phosphate transglycosylase (197) activities are readily obtainable from dried peas, as had been confirmed by other work in this department. Many other enzymic activities are possessed by this preparation and it was decided to

use it in tests for the present purpose.

A quantity of the flour obtained by hammer milling dried peas (var. Maple) was extracted with dilute sodium bicarbonate and the supernatant from the extraction treated with magnesium sulphate (after Elliott, 196) to precipitate mucilaginous components (Expt. 76). The protein fraction precipitated with ammonium sulphate to 50% saturation was retained, dissolved in water, the pH adjusted to 7.2 and dialysed overnight against two changes of distilled water. The impermeate was either used directly or was freeze dried and stored at 0° until required.

Potato juice preparations have been shown to possess several carbohydrases. Thus phosphorylase (13), Q-enzyme (8), D-enzyme (7) and starch synthetase (92, 94) have all been demonstrated. The methods of obtaining the juice used in this work were those previously used by Rees (198). In one of these methods (Expt. 77b) washed, peeled potatoes were sliced into dithionite solution, allowed to soak for 15 minutes and minced with a hand machine. After squeezing through muslin, the juice was centrifuged and then subjected to a fractionation either with ammonium sulphate (Expt. 78a) or cold ethanol (Expt. 78b).

The alternative was to treat the minced tissue with charcoal (Expt. 77a), whereupon the juice was recovered in a similar fashion to the above. High speed centrifugation was required to

remove colloidal charcoal, but the juice obtained resisted oxidation and consequent darkening by virtue of the removal of phenolic substrate by the charcoal.

Two essentially distinct methods for obtaining starch grains with associated enzymic activity for the synthesis of further starch have been described (Expt. 79a and b). Leloir and his colleagues have used preparations obtained by merely grinding potato tissue in a mortar with water containing a small amount of reducing agent (cysteine, dithionite) to delay browning (Expt. 79a). The liquid from the pulped tissue, together with starch and some small debris, is then strained through muslin and by repeated decantations with further quantities of water, washed starch grains free of debris are obtained. These are then thrown into excess cold acetone, the process being repeated until the grains are in a suitable conditions for storing in a desiccator until required.

Pottinger and Oliver have used a procedure which differs from the above in that the original potato tissue is blended for a brief period in 0.5M sucrose buffered to pH 7 and containing reducing agent. Thereafter, as described (Expt. 79b), the method is similar to that of Leloir with the difference that treatment of the grains with water is always avoided in favour of the sucrose buffer medium.

Fuller accounts of the preparations of these enzyme systems are provided in the Experimental Section (Expts. 75 - 79).

(2) Treatment of nucleotide-sugar substrates with the soluble enzyme systems.

As this aspect of the work was purely of an exploratory nature it was decided to limit assay methods to one or occasionally two per trial. Since the experience of other workers had been that a transfer of one unit to a priming molecule was the customary positive result, it was felt that a reliance upon paper chromatography as the main assay system was not unjustified. Thus, in a particular digest containing, for example, a three unit priming dextrin, the appearance of a four unit product as evidenced by chromatography against suitable control substances would be positive evidence for transfer reactions of the hoped-for type. Assay methods depending upon the liberation of free nucleoside diphosphates are somewhat dubious, because crude enzyme preparations may contain hydrolytic enzymes which could account for their production.

In the event of a system being present which resulted in the formation of a highly polymeric product, it was thought that techniques of preparing the samples for chromatography would not lead to a failure to detect these; in any case, the alternative of carrying out assays on alcohol precipitable material was so unreliable that it was not thought profitable to include such an assay at this stage. Occasionally, depending on the particular system, checks

were carried out with I_2/KI solutions to indicate synthesis of α-1-4 glucosyl linkages.

Since the main test criterion was paper chromatography, the use of interfering buffer salt components which can cause streaking had to be avoided. Generally however, the enzyme preparations contained sufficient protein to maintain the digests at a pH of near neutrality. In addition, the nucleotide-sugar substrates themselves, contributed to maintaining this pH.

For convenience of manipulation a standard digest was devised which contained :-

(a) 0.1ml of nucleotide-sugar substrate solution containing 3 or occasionally 6mg nucleotide,

(b) 0.3ml of enzyme preparation,

(c) 0.05ml of 0.05M magnesium sulphate and

(d) 0.05ml of water, priming species, or other addition in a total digest volume of 0.5ml.

0.1ml samples were withdrawn at intervals and delivered into 0.2ml ethanol, or alternatively, deproteinised with zinc sulphate-barium hydroxide solutions after Somogyi (199).

The protein free solutions were applied to chromatograms and run in one or other of the solvent systems described (G.M. 1, solvents 3, 4 and 5). After development the papers were examined under UV light and sprayed as suitable with silver (200), benzidine-

TCA (202) or occasionally with orcinol (201) reagents (C.H. 4-3).

The nucleotide-sugar substrates were tested with the wheat scutella (Expt. 80), the dried pea (Expt. 81) and the potato juice (Expt. 82) enzyme preparations separately. Tables IX, X and XI respectively summarise the results, if any, obtained by chromatography and by other tests applied.

As can be seen the results were in the main negative or at best inconclusive with all the substrates tested.

Treatment of the majority of chromatograms with spray reagent revealed the presence of traces of the sugar moiety derived from the nucleotide, in free form, presumably having been liberated by weak hydrolytic activities.

There was occasionally the appearance of material on the starting line of the chromatogram which showed an increase in intensity of stain with the spray reagent, as the time of incubation increased. For example, incubation of UDP-β-glucose with the scutellum enzyme gave such an effect even in the absence of primer but there were no additional indications of lower molecular weight intermediates having been formed concomitantly. A disadvantage possessed by this enzyme preparation over the others in digests intended to test for α-1-4 glucosyl transfers was the high amylase activity of the extracts.

Potato juice with ADP-glucose as substrate, rather interestingly and uniquely gave rise to a fast moving sugar tentatively

TABLE IX.

TRIALS WITH SCUTELLA PREPARATIONS.

SUBSTRATE	PRIMER (ACCEPTOR) ADDED	PAPER CHROMATOGRAPHIC RESULTS	OTHER TESTS
UDP-α-glucose	None, maltose, maltotriose, cellobiose.	No sugar spot other than glucose or added primer.	Negative I_2/KI.
UDP-β-glucose	None, maltose, maltotriose, cellobiose.	Spot on base line with all solvents plus a spot with $R_{glucose}$ 1.2(solvent 4).	Negative I_2/KI.
UDP-α-mannose	None.	Spot with $R_{glucose}$ 1.4.	- -
UDP-α-xylose	None.	Only xylose.	- -
UDP-l-fructose	None, sucrose, glucose.	No sugar spot other than fructose, glucose or sucrose.	- -
UDP-α-galactose	None.	Only galactose.	- -
ADP-α-glucose	None, maltose, maltotriose, cellobiose.	Fast spot with benzidine unaffected by primer($R_{glucose}$ 0.6)	Negative I_2/KI.
ADP-α-mannose	None.	Only mannose.	- -
ADP-α-xylose	None.	Only xylose.	- -
ADP-l-fructose	None, sucrose, glucose.	Only fructose, glucose, sucrose.	- -
GDP-α-glucose	None, maltose, maltotriose, cellobiose.	2 faint benzidine positive spots $R_{glucose}$ 0.50 & 0.35.	Negative I_2/KI
GDP-α-mannose	None.	Only mannose.	- -
CDP-α-glucose	None, maltose, maltotriose, cellobiose.	No sugar spot other than glucose or added primer.	Negative I_2/KI.

TABLE I.

TRIALS WITH DRIED PEA PREPARATIONS.

SUBSTRATE	PRIMER (ACCEPTOR) ADDED	PAPER CHROMATOGRAPHIC RESULTS	OTHER TESTS
UDP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	Besides glucose and added primer sucrose was present with F-6-P	Negative I_2/KI.
UDP-β-glucose	None, maltose, cellobiose, fructose-6-phosphate.	Only glucose and added primer vose revealed.	Negative I_2/KI.
UDP-α-mannose	None.	Only mannose.	- -
UDP-α-xylose	None.	Only xylose.	- -
UDP-l-fructose	None, sucrose, glucose, glucose-6-phosphate.	No sugar spot other than fructose, glucose or sucrose.	- -
UDP-α-galactose	None.	Only galactose.	- -
ADP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	No recognisable spot other than glucose or added primer.	Negative I_2/KI.
ADP-α-mannose	None.	Only mannose.	- -
ADP-α-xylose	None.	Only xylose.	- -
ADP-l-fructose	None, sucrose, glucose, glucose-6-phosphate.	No sugar spot other than fructose, glucose or sucrose.	- -
GDP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	No sugar spot other than glucose or added primer was revealed.	Negative I_2/KI
GDP-α-mannose	None.	Only mannose.	- -
CDP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	No sugar spot other than glucose or added primer.	Negative I_2/KI

TABLE XI.

TRIALS WITH FRACTIONATED POTATO JUICE PREPARATIONS.

SUBSTRATE	PRIMER (ACCEPTOR) ADDED	PAPER CHROMATOGRAPHIC RESULTS	OTHER TESTS
UDP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	No other sugars besides glucose and primer were revealed.	Negative I_2/KI.
UDP-β-glucose	None, maltose, cellobiose, fructose-6-phosphate.	Besides glucose & primer a faint spot on base-line was revealed.	Negative I_2/KI.
UDP-α-mannose	None.	At least two faint hexose spots.	- -
UDP-α-xylose	None.	Only xylose.	- -
UDP-l-fructose	None, sucrose, glucose, glucose-6-phosphate.	No sugar spot other than fructose, glucose or sucrose.	- -
UDP-α-galactose	None.	Only galactose.	- -
ADP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	Besides glucose & primer a pentose spot (ribose?) was revealed.	Negative I_2/KI.
ADP-α-mannose	None.	Only mannose.	- -
ADP-α-xylose	None.	Only xylose.	- -
ADP-l-fructose	None, sucrose, glucose, glucose-6-phosphate.	No sugars were revealed other than fructose, glucose or sucrose.	- -
GDP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	Besides glucose & primer faint low R_F spots were revealed.	Negative I_2/KI.
GDP-α-mannose	None.	Only mannose.	- -
CDP-α-glucose	None, maltose, cellobiose, fructose-6-phosphate.	No other sugars besides glucose and primer were revealed.	Negative I_2/KI.

identified as ribose. The amount of this sugar increased with time of digestion and its formation can possibly be accounted for by the action of specific hydrolytic enzymes upon the nucleotide-sugar substrate. At this stage, however, the existence of a more interesting sequence of events cannot be excluded. This observation was not meanwhile pursued further.

Treatment of GDP-glucose with the scutellum preparation resulted in the formation of two compounds of $R_{glucose}$ 0.50 and 0.35 respectively. These fluoresced white in UV light after spraying of the chromatograms with benzidine-TCA reagent but the amounts formed were too small to encourage any immediate attempts to examine them further.

(3) The starch grain system.

The demonstration of starch synthesising activity by whole starch grains poses a surprisingly difficult technical problem because of the need to show that small increases to a comparatively large amount of basal material have occurred.

Leloir and co-workers (92) on the one hand, and Pettinger and Oliver (94) on the other, have utilised two distinct methods to achieve this end.

Leloir incubated 5mg of whole grains with 0.3 micromoles

of UDP-glucose (0.18mg containing ~0.06mg glucose) radioactively labelled in the sugar moiety. Thus, even if 100% incorporation had occurred the increase in amount of starch would be somewhat greater than 1%. Results quoted indicate that the transfers obtained are in the region of 10%, hence the increase in amount of starch is correspondingly less.

Bearing in mind the difficulty of weighing accurately 5mg of starch grains reproducibly, it would be quite impossible to estimate increases of the order of 0.1% by conventional analytical methods. The use of radioactive techniques diminishes the force of this criticism and the isolation by Leloir of radioactively labelled maltose after amylolysis of the solubilised starch grains confirms that transfer has occurred.

Pottinger and Oliver on the other hand, make an estimate of the amount of synthesis by a direct method depending on precipitation of total starch (original, starter starch and newly formed) with iodine followed by anthrone determination. It is somewhat difficult to ascertain from the data provided in their paper what precisely is the percentage increase in total starch achieved by their preparations. Thus, although they refer to the volume of the starch grain suspension used, no details of the starch content of this suspension are provided. A table referring to the specific activities of their preparations, makes no comment upon the fact that one experiment appears to have

been far more successful than two others listed. If it is assumed that a specific activity of five, equivalent to an incorporation of 5µ.moles glucose/hour/mg of starch grains, means that 5 x 0.18mg = 0.9mg of glucose are incorporated by a milligram of starch grains in one hour, then clearly this means that the amount of starch has virtually doubled in this time. Yet nowhere in the text is this startling fact commented upon. If this is so however, their assay method is capable of detecting such differences with ease. Cabib (33) has criticised this assay technique, mainly on the grounds of the lack of specific methods for the enzyme determinations.

In the present work, the assay system used was mainly based upon that of Pottinger and Oliver. The two techniques (Expt. 79a and b) of obtaining starch grains were utilised in different experiments.

In early trials (Expt. 83a) maltodextrins were included in the digests but contrary to the view of Leloir, no higher dextrins were detected by paper chromatography. This point was not pursued further at this stage.

In a typical experiment (Expt. 83b), in a final digest volume of 0.5ml, 3 to 6mg of UDP-glucose (5-10µ.moles equivalent to 1-2mg glucose), together with additions of glucose-6-phosphate, magnesium sulphate and starter starch were placed in a digest tube and amounts of the order of 1-2mg of whole starch grains were added.

Thus, assuming 100% transfer of nucleotide-glucose to the grains, allowance was being made for 100 to 200% increases. Allowing for more realistic values of 5 to 10%, easily detectable increases in total starch should still be possible.

Some of the results actually obtained are shown in Fig. 19. Clearly, the method as performed here is of poor reproducibility, or alternatively, the kinetics of apparent starch formation by the system are complex.

As mentioned earlier the difficulties of pipetting quantities of starch grains to reproducibility standards of 1 to 2% are considerable. The use of larger volumes of more dilute grain suspensions did not materially improve accuracy.

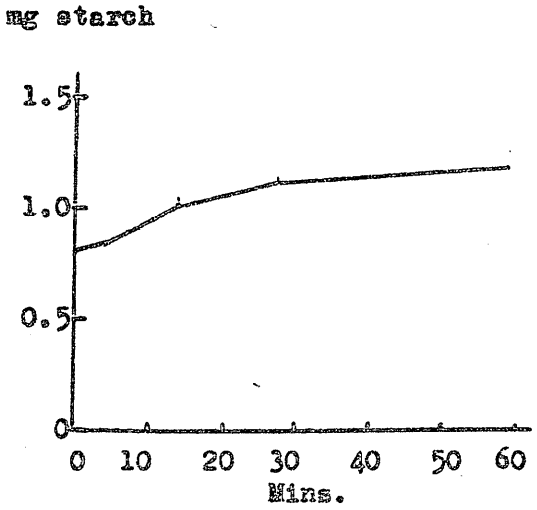
Despite the somewhat unpredictable course of the progress curves shown certain consistencies still emerge.

For example, in the complete system a net increase of starch (20 to 50%) could almost invariably be demonstrated. During this time however, control experiments not containing added nucleotide would show starch increases of about half this amount. The presence of glucose-6-phosphate also has a considerable bearing on the results obtained, the total starch synthesised being substantially diminished in its absence. In the trials attempted the starch precipitated by iodine after 60 minutes was approximately the same whether UDP-glucose or ADP-glucose was used as glucose donor. However, the patterns

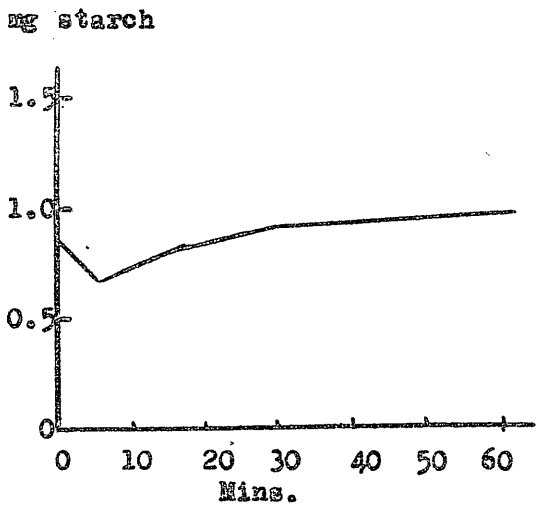
F I G. 19.

EXAMPLES OF STARCH GRAIN ENZYME DIGESTS.

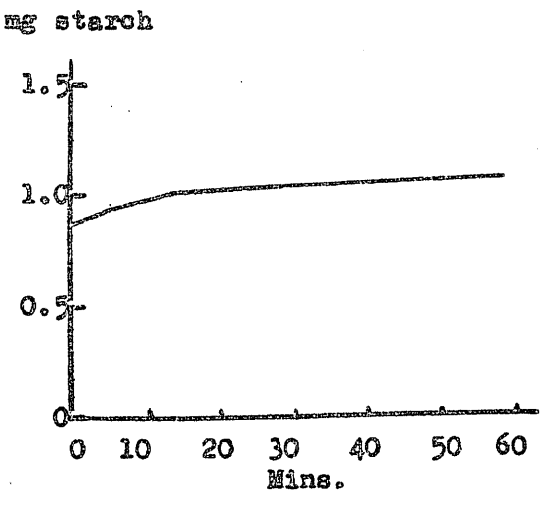
UDP-glucose, glucose-6-phosphate, starch grains.



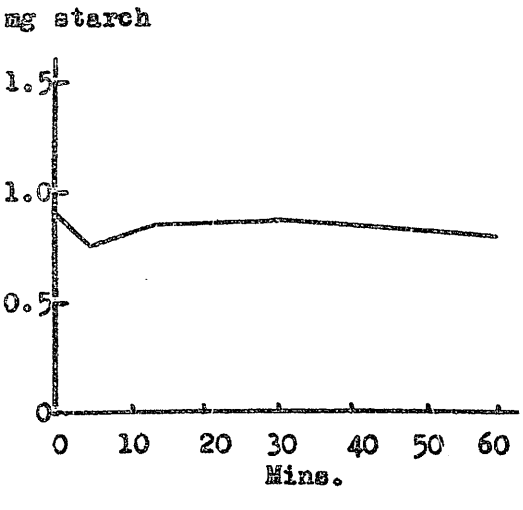
UDP-glucose, starch grains.



ADP-glucose, glucose-6-phosphate, starch grains.



Starch grains.



obtained with time were substantially different (Fig. 19).

These results, not satisfactorily explicable solely on the basis of experimental uncertainties, reinforced the view that a more fundamental examination of starch grains for the presence or otherwise of endogenous nucleotide-substrates etc. which could conceivably interfere in the above trials was now advisable.

These investigations are described in Parts III and IV.

PART III.

A SEARCH FOR NATURALLY OCCURRING NUCLEOTIDE-SUBSTRATES IN WHOLE STARCH GRAINS.

INTRODUCTION.

Repeated reference has been made previously in this Thesis to work which indicates that enzymic activity, capable of bringing about starch synthesis, resides upon the surface of, or within, the starch grain (92, 94). Even the latest proposals of Frydman and Cardini (95) which relegate the role of the grain to acting as "acceptor" for donated glucose residues by a soluble enzyme, leave plenty of scope for the possibility that this process may be highly selective and complex. Thus there are as yet no experimentally established grounds for insisting that the formation of this physiologically organised entity occurs by a simple transfer from a donor species to the non-reducing ends of amylose or amylopectin chains which may be suitably orientated to act as acceptors on the surface of the grains. Donation of glucosyl radicals to intermediates in the grain forming process, which could possess the ability to facilitate the assembly of morphologically uniform grains from an aqueous environment, cannot be excluded.

Clearly, the presence or otherwise of nucleotides and nucleotide-substrates within starch grains needed to be established. Some explanation for the erratic results obtained with the starch grain enzyme preparations, described in Part II, would also be obtained by positive findings. If, of the mono-, di- and tri-phosphates of the four nucleosides together with their glucosyl or related derivatives, only a few members were consistently found, this would be presumptive evidence for some more or less direct connection between their presence and the formation of starch.

(1) Choice of extracting solvent and tests for a nucleotide fraction in various starches.

In preliminary trials, commercial preparations of potato starch grains (Hopkin and Williams) were used. These trials gave immediate indications of the presence of nucleotide material even when distilled water was used as the extractant. Thus, when 200g quantities of starch grains were packed into filter funnels and eluted with water, the clear colourless eluate gave a UV spectrum strongly reminiscent of a typical nucleotide. Examination of subsequent fractions revealed that the process of "desorption" of nucleotide was not complete after several hours, although the amount of UV absorbing material removed decreased slowly with time.

Due to the slow rate of removal of the compounds responsible for the UV absorption by water and because of the hazards of bacterial contamination, aqueous mixtures of organic solvents were tested and it was finally decided that a 50% aqueous methanol solution was the most suitable. This solvent appeared to penetrate the grains rapidly, gave good flow rates in column work, was a good solvent for a wide range of compounds and did not itself absorb in the 220 to 300mμ range of the ultra-violet.

Tests with commercial preparations of wheat, rice and maize starches, in addition to other samples of potato starch, gave results which showed that UV absorbing compounds with spectra characteristic of nucleotides were obtainable in every case. Occasionally certain starches gave patterns where the characteristic minimum at 230mμ was partially suppressed or the spectrum was otherwise distorted, but in every case a pattern reasonably attributable to an impure nucleotide mixture was obtained.

Freshly prepared potato starch, obtained as described by Leloir (92) for his grain enzyme preparations but excluding the final acetone treatment, was also submitted to the elution. As with all other samples tested, these also gave substantial UV readings upon elution with the methanol-water solvent.

Typical results of the elution of (a) a commercial preparation and (b) a freshly prepared potato starch (Expt. 84a and b)

F I G. 20.

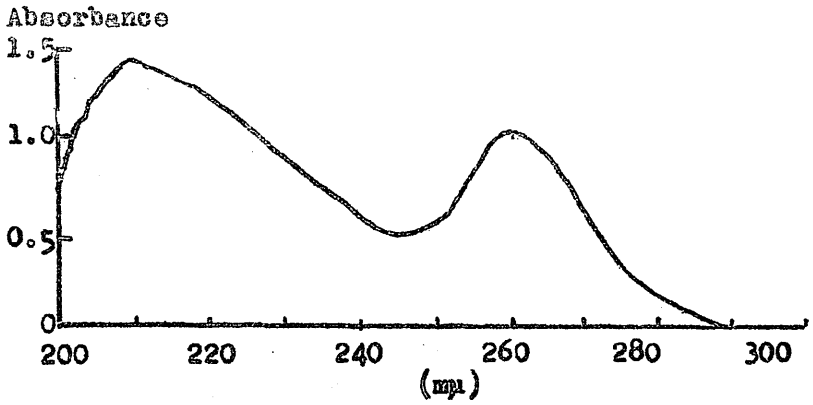
(a) COMMERCIAL STARCH.

TABLE XII.

Ftn.No.	OD_{260}	Ftn.No.	OD_{260}	Ftn.No.	OD_{260}
1	0.68	10	0.70	19	0.32
2	0.75	11	0.64	20*	0.29
3	0.80	12	0.56	21	1.14
4	0.81	13	0.51	22	1.14
5	0.82	14	0.47	23	0.71
6	0.81	15	0.42	24	0.36
7	0.82	16	0.41	25	0.26
8	0.84	17	0.38	26	0.22
9	0.80	18	0.36	27	0.20

Total OD units extracted 525 (2,625 units/kilo).
* Elution discontinued at this point.

UV spectrum (Ftn.3).



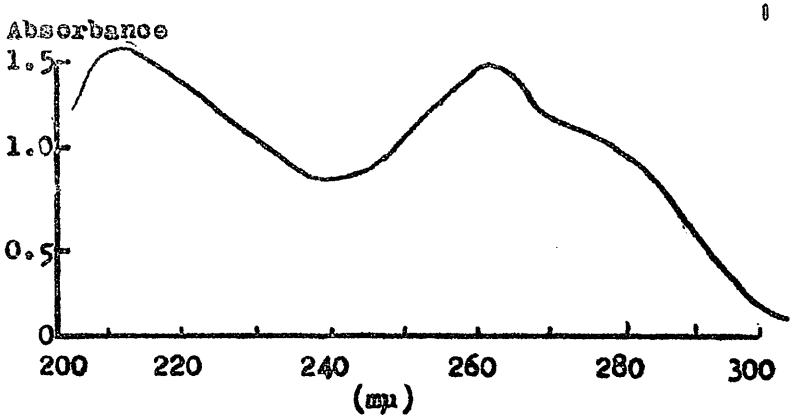
(b) FRESH STARCH.

TABLE XIII.

Ftn.No.	OD_{260}	Ftn.No.	OD_{260}	Ftn.No.	OD_{260}
1	1.48	8	1.10	15	0.41
2	1.53	9	0.94	16	0.38
3	1.46	10	0.88	17	0.34
4	1.41	11	0.74	18	0.32
5	1.33	12	0.65	19	0.28
6	1.21	13	0.56	20	0.26
7	1.16	14	0.47	21	0.25

Total OD units extracted 514 (2,570 units /kilo).

UV spectrum (Ftn.3.).



are provided in Tables XII and XIII. Fig. 20 shows the UV pattern of representative fractions from each of these starches. These values cannot take into account the losses of material inevitably sustained during the water processing of the commercial starches and indeed of the laboratory prepared samples.

(2) Column versus batch techniques in large scale extractions.

Suitable equipment to permit the application of large-scale column techniques for the recovery of sufficient quantities to allow of unequivocal identification by ion exchange chromatography and degradative chemical techniques was unavailable at this time. Hence a batchwise shaking method was adopted.

In a typical extraction of commercial potato starch by this technique (Expt. 85), 3 kilograms of the starch were shaken for 18 hours with 50% methanol-water giving 4.6 litres of supernatant with an OD_{260} of 1.0. Subsequent extractions for similar periods gave (a) 3.6 litres of supernatant with an OD_{260} of 0.61 and (b) 3.2 litres of supernatant with an OD_{260} of 0.23 (Table XIVa). At this stage the extraction was discontinued, the supernatant bulked and the volumes reduced by vacuum distillation. These figures, generally confirmed by further experience (Table XIV), indicated that some 2,300 OD_{260} units were available upon extraction of each kilogram of

the commercial potato starch, with the priviso that it did not all consist of nucleotide material. This became evident when the extracts were subjected to preliminary ion exchange treatments.

TABLE XIV.

(a) 3 kilos starch.			
Extn.	Vol. of supernatant.	OD_{260}	Total OD units.
1a	4.6 litres	1.00	4,600
2a	3.6 litres	0.61	2,196
3a	3.2 litres	0.23	736
Total OD units extracted = 7,532 (2,511/kilo.).			
(b) 3 kilos starch.			
1b	3.6 litres	0.93	3,355
1b	3.78 litres	0.61	2,308
1c	3.86 litres	0.29	1,120
Total OD units extracted = 6,783 (2,261/kilo.).			

(3) The selecting of optimal conditions for separating the nucleotide components.

In one early trial (Expt. 86a), a quantity of concentrated

extract, obtained as described above, was applied to a cation exchange Dowex 50 resin in free acid form with the intention of converting the salt forms into free acids. Of 9,500 OD_{260} units applied the resin retained some 3,000 units which drastic measures such as elution with 1N ammonium hydroxide failed to remove. This fraction clearly did not conform with the expected behaviour of any customary nucleotide.

The remaining fraction, representing two thirds of the optical density, upon submission to the usual ion exchange chromatographic procedures for the separation of nucleotides, already described (Part I, Section V), was only poorly resolved into two unsatisfactorily flat peaks (Fig. 21a). Thus, it was decided to abandon these direct ion exchange methods in favour of ones involving some preliminary separation of interfering impurities.

A method widely used since its introduction by Leloir (35) to isolate UDP-glucose from yeast extracts, is to precipitate all the nucleotides in an extract as their mercury derivatives at a pH of 3.5 from a 50% ethanol solution. Of various alternatives such as charcoal adsorption, barium fractionation etc. other work in this laboratory on the isolation of bracken nucleotides had demonstrated the effectiveness and reliability of the mercury method.

Consequently, sufficient commercial potato starch to provide 23,000 OD_{260} units (10 Kilos.) was extracted with the 50%

methanol-water mixture and after concentration to remove methanol and to provide a convenient volume an equal quantity of ethanol was added (Expt. 86b). After adjustment of the pH to 3.7 the appropriate amount of freshly prepared mercuric acetate reagent was added to the cold solution and the precipitate gathered and washed after one hour. Dispersion of the precipitate by homogenisation allowed its decomposition with hydrogen sulphide to proceed smoothly, whereupon the supernatant obtained after removal of the mercuric sulphide was aerated to remove excess hydrogen sulphide.

Optical density measurement at 260mμ revealed that some 8,500 units, representing 40% of the original value was still available at this stage.

Thereafter this solution was applied to an anion exchange Dowex I resin in chloride form for gradient elution separation by a lithium chloride system as described elsewhere (p.140). Of the 8,500 units applied some 1,800 units were not held by the column at the pH of application (pH 7.0) and presumably consisted of bases and nucleosides.

The lithium chloride gradient applied to this column resulted in the appearance of two large peaks as depicted in Fig. 21b and it was realised that the conditions for separation needed improvement. Nevertheless, chromatography of material isolated (Expt. 87, Table XV) from these two main peaks revealed that the

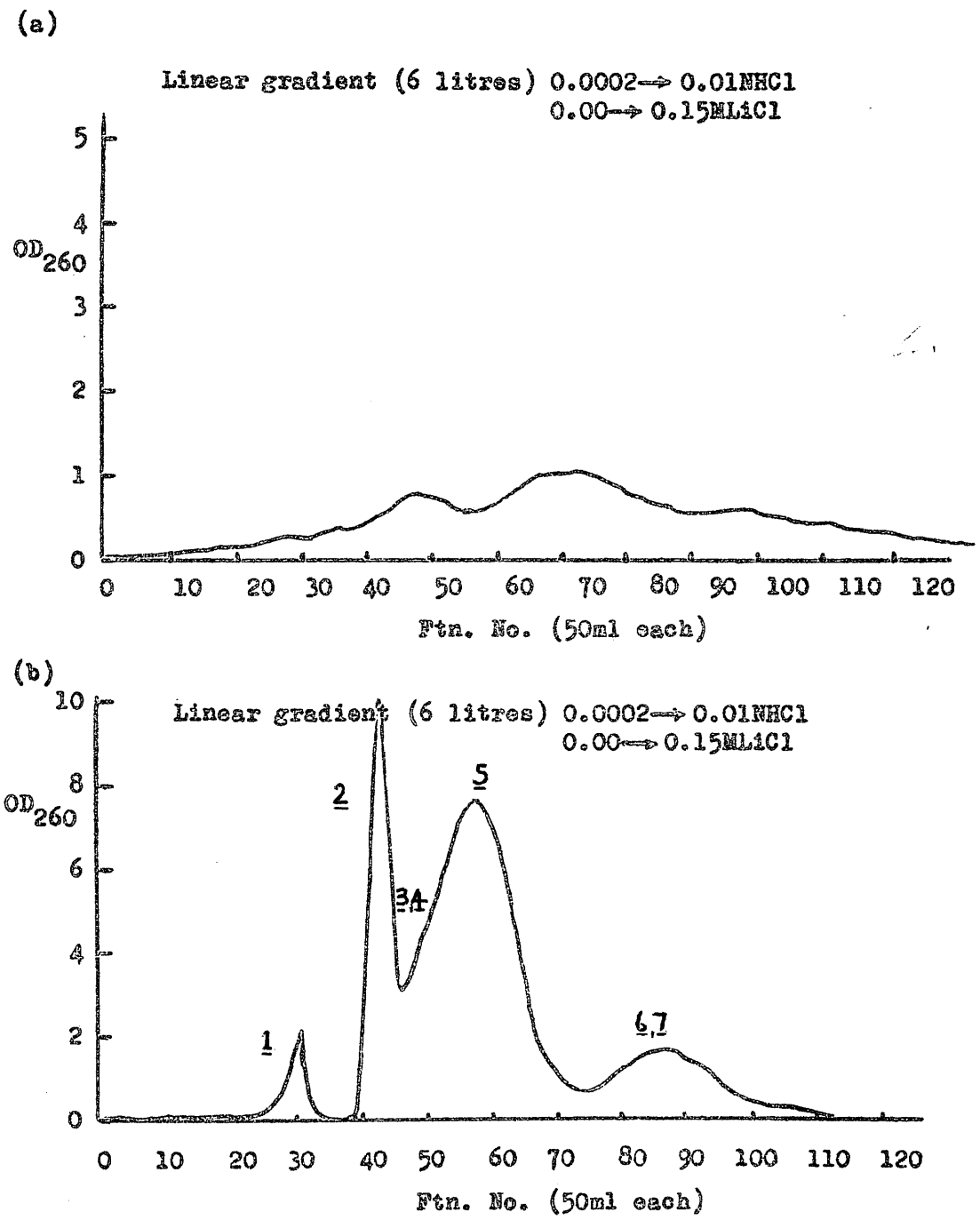
predominant base in each case was adenine and paper electrophoresis of samples from each peak tentatively revealed that the major components were adenosine monophosphate and adenosine diphosphate respectively. The smaller peak which preceded the two larger ones, labelled Peak I in Fig. 21b, accounted for 350 of the OD_{260} units and gave 33mg of lithium salt derivative when subjected to this procedure for its isolation (G.M. 7-1b). It also gave the spectrum characteristics of an adenine derivative and this was confirmed by hydrolysis and chromatography. The failure of the resin to retain this compound until after adenosine monophosphate had been eluted gave rise to the suspicion that it might be nicotinamide-adenine dinucleotide (NAD). Since a fairly specific simple test for this compound exists (205), consisting of the formation of a cyanide derivative detectable by a change in spectrum at 325mμ, the test was applied and was found positive (Expt. 87).

Hence, this first full experiment revealed that at least three nucleotide constituents were present in potato starch grains viz. nicotinamide-adenine dinucleotide, adenosine monophosphate and adenosine diphosphate and that as a minimum one kilogram of starch contained amounts of these of the orders- 2.5, 7.5 and 19mg respectively.

As explained, one defect of the separation discussed above, was the steepness of the applied gradient for elution and several

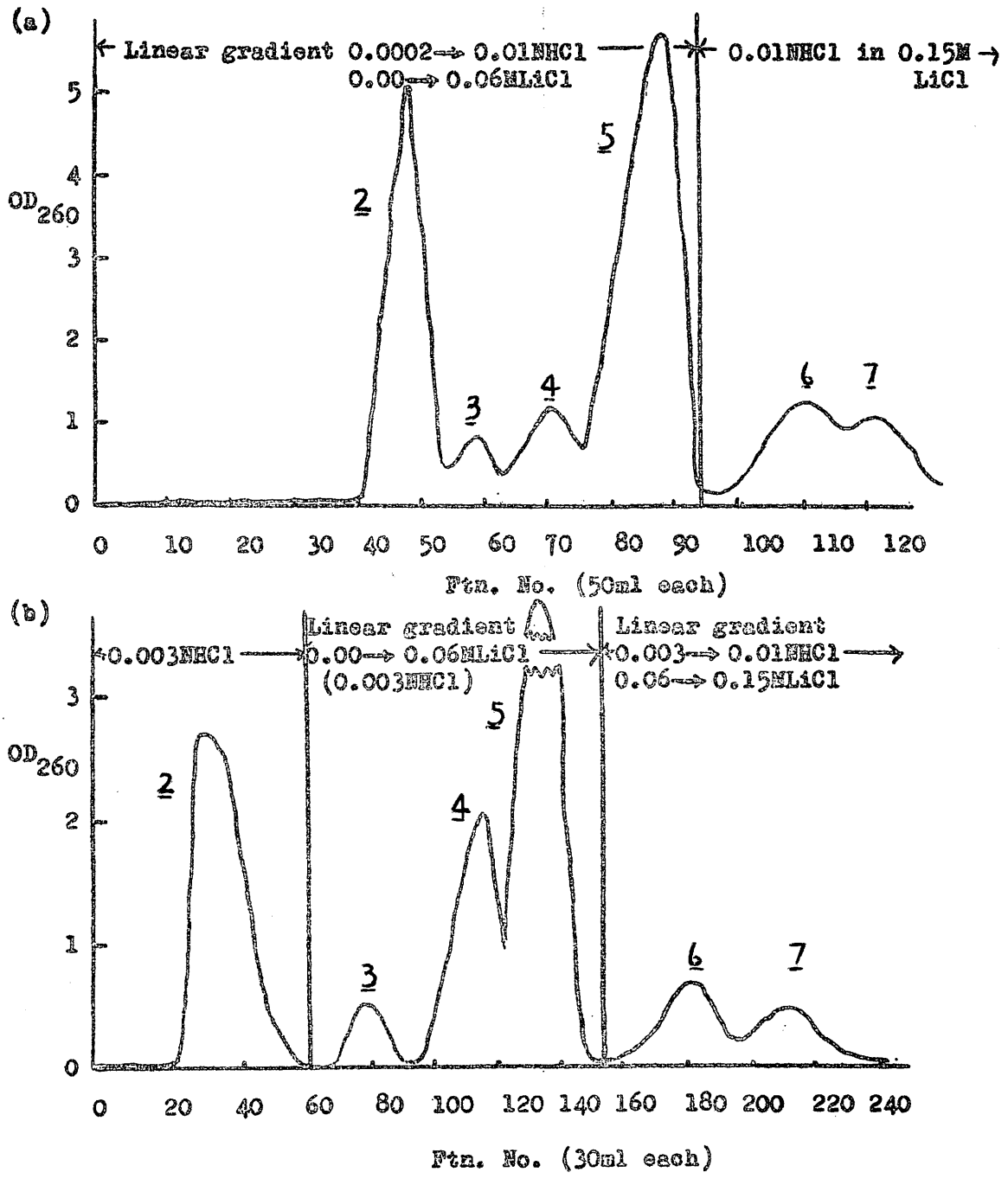
F I G. 21.

ELUTION OF STARCH GRAIN NUCLEOTIDES FROM DOWEX 1 x 4 RESIN.



F I G. 22.

ELUTION OF STARCH GRAIN NUCLEOTIDES FROM DOWEX 1 x 4 RESIN.



separations on other similar starch extracts were carried out to arrive at optimal conditions.

The elution pattern illustrated in Fig. 22a (Expt. 86c), which is typical of several obtained, demonstrates the improvement effected in the technique by halving the lithium chloride concentration in the reservoir flask. Thus, the reservoir in this experiment contained three litres of 0.01N HCl in 0.06N lithium chloride and the mixing flask, in turn, contained three litres of 0.0002N HCl. In addition, the column dimensions were varied, the resin length being increased by 50%.

(4) Identification of the components.

The fractions, corresponding to the seven major peaks (Figs. 21 and 22), were bulked as shown in the figures. As previously described (Part I, Section V) much information concerning the identity of a particular compound can be gleaned from its breakthrough point in such a separation. In addition, however, samples were taken for hydrolysis and paper chromatographic examination in solvents suitable for nucleotides and sugars (after hydrolysis). Phosphate analyses, both total and after partial acid hydrolysis, tended to be unreliable if carried out directly on the eluates, especially in the region of elution of adenosine monophosphate.

This was due to the simultaneous elution of comparatively large quantities of inorganic phosphate which rendered direct analysis meaningless. As an illustration of the magnitude of this interference a sample from Peak 2 (subsequently shown to be pure A5'P) which contained 20µg of AMP as assessed by optical density, gave values of 200µg of phosphate before acid hydrolysis as determined by the King method (C.M. 6-2). There was also a further uncertainty at other points along the elution spectrum caused by the likelihood of there being sugar phosphates as impurities in some samples.

It was possible, however, to eliminate most of these interfering substances by isolating the nucleotide peaks separately as their lithium salt derivatives. To this end each fraction was carefully evaporated to dryness under vacuum with a rotary evaporator at bath temperatures below 35°, the residue being finally dried by co-distillation of traces of water with dry methanol. A solution of the lithium nucleotide(s) in methanol was then treated with excess dry acetone and the lithium nucleotide residue, largely free of impurities, was obtained (C.M. 7-1b).

At this stage nucleotide from each peak was carefully examined in the ethanol-ammonium acetate solvents, described by Leloir, for homogeneity (Expt. 87, Table XV). By this means and also by analysis it was established that material from Peak 2 was solely adenosine-5'-phosphate. Similarly Peak 3 was shown to be

TABLE XV.

PEAK	PAPER CHROMATOGRAPHY		CONCN.	BASE PRESENT		PHOSPHATE		PENTOSE	OTHER
	SOL.1. (pH3.8)	SOL.2. pH7.5		OD RATIOS		PERCHLORIC	TOTAL	ACID LABILE		TESTS
	R_f	R_f	OD_{260}	$\frac{280}{260}$ pH7	$\frac{250}{260}$ pH7	DIGESTION SOL. 5.			
1 (DPN)	0.07	0.16	1.13	0.24	0.81	Adenine	2.31	- - - -	2.52	Cyanide complex
2 (AMP)	0.36	0.25	2.40	0.20	0.79	Adenine	High inorganic phosphate concn.		2.64	- -
3 (UMP)	0.40	0.28	2.49	0.34	0.72	Uracil	2.62	- - - -	0.75*	- -
4 (ADP-ribose)	0.14	0.42	1.32	0.22	0.80	Adenine	2.71	- - - -	2.80	- -
(ADP-glucose)	0.12	0.34	1.26	0.19	0.78	Adenine	2.57	1.34	- -	Glucose (G.M.8)
(GMP)	0.19	0.13	2.30	0.69	0.92	Guanine	2.43	- - -	2.54	- -
5 (ADP)	0.10	0.11	2.05	0.17	0.78	Adenine	4.08	2.11	2.30	- -
6 & 7 (?)	--	--	--	--	--	--	--	--	--	- -

All results expressed in µmoles.

*Pyrimidine-ribose bonds are not completely hydrolysed under the conditions of reaction.

uncontaminated uridine monophosphate.

Peak 4 contained three nucleotides and to establish their identity a quantity of solid was dissolved in water and the solution applied as bands to sheets of Whatman No. 3MM thick paper. Development in Leloir's pH 7.5 solvent (C.M. I, solvent 2) and location of the bands under UV light permitted recovery of the three components separately by elution of the appropriate regions cut from the original sheets (Fig. 23).

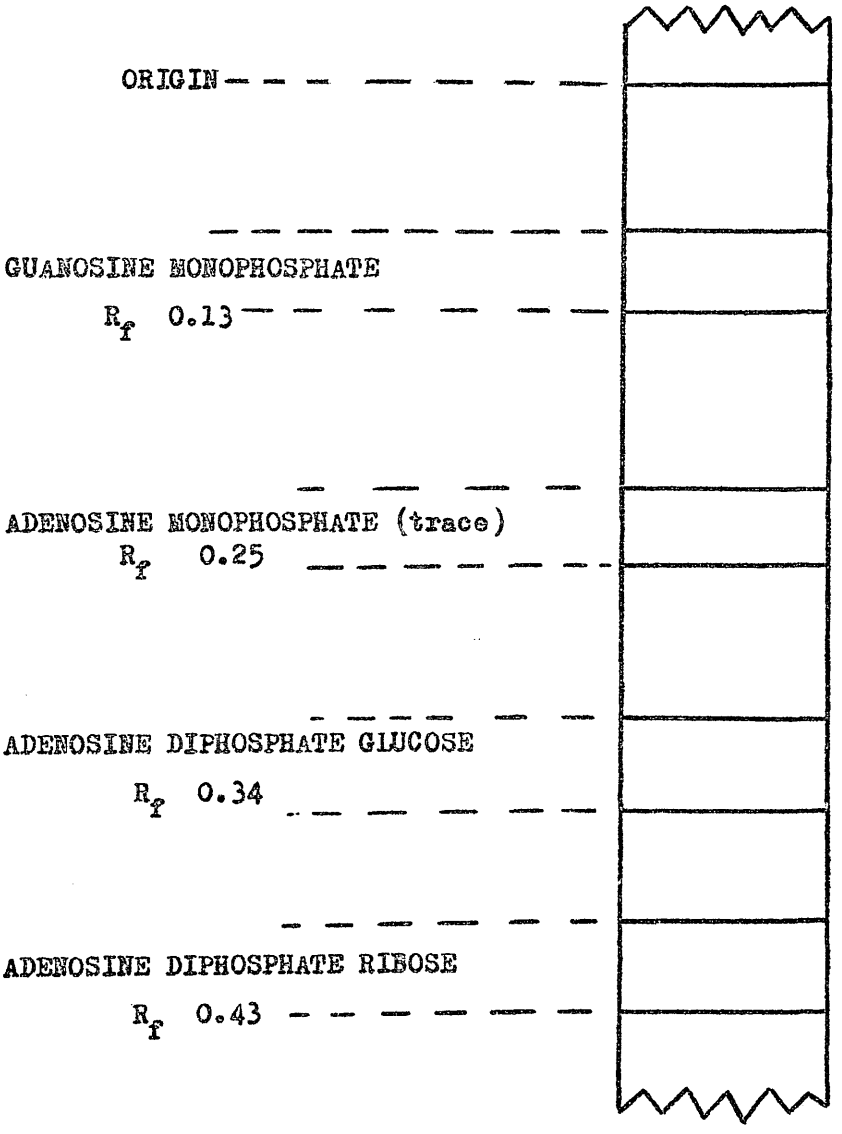
The fastest running component (R_f 0.42) proved upon hydrolysis and analysis for phosphate, base and pentose to be adenosine diphosphate ribose (ADP-ribose). It is likely that this nucleotide arises as the result of the partial degradation of nicotinamide-adenine dinucleotide (NAD). It is worthy of note that in this particular experiment and contrary to a result described previously no NAD was separately found.

The material recovered from the band of intermediate R_f (0.34) gave glucose upon hydrolysis with N/50 acid for ten minutes at 100°. It also possessed one phosphate group susceptible to hydrolysis under the same conditions. This was 52% of the total phosphate found. The base, by its spectral characteristics and chromatography, was shown to be adenine.

The original material of the band co-chromatographed in two solvents with authentic, chemically synthesised adenosine di-

F I G. 23.

RESOLUTION OF PEAK 4 USING LELOIR'S PH 7.5 SOLVENT AND WHATMAN NO. 3MM PAPER (DEVELOPED FOR 42 HOURS).



phosphate glucose and a comparison of its chromatographic behaviour with each of the four ∝-linked glucose containing nucleoside diphosphates available (Part I, Section V) eliminated all possibilities of it being mistaken for these alternative analogues (Table XVI).

TABLE XVI.

NUCLEOTIDE	R_f	
	pH 7.5	pH 3.8
Unknown	0.34	0.12
ADP-∝-glucose	0.34	0.12
UDP-∝-glucose	0.39	0.14
GDP-∝-glucose	0.21	0.08
CDP-∝-glucose	0.30	0.10

This left little room for doubt that the nucleotide was in fact adenosine diphosphate glucose. A further item of observation served to confirm this opinion. In the original elution from the Dowex column (Fig. 22) the nucleotide was eluted at a point entirely consistent with it being ADP-glucose (Fig. 22a, Fractions 58 to 68). UDP-glucose on the other hand would not be expected to break through the column before Fraction 86, following elution of ADP. This

would also apply to GDP-glucose and the remaining possibility viz. CDP-glucose would have been eluted prior to UMP.

The slowest running component (R_f 0.13) which was the third constituent of Peak 4 was identified as guanosine-5'-phosphate (G5'P).

Peak 5 was shown by UV spectrophotometry, phosphate analysis (total and labile) and by chromatography to consist entirely and solely of adenosine diphosphate (ADP).

The components in Peaks 6 and 7 were not identified in that the spectra were anomalous and did not correspond with those of typical nucleotides. Attempted paper chromatography by the usual solvents (C.M. 1, solvents 1 and 2) also failed and the components were not dislodged from the starting line. Nevertheless the materials contained organically bound phosphate and were isolatable by the usual lithium salt procedure.

The analytical data obtained for the seven peaks described above are summarised in Table XV.

As stated earlier, other similar confirmatory separations on methanol-water extracts from commercial potato starch grains were carried out with results essentially similar to those discussed above.

In one trial an attempt was made to recover the nucleotide from a starch paste after removal of amylose and amylopectin by ethanol precipitation. This technique was abandoned after it was found that the concentrated supernatants invariably contained some

interfering starch residues. In addition the technique was tedious and the liquid volumes required inconveniently large.

Examination of fresh potato starch samples and other commercial starches such as wheat, rice and maize, were limited to establishing that all such starches contained nucleotide material as evidenced by UV spectra of samples from methanol-water eluates.

Thus detailed information of the identity of the nucleotide components of starches is only available for commercial potato starch.

From the results of several experiments it appears that quantitatively the two nucleotides present in greatest amount are AMP and ADP. Small but significant amounts of UMP and GMP are also found. The only sugar containing nucleotide appears to be ADP-glucose and this finding may have a bearing upon Leloir's observation (93) that in his starch grain system ADP-glucose was ten times as effective as UDP-glucose in the role of glucose donor. The small amount of ADP-glucose found may not be unexpected in that evidence has been presented (206) indicating that the equilibrium for the starch synthetase enzyme favours polymer formation with consequent liberation of ADP. In this context it is worth noting that the quantity of ADP found was unexpectedly large (Figs. 21 and 22).

Especial care was taken to examine relevant fractions for the presence of UDP-glucose but in no case was there evidence for its existence. Indeed the only uridine compound found was UMP and the

amount of this represented only 3% of the total UV_{260} held on the column and was only little greater than the amount of GMP also found.

These results do not, of course, offer conclusive proof of an involvement of these compounds in starch synthesis or starch grain formation. It could be argued that if grain formation occurs by some accretion process or by some sequence which involves polymerisation in a homogeneous liquid phase followed by "crystallisation" with concomitant occlusion of other entities in the vicinity then these entities would be many and various. The fact that the few nucleotides found were predominantly of the adenine series and that the sole sugar containing nucleotide was ADP-glucose tends to the conclusion that these compounds are probably involved in some mechanistic way.

Perhaps the most unexpected member of the nucleotide group of substances found was nicotinamide-adenine dinucleotide. At the present state of knowledge it would be premature to propose a role for this in the metabolism of the starch grain but a search for dehydrogenases associated with the grain, capable of using this co-factor for the oxidation and reduction of grain components, might be profitably undertaken.

PART IV.

LIPIDS AND THE STARCH GRAIN PROBLEM.

INTRODUCTION.

Any refined model to explain the mechanism of formation of a starch grain must state precisely what initiates the formation of the grain, what intermediates are involved, with the order in which they participate, what species act as glucose donors, the conditions under which the process works and the nature of the catalysts required.

It must be presumed that at some point in the process, material which was once in true solution, deposits. This deposition may occur on some unspecific nucleating material but it is more reasonable to assume that there is some starch grain precursor or particle which favours these events.

Badenhuizen pictures the formation of a starch grain as occurring by the coming together of "precursors" in the plastids with the formation of coacervate droplets which are deposited on the growing grain by a process of apposition (207).

This model is lacking in detail but it does contribute to an understanding of the reasons for the appearance of amorphous

layers and shells in cereal starch grains and why these differentiated layers disappear under conditions of constant illumination. Thus it is argued that under these latter conditions coacervate droplet formation is continuous and uninterrupted, leading to uniform deposition and hence uniform hydration. There is, therefore, no differentiation into crystalline regions (shells) and amorphous regions.

The model also predicts that stroma material would be expected to be included in the grain during growth by a process of intussusception.

Some evidence for the accumulation of "precursor material with reducing properties" as droplets has been obtained by histological techniques and by electron microscopy (208).

Although Badenhuizen does not so state, it is difficult to envisage the development of a droplet in an aqueous environment unless there is some degree of phase difference between the droplet and its surroundings. Thus a droplet suggests an interface, which, in turn implies a degree of organisation and possibly a lipid surface.

It is useful from the viewpoint of constructing a model to assume that there is some degree of organisation of metabolites even in the liquid phase. Of relevance is the appreciation of the role of lipid materials in contributing to the structures of cellular components and membranes as envisaged by Benson (209). Conventional lipids such as triglycerides would not be expected to act other than

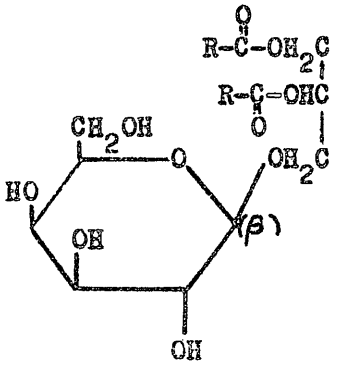
as generally impermeable layers, but if they are modified to contain a polar group then the attributes of partial aqueous and partial lipoid solubility are conferred. The characterisation of some glycolipid constituents of wheat flour by Carter in 1956 (210) led to the first real understanding of the great potential importance of these compounds in biological systems. Carter identified the compounds I, II and III, the structures of which are shown in Fig. 24.

In photosynthetic studies with Chlorella, Benson (211) observed that galactolipids and sulpholipids accounted for up to 50% of the total lipid material of the chloroplasts (212). He identified the compounds I, II and IV (Fig. 24) viz. the original mono- and di-galactodiglycerides of Carter, together with a sulphonated glucose diglyceride. This latter compound, the structure of which was elucidated by Benson, possesses the surface active properties of the glycolipids and, in addition, is negatively charged at physiological pH's. Benson appreciated that the presence of such molecules, suitably orientated, could endow surfaces with special properties (209). Aggregation of subcellular particles, the ability of charged ions to penetrate membranes, orientation of proteins and other macromolecules to each other, aqueous solubility of hydrophobic substances and the mechanism of heterogeneous enzyme reactions are all phenomena which could conceivably be largely affected by these surface active lipids.

It was, therefore, of direct interest in the present study

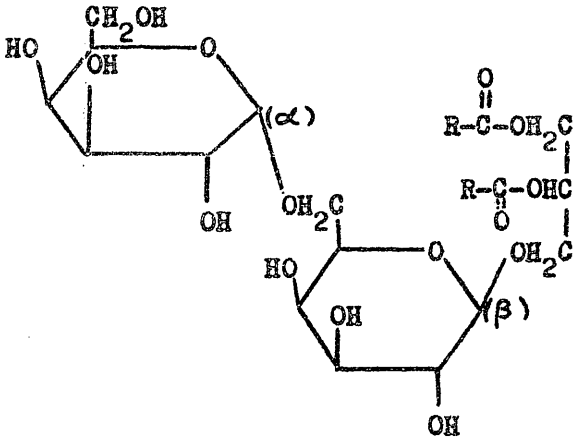
F I G. 24.

G L Y C O L I P I D S. (examples)



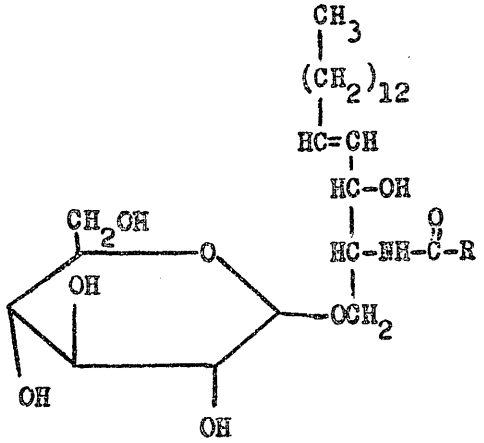
(I)

MONOGALACTO-DIGLYCERIDE



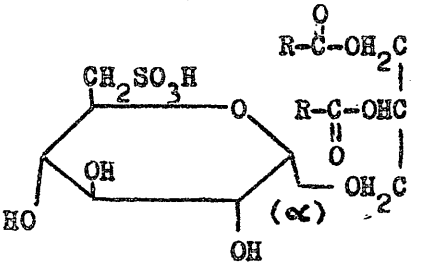
(II)

DIGALACTO-DIGLYCERIDE



(III)

GLUCO-CEREBOSIDE



(IV)

SULPHO-LIPID

to inquire whether starch grains contained compounds of similar character. Under the licence permissible in attempting to conceive of a model for the grain formation process it was intriguing to speculate that such compounds could play a more direct role than those mentioned above. Thus in addition to the advantages conferred upon a solid-liquid interfacial enzyme reaction by a component possessing detergent properties it is not inconceivable that a sugar containing lipid might itself participate directly either as donor or acceptor in a sugar transferring sequence of events. This participation might precede or alternatively succeed the stage where sugar is transferred from a nucleotide such as ADP-glucose.

Of interest in this connection is a report by Neufeld (213) that spinach leaf chloroplasts suspended in a solution containing radioactively labelled galactose-1-phosphate and UTP (presumably serving as a source of UDP-galactose) resulted in the formation of sugar labelled galactolipid. In addition there were some indications that a small amount of label appeared in an oligogalactose of chain length comprising three or four units. A fuller account of this work would be interesting.

References to the discovery of other members of the glycolipid series are currently appearing. For example Lennarz (214) has described the isolation and tentative characterisation of a mannodiglyceride which is probably analogous to Compound I, Fig. 24.

The studies carried out by Colvin (215) on a cellulose synthesising system in Acetobacter xylinum have given rise to some interesting observations. He found that treatment of an 80% ethanol extract obtained from the organism, with a crude protein preparation from the same organism resulted in the formation of cellulose fibres, identified mainly by physical characteristics such as the X-ray pattern. The nature of the precursor(s) in the ethanol extract was not established. However, he has also obtained somewhat similar results by using an ethanol extract obtained from plant material (216). The enzyme preparation was in each case derived from the Acetobacter.

In what follows an account is given of work which establishes that potato starch grains do, in fact, contain appreciable quantities of at least two and possibly three glycolipid components.

(1) A note on the extraction of lipid material from plant tissues.

The traditional solvents used to extract the lipid fraction from materials such as feeding stuffs for purposes of analysis have generally been ether, low boiling fractions of petroleum ether or mixtures of these.

Where it is necessary to extract materials which cannot be oven dried or alternatively where the purpose, at least in part, is to attempt to isolate lipids possessing some hydrophilic properties then

these solvents are not entirely suitable.

Thus, for example, Schoch in his examination of lipid components of certain starches, carried out in 1942 (217), appreciated this difficulty and after some preliminary experiments adopted the use of 80% methanol or 80% dioxan for the purpose. He found that the amounts of lipid material extracted were consistently higher with these solvents than with other alternatives.

For other plant tissues mixtures of methanol and chloroform have frequently been used for glycolipid extraction. It is claimed that such mixtures (generally two parts chloroform : one part methanol, v/v) reduce the amount of unwanted sugars, amino acids etc. that are extracted.

(2) <u>The extraction of lipids from potato starch grains.</u>

In the present work it was felt that no advantage was to be gained by any substantial modification of the extraction technique used by Schoch, despite the fact that his examination of the lipid fraction of potato starch by the methods then available had not led to a detection of any components other than conventional free fatty acids.

Thus, as described (Expts. 88 and 89) two methods for obtaining lipid extracts were used. In the first a batchwise

method (Expt. 88), two kilograms of commercial potato starch were refluxed with 5 litres of 90% methanol for about three hours. The water content of the starch (ca 20%) reduced the effective concentration of the methanol to about 83%.

The other method (Expt. 89) which experience showed to be superior in extracting larger amounts of lipid and which was less drastic than the refluxing technique involved packing the starch grains into a wide diameter (9") visible flow pipeline to a height of about twelve inches on a cotton wool plug. This starch column which was jacketed with a heating tape device (Electrothermal Ltd.) was then continuously eluted with quantities of 90% methanol separately warmed to a few degrees below the boiling point. The eluate was reheated and recycolised, the process being continued for about three hours. At the end of this time the methanol extract was concentrated under vacuum and the concentrate stored.

(3) A note on the "purity" of lipid extracts.

Traditionally, the "lipid fraction" has always referred to a wider group of compounds than the mono-, di-, and triglycerides. The recognition that ether and petroleum ether solvents also extracted fatty acids, steroids, waxes, fat soluble vitamins etc. led to the term "lipid" being applied also to these materials although this was

not justified from the point of view of structure. Solvents with more polar properties such as are now used for reasons already discussed can extract an additional number of compounds and the problem of deciding whether or not to regard certain of these as lipids even in the broad sense becomes meaningful. Thus the original sugar containing compound discovered by Carter (Fig. 24, I) can confidently be classified as a lipid because the glycerol is still doubly esterified with fatty acids as is the case with the phospholipids. However saponification of this would give rise to a glyceryl-galactoside which might more appropriately be regarded as a sugar.

It follows that a fractionation technique designed to exclude sharply all compounds not possessing certain characteristics could well result in a failure to detect important constituents unless all fractions are examined. Hence there is a need to evaluate the potentialities of any method most carefully.

Since the particular purpose of the present work was to attempt the identification of sugar containing lipid entities in starch grains it was felt that the limits should be set to include any sugar containing materials extracted excluding only those such as free sugars, oligosaccharides etc., compounds traditionally regarded as being primarily classifiable as carbohydrates.

Indeed the first serious difficulty of work on glycolipids is to decide with certainty whether lipid components thought to

contain bound sugar are not in fact physical associations of conventional lipids with free sugars. To distinguish between these two possibilities is not always easy because organic solutions of lipids can frequently entrap aqueous droplets referred to as micelles. Aqueous extraction of micelle containing extracts in separatory funnels seldom succeeds in breaking the micelles which are objected to because the inner aqueous region contains dissolved hydrophilic substances such as free sugars. Hanahan (218) believes that the micelles are stabilised by the presence of polar lipids which orientate over the surface of the droplets offering a hydrophobic surface to the organic phase in a way almost precisely converse to the action of soaps upon oil droplets in water.

Submission of micelle containing organic extracts to subsequent fractionation and characterisation procedures could thus well lead to the erroneous conclusion that glycolipids are present and methods have been devised to overcome this problem.

Two procedures, devised by Folch (219, 220), have been used by most workers in this field. In the first (219) the lipid mixture is dissolved in chloroform-methanol (2:1, v/v) and water in amount equal to a fifth of the volume of the organic phase added. By virtue of the presence of the methanol which has good organic phase and water solubility the micelles are slowly dispersed upon shaking with consequent removal of micelle entrapped sugars etc.

A repetition of this treatment is claimed usually to suffice for the purpose.

The alternative procedure (220) is to place the chloroform-methanol solution of mixed lipids into a dialysis bag and to perform the dialysis of this solution against water, with agitation, for several days. Methanol and methanol-water soluble components escape through the membrane but the chloroform and components preferentially soluble in it are retained. In addition certain constituents soluble in the original mixture but insoluble in chloroform do not pass through and remain as an oily phase suspended in the chloroform-water within the bag.

Use of either of these procedures entails some risk of the loss of interesting methanol-water soluble components but in each case, of course, this phase can be separately examined.

Before submitting the starch grain lipids to any of the fractionation procedures to be discussed it was desirable to establish that components of interest were in fact present. This was of particular concern since Schoch in 1942 (217) had stated that the lipid fraction of potato starch consisted almost entirely of free fatty acids. Thus portions of the extracted lipid material were treated by the Folch techniques (Expt. 90). Paper chromatography of the original lipid mixture on the one hand and the Folch treated dialysed lipid on the other in the solvent butanol-pyridine-water

enabled the success of the Folch technique to be assessed. Thus untreated extracts were shown to contain large quantities of free sugars, mainly glucose, fructose and sucrose. In this solvent the lipids were carried with the front and did not interfere unduly. Repetition of the Folch treatment until no traces of free sugar remained gave material which could confidently be regarded as being suitable for further investigation.

When this treated lipid mixture was saponified and acid hydrolysed (Expt. 90), paper chromatography showed that bound sugars liberated by the hydrolysis treatments and identified tentatively as being galactose and glucose, together with glycerol, were present. This established that potato starch grain lipid extracts did in fact contain glycolipid components.

As will be seen subsequently, column separation techniques were applied which rendered it unnecessary to apply the time consuming Folch technique for removal of free sugars. At this stage, however, use of the technique enabled a positive decision to be made concerning the presence of the glycolipids.

(4) Some fractionation techniques currently used in lipid research.

Only in comparatively rare instances are manual solvent partition methods now used to separate lipids. These methods

depended, for example, upon the belief that phospholipids were insoluble in acetone, hence a warm acetone extraction of a lipid residue was claimed to dissolve all components except these. It has since been shown that this claim is fallacious and phospholipids containing a high percentage of unsaturated diglyceride are in fact acetone soluble.

Another method extensively used in the past involved a partitioning of lipid constituents between heptane and methanol. This method utilised by Carter (210) in his preliminary purification of two of the compounds shown in Fig. 24 (I and II), relies upon the methanol phase being a more attractive solvent for polar constituents, the heptane on the other hand preferentially dissolving the non-polar entities. The method suffers from the practical disadvantages that the presence of some constituents may delay phase separation unduly and also from the fact that frequently inconveniently large quantities of interfacial material collect. This material is usually described as being lipoprotein in nature, but there is always the risk that it has associated with it some non-protein components.

The persistence of the micelles earlier referred to in the batchwise techniques of solvent separations was a further serious disadvantage.

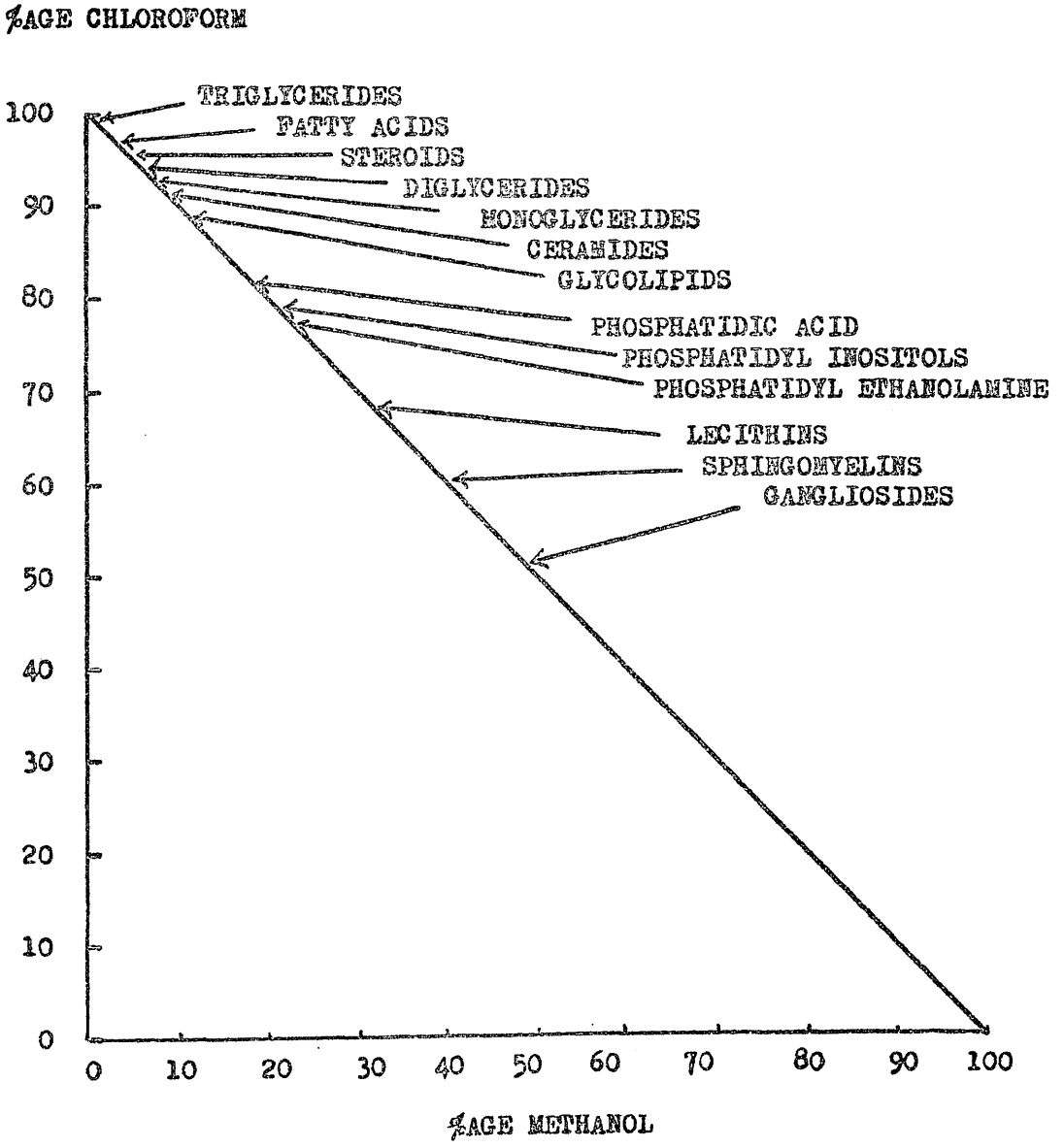
The discovery that silicic acid as a material for column chromatography possessed the ability to dissipate the micelles when

used in conjunction with certain organic solvents has led to the almost complete abandonment of the earlier methods (221). Silicic acids prepared by various techniques and pretreated differently vary in their ability to accomplish the destruction of the micelles and the product supplied by the Mallinckrodt Chemical Co. which is highly effective in this role as well as being otherwise suitable is now widely used generally. Using this particular silicic acid in column form suspended in chloroform it is possible to fractionate a lipid mixture into polar and non-polar fractions merely by pouring a chloroform solution of the mixture through the column (222). Thus triglycerides and free fatty acids (unionised in chloroform) will pass directly through the column without retention although even these can be separated by using a more non-polar liquid phase such as heptane and effecting separations with heptane-ether mixtures.

Addition of slowly increasing amounts of methanol to the original chloroform displaces in turn steroids, ceramides, glycolipids, phospholipids, free sugars and gangliosides (Fig. 25). Hence separation depends mainly upon the degree of polarity exhibited by the various components. Experience has shown (222) that the influence of the degree of unsaturation upon the point of elution is fortunately minimal. Thus, for example, both saturated and unsaturated lecithins emerge from the column in the same region without cross contaminating other fractions of different character.

F I G. 25.

THE ORDER OF ELUTION OF LIPIDS FROM SILICIC ACID COLUMNS WITH CHLOROFORM-METHANOL MIXTURES.



The most puzzling feature of the silicic acid separations is the behaviour of the free sugars. Such judgment as can be applied to the situation would lead to the prediction that free sugars would be retained firmly by the silicic acid, to be removed if at all by high methanol concentrations. Yet the consistent experience is that they are removed by 20% methanol in chloroform. The point is discussed in detail by Wren (222).

A basically similar technique which can be used in conjunction with the above is thin layer chromatography using silica gel as the adsorbent and methanol-chloroform mixtures for development (223). The fact that separations can be effected in about one hour and that the spots can be readily located by examination of rhodamine treated plates under UV light (G.M. 5) makes it possible to use this auxiliary method as a check upon the effectiveness of the larger scale column separations.

Svennerholm et al (224) and separately Rouser and his collaborators (225) have described a technique involving the use of DEAE-cellulose column chromatography. This technique can be applied to crude lipid extracts directly or alternatively can be used to resolve even further the fractions obtained by the silicic acid column method.

The recommendations of these two groups of workers differ slightly in detail but both methods require that the cellulose

exchanger be used in an anhydrous organic solvent composed of chloroform and methanol. The exchanger in either acetate or hydroxyl form exhibits no affinity for uncharged components of the lipid mixture applied to the column in the same anhydrous solvent mixture. Thus tri-, di- and monoglycerides, glycolipids and even lecithins are reported to run directly through the column. It is implied in the publications referred to that free fatty acids are retained by the column under these conditions; trials carried out in this work with stearic and oleic acids, however, led to the converse finding that these acids are also eluted.

After complete removal of the above listed entities a further fraction can be eluted by addition of acetic acid to the chloroform-methanol mixture. These authors regard this fraction as representing free fatty acids, mildly acidic lipids, free sugars and other non-lipid impurities. Again, as will be mentioned later, results obtained in the present investigation indicated that sugar containing material in addition to any free sugars was to be found in this fraction.

Following upon the acetic acid treatment removal of charged lipids may be accomplished by subjecting the column to elution with chloroform-methanol containing about 5% by volume of an aqueous solution of lithium chloride. This salt which can ionise in methanol displaces components such as phosphatidic acid, sulpho-

lipids etc.

It is likely that the success of the DEAE-cellulose method in achieving fractionation of lipids, as described above, does not depend solely upon its ion exchange properties. The final step involving elution with lithium chloride may be wholly an ion exchange effect, but the results obtained in the intermediate stage when acetic acid is a component of the eluting solvent indicate a balance between ion exchange and adsorption effects.

(5) The fractionation of the starch lipid extracts.

It has already been stated that early trials on starch grain lipid mixtures had established the presence of glycolipid material (Part IV, (3), Expt. 90). Many other preliminary experiments to test the utility of the silicic acid and DEAE-cellulose fractionation techniques when applied to starch lipids in particular were also necessary. Thus it will be appreciated that the precise way in which these techniques are applied to greatest advantage will depend upon such factors as solubility, the ratio of hydrophilic to hydrophobic content etc. This essential information together with the required experience in the setting up and use of the various columns was obtained by performing many initial experiments which will not be detailed here. What follows is an account of a typical

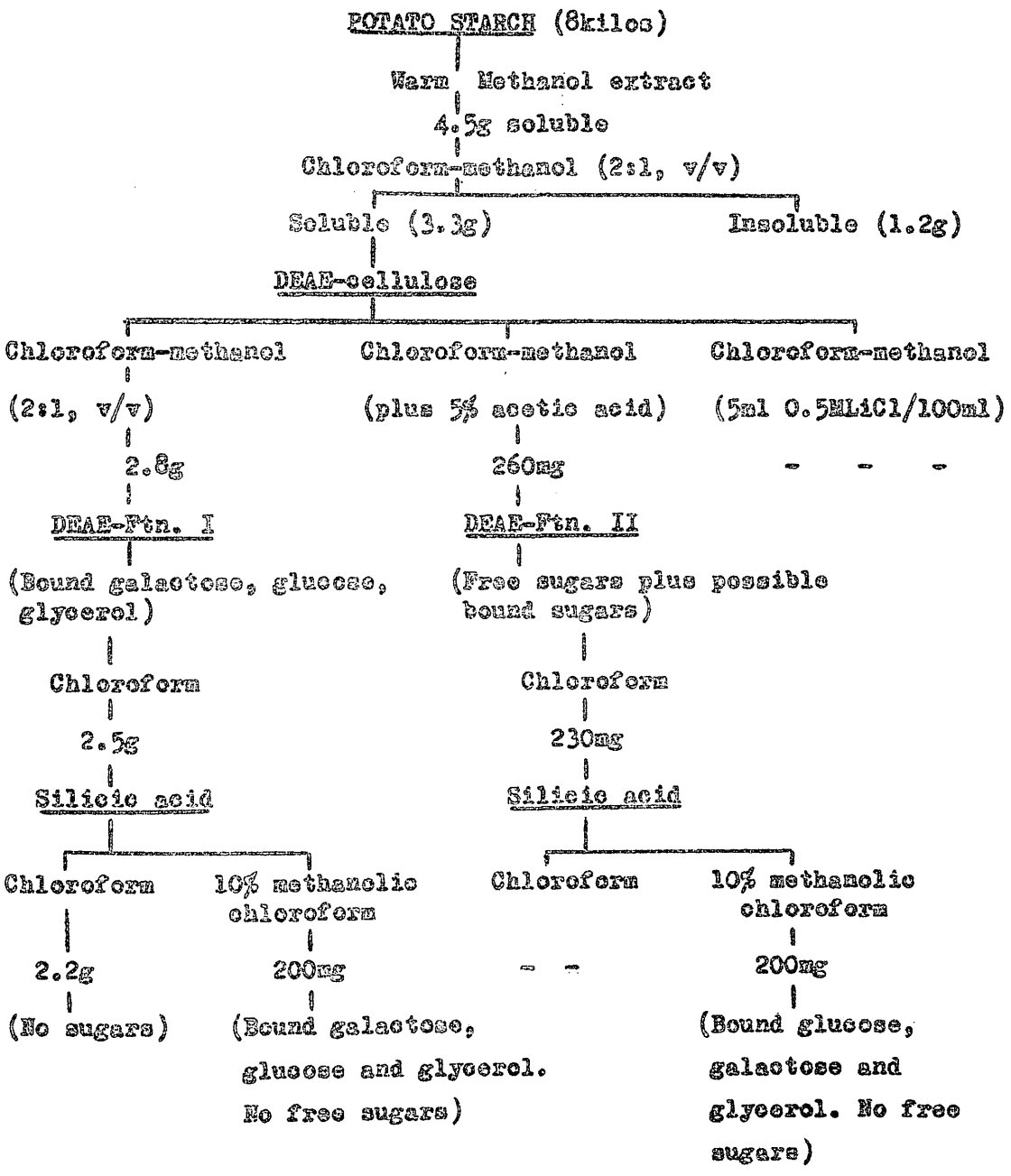
sequence of operations which preliminary experience had shown to be the most effective in achieving the desired separations. The findings detailed here were confirmed by duplicate experiments and by the results obtained in the less comprehensive trials mentioned.

4.5g of lipid obtained by the column technique of starch grain extraction (Expt. 89, Fig. 26) was triturated repeatedly with quantities of chloroform-methanol (2:1, v/v) and the 1.2g which failed to dissolve was discarded. The 3.3g which had dissolved was applied to a DEAE-cellulose column (Expt. 91) and of this 2.8g passed directly through without retention (referred to as DEAE Fraction I). Elution with chloroform-methanol containing 5% acetic acid effected the removal of some 260mg of material (DEAE Fraction II). The lithium chloride treatment however did not appear to elute any further lipid components, implying that potato starch does not contain free phosphatidic acid and similar charged compounds.

Acid hydrolysis (G.M. 12) of DEAE Fraction I under conditions which would totally hydrolyse glycerides and glycosidic linkages, followed by paper chromatography released galactose, glucose and a small quantity of glycerol. Free sugars were absent from this fraction as evidenced by control experiments. It appeared however that the amounts of sugars and glycerol released were considerably less than the equivalent of the lipid submitted to hydrolysis, indicating possibly that the bulk of this fraction consisted of

F I G. 26.

FLOW SHEET FOR FRACTIONATION OF COMMERCIAL POTATO STARCH.



free fatty acids.

A similar examination of DEAE Fraction II was less informative as it was found that free sugars (glucose and some fructose) contaminated this fraction. However there were indications that acid hydrolysis resulted in the appearance of additional quantities of glucose and galactose together with glycerol.

Each of these two fractions was now submitted to the silicic acid separation technique (Fig. 26). 2.5g of DEAE Fraction I was dissolved in dry chloroform (Expt. 91) and the solution applied to a column of silicic acid. 88% or 2.2g of this total passed directly through the column. Chromatography of the acid hydrolysis products of this fraction showed the complete absence of sugar containing components and the liberation of only trace amounts of glycerol indicated that the fraction was almost wholly composed of fatty acids. Thus, Schoch's claim that the lipid fraction of potato starch is mainly fatty acid is at least partly confirmed here in that an original 3.3g of chloroform soluble lipid contains the equivalent of 2.5g of mixed fatty acid i.e. 75% (Fig. 26).

The silicic acid column was now treated with a 10% solution of methanol in chloroform and elution continued until no further material emerged. Increasing the methanol content of the eluting solvent also failed to displace further material.

The solid from this elution accounted for 200mg and paper

chromatography of an acid hydrolysate gave substantial amounts of galactose and somewhat smaller quantities of glucose and glycerol. A discussion of the results of further examination of the materials comprising this fraction is deferred until later.

230mg of DEAE Fraction II in chloroform was meanwhile applied to a second silicic acid column (Expt. 91). On this occasion virtually all the material applied was held, but continuation of the elution with a 10% solution of methanol in chloroform resulted in the displacement of some 200mg of the original 230mg. As with the previous silicic acid column little or no lipid material was recoverable from the column by increasing the methanol content of the eluting solution beyond 10%, the sole result of such attempts being to displace the free sugars which the DEAE Fraction II uniquely contained. Hence the only fraction requiring examination here was that obtained by eluting the column with 10% methanol in chloroform.

In agreement with reports (222) this fraction was shown by chromatography to be uncontaminated with free sugars thus confirming the efficacy of the technique to accomplish this purification step. Acid hydrolysis and paper chromatography showed the presence in the hydrolysate of substantial quantities of glucose, together with smaller amounts of galactose and glycerol.

Thus, to summarise, it can be seen that the whole sequence of the separation detailed above and in Fig. 26 resulted in the

isolation of two fractions of interest accounting for about 15% of the total lipid extracted. Acid hydrolysis of each of these gave glucose, galactose and glycerol but the one earlier described gave a higher amount of galactose than of glucose upon hydrolysis, whereas the fraction referred to above yielded relatively more glucose than galactose.

The presence of galactose in each of these fractions held open the possibility that compounds analogous or even identical to those described by Carter (Fig. 24, I and II) were present. The fraction derived from DEAE Fraction I would be the more likely to contain such compounds, but modified structures of similar nature could conceivably appear in either. The glucose found could be derived from glucose analogues of the Carter compounds present as mixtures, or alternatively glucose-galactose oligosaccharide moieties could pre-exist in bound lipid form. There are various other possibilities. For example, glucose containing cerebrosides (Fig. 24, III) are known in higher plants (221) and animals (230) and galactose analogues have also been reported in animal tissues (231).

Whilst the full identification of the components of these two fractions has not been completed the results of experiments discussed below suggest various possibilities.

As stated, each of the two fractions on acid hydrolysis gave glucose, galactose and glycerol. Each fraction was separately

submitted to saponification (Expt. 92b). The aqueous hydrolysates were each neutralised with sulphuric acid and then extracted with chloroform-methanol mixtures (2:1, v/v). This was done to recover from the aqueous phase fatty acids cleaved from glyceride linkage or else to extract components which had resisted saponification.

Meanwhile the aqueous phase in each case was concentrated and samples retained for chromatography. Acid hydrolysis of portions of the remainder under conditions suitable for breaking glycosidic bonds released galactose, glycerol and a trace of glucose, as revealed by paper chromatography.

Thus the two fractions contained components which behaved towards saponification and acid hydrolysis in a manner entirely consistent with their being compounds of the type described by Carter (Fig. 24, I and II). The trace of glucose may have arisen because of contamination, or else may be due to the presence of glucose containing analogues of Carter type compounds.

The saponification and organic extraction steps had removed all but a trace of the glucose containing substances. Consequently, the chloroform-methanol solvents were removed in each case to provide brown oils, which upon hydrolysis with N acid in sealed tubes for six hours followed by neutralisation and chromatography gave glucose with no galactose or glycerol. The likelihood therefore, is that both fractions contain glucose bound to a moiety other than glycerol and

that the original compound either survives saponification or else gives rise upon alkaline hydrolysis to a glucose containing entity which is preferentially soluble in the organic phase.

The similarity and behaviour of the two fractions requires comment. Both contain Carter type compounds and each has glucose containing components which may be resistant to saponification. If the lipid fractionation procedure had depended entirely upon a silicic acid technique, with omission of the DEAE-cellulose stage, no fractionation would in fact have been obtained. It is thus possible that the separation resulting from the use of the DEAE-cellulose may have arisen because of some side chain difference between compounds of essentially the same character, these differences causing changes in polarity to which the column was sensitive.

(6) Some results obtained with fresh potato starch.

It was felt that the results discussed in (5) above which proved the presence of glucose and galactose containing lipids in starch grains also had a bearing upon the enzymic experiments described in Part II. It will be recalled that the Leloir method of obtaining starch synthetase involved treatment of freshly prepared grains with acetone and that the Pottinger and Oliver technique, alleged to give grains with much greater activity, avoided this

treatment. Although several explanations for the difference in activity are possible the present results suggest one likelihood viz. that the isotonic extraction technique of Pottinger and Oliver minimises the loss of components which might be involved in starch biosynthesis.

Hence, starch grains obtained from potatoes by Leloir's technique (Expt. 79a) were deliberately extracted with acetone in order to investigate this point. Room temperature conditions were used for convenience, 500g of fresh grains were shaken with 1.5 litres of acetone for two hours and the extraction repeated twice (Expt. 93). A total of 560mg of extractable solids was obtained.

This material, dissolved in chloroform, was applied to a silicic acid column (Expt. 93). Of the material applied 80% was not retained and examination proved it to contain no free or bound sugars. A 10% methanol in chloroform solution removed the whole of the remainder and acid hydrolysis followed by paper chromatography of a sample showed the presence of bound glucose with traces only of bound galactose and glycerol. Hence it appears that the glycolipid fraction from fresh starch may be qualitatively very different from that of aged samples. It is intended to investigate this and other points more fully at another time.

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General Methods and Procedures.

(abbreviated G.M.)

G.M. 1. Paper chromatography.

For descending development of chromatograms, samples were applied at intervals of 2½cm along a line 7cm from the edge of the filter paper sheet which was supported on a frame between three glass rods in such a manner that the end of the paper dipped into the developing solvent. When the development was prolonged and the solvent allowed to run off the end of the paper, the end was serrated to encourage an even flow.

For ascending development, the samples were applied at intervals of 2½cm along a line 3cm from the lower edge of the filter paper sheet which was held in a Teflon frame with the lower edge submerged in the solvent.

The following solvents were used:-

(1) Ethyl alcohol (95%) - M ammonium acetate (7.5:3, v/v) pH 3.8.

(2) Ethyl alcohol (95%) - M ammonium acetate (7.5:3, v/v) pH 7.5.

(3) n-butanol-pyridine-water (6:4:3, v/v/v).

(4) Propanol-ethyl acetate-water (6:1:3, v/v/v).

(5) Phenol-water-ammonia (0.88) (160:40:1, v/v/v).

(6) Isopropanol-conc. HCl (12N) (65:16.7, v/v).

G.M. 2. Electrophoresis on filter paper strips.

The technique used was essentially that of Markham and Smith (227).

The samples were applied along a line 10cm from one end of a filter paper strip 45cm long. The paper was carefully impregnated with the buffer solution to be used, by approaching the line containing the samples to be analysed, from both sides thereby minimising fears of diffusion. After removal of excess buffer the middle portion of the paper was suspended in a container of dry carbon tetrachloride and the ends placed in two similar jars both containing the conducting buffer and the electrodes. Current was passed through at a set voltage for a prescribed period after which the apparatus was switched off and the paper removed and dried.

The buffers used and the conditions applied were as follows:-

(1) 0.05M borax solution ($Na_2B_4O_7$, $10H_2O$) pH 9.6 for 1½ hours at 900 volts.

(2) 0.02M phosphate buffer solution pH 7.5 for 1 hour at 600 volts.

G.M. 3. Thin layer chromatography.

Chromatoplates of cellulose and silica gel were both used in preliminary studies and were prepared using the equipment supplied by Messrs. Shandon Instrument Co. Ltd.

The samples in trace amounts were carefully applied with

micro pipettes along a line 1cm from the bottom edge of the plate. This was then placed in a sealed tank lined with filter paper to aid equilibrium with the bottom edge immersed in the solvent.

The solvents used were:-

(1) The water poor phase from the mixture tertiary amyl alcohol-water-p-toluene-sulphonic acid (60:30:2, v/v/w).

(2) Isobutyric acid-ammonia (0.88)-water (66:1:33, v/v/v).

(3) 8% methanolic chloroform.

G.H. 4. Detection of compounds on filter paper.

(1) Nucleotides.

This was accomplished using a UV Hanovia "Chromatolite" lamp.

(2) Phosphate esters.

Acid molybdate reagent:- Made up freshly as required from a stock solution of ammonium molybdate in water (1g/8ml):- 8ml of ammonium molybdate solution, 3ml conc. HCl and 3ml 72% perchloric acid were diluted with 90ml of acetone.

Procedure:- The paper was sprayed lightly with the above reagent, dried at 100° (1-2 mins.), then placed under a UV lamp for 10 - 15 mins. or until a background colour began to develop.

(3) Sugars.

(a) Alkaline silver nitrate.

Reagents:-

(1) Silver nitrate (0.5ml of a saturated water solution) was diluted with acetone (100ml) and water added until the precipitate formed just redissolved.

(2) Caustic soda (2g) was dissolved in water (5ml) and diluted to 100ml with ethanol to give a 0.5N alcoholic solution.

Procedure:-

The paper was dipped rapidly through the silver nitrate reagent, allowed to dry in air (15 mins.), then dipped through the caustic soda solution and left until the spots had developed the required intensity or until the background colour showed signs of becoming too pronounced.

The excess reagents were removed by washing with ammonia solution (6N) and finally with constant running water for two hours.

(b) Benzidine-TCA.

Reagent:-

Benzidine (0.5g) was dissolved in glacial acetic acid (10ml) and the solution mixed with trichloroacetic acid (TCA, 10ml of 40% w/v) and ethanol (80ml).

Procedure:-

The reagent was sprayed lightly on the paper in a well

ventilated fume cupboard. The sprayed paper was then heated at 100° until the spots reached maximum intensity.

(c) Orcinol-TCA.

Reagent:-

Orcinol (0.5g) and trichloroacetic acid (TCA, 15g) were dissolved in water saturated n-butyl alcohol (100ml) and the solution stored at 0°. The reagent was discarded after 14 days.

Procedure:-

The paper was sprayed lightly with reagent and heated at 105° until the spots reached maximum intensity.

G.M. 5. Detection of compounds on thin layer plates.

The reagents described above for filter paper were all suitable for thin layer plates with at most only slight modifications.

For lipid components using silica gel the plates were either sprayed with conc. sulphuric acid and heated directly whereby the lipids were revealed as charred spots or else use was made of the fluorescence quenching under UV light observed when a water soluble dye, rhodamine, was incorporated into the gel during the preparation of the plates. A concentration of 0.1g rhodamine/30g dry silica gel was suitable for this purpose.

G.M. 6. The estimation and identification in solution of (1) nucleotides, (2) phosphate esters (general) and (3) sugars.

(1) Nucleotides.

Estimation of concentrations:- Due to the characteristic absorption spectra of the pyrimidine and purine rings of nucleotides, their concentrations can be calculated from their absorption maxima.

Uridine derivatives - 1 mole - 10,000 OD units.

Adenosine derivatives - 1 mole - 15,400 OD units.

Guanosine derivatives - 1 mole - 13,700 OD units.

Cytidine derivatives - 1 mole - 9,000 OD units.

OD units are obtained by multiplying the absorption of a given solution at its maximum (generally assumed to be 260mμ for nucleotides), measured in a 1cm cell, by its volume (ml). If the requirement is to determine the concentration of nucleotide in a given solution the expression:- Molarity = OD/ε where ε is the molar absorbance, may be used.

Identification:- As an alternative to plotting the complete spectrum of each nucleotide sample the base present can be identified from the absorbancy ratios 250/260 and 280/260 which are characteristic of the base present. These ratios at neutral pH are:-

	250/260.	280/260.		250/260.	280/260.
Uridine	0.73	0.39	Guanosine	0.90	0.68
Adenosine	0.79	0.16	Cytidine	0.84	0.98

Identification of base after perchloric acid digestion.

A dry sample of nucleotide (5mg) was intimately mixed with perchloric acid (0.1ml of 72%, 12N) in a small stoppered test tube and heated at 100° for 60 mins. After cooling, water (0.4ml) was added, the contents mixed and the supernatant collected after centrifugation. The solution was spotted on a paper chromatogram and developed in isopropanol-HCl (22%, C.M.1, solvent 6) and the R_f of the bases measured under UV light.

(2) Phosphate esters (general).

The determination of inorganic, acid labile and ester phosphate was carried out according to the method of King (189).

Reagents:-

(a) 72% perchloric acid.

(b) 5% ammonium molybdate.

(c) 1-amino-2-naphtholsulphonic acid (amidol, 0.5g) and anhydrous sodium bisulphite (30g) were dissolved in water (250ml) and the solution filtered if required. The solution was stable for about two weeks.

Procedure:-

(a) Inorganic phosphate.

To an aqueous solution of the sample containing 10-90µg phosphorus, solutions (a) (1ml), (b) (1ml) and (c) (0.5ml) were carefully added and the volume diluted to 15ml in a pre-calibrated

test tube. The contents were mixed and the tube allowed to stand at room temperature for 15 mins. after which the blue colour produced was measured with an EEL colorimeter (filter 608, 660mμ) and the phosphorus content estimated by the use of a standard curve prepared under identical conditions.

(b) Acid labile phosphate.

Acid labile phosphate was determined as inorganic phosphate after heating the sample with N HCl in a boiling water bath for 7 mins.

(c) Ester phosphate.

Ester phosphate was determined as inorganic phosphate after removal of the organic matter by a wet digestion process with 72% perchloric acid. The digestion was carried out by heating the sample (1ml) with solution (a) (1.2ml) until the charred material had disappeared, to give a clear solution. The solution was then cooled and without adding more solution (a) the inorganic phosphate was determined as before.

(3) Sugars.

(a) Reducing power by the Somogyi (1952) - Nelson colorimetric method (190).

Reagents:-

Copper solution. Rochelle salt (12g) and anhydrous sodium carbonate (24g) were dissolved in water (250ml). A solution of copper sulphate (4g of pentahydrate) in water was added followed by sodium bicarbonate

(16g). A solution of anhydrous sodium sulphate (180g) in water (500ml) was boiled to expel air, then the two solutions were combined and diluted to 1 litre. After standing for one week the clear supernatant was used. The solution was stored at a slightly elevated temperature(37°).

Arsenomolybdate solution. Ammonium molybdate (25g) was dissolved in distilled water (450ml) and conc. sulphuric acid (21ml) added. Disodium hydrogen arsenate (3g) in water (25ml) was added and the solution kept at 37° for 24 - 48 hours. The solution was stabilised by storage in a brown glass bottle.

Procedure:-

Aliquots of the sugar solution (1ml) and copper reagent (1ml) were mixed in narrow pyrex tubes and heated in a boiling water bath for 20 mins., the tubes being loosely stoppered with marbles. After a cooling period of 5 mins. in a bath of cold water arsenomolybdate reagent (1ml) was added with shaking. The resulting solution was made up to 10ml and after 10 mins. the colour produced was measured with an EEL colorimeter (filter 608, 660mμ) and the results compared with equivalent standards, after correction for blank estimations.

(b) Free and combined fructose by the method of Cole as recorded by Bacon and Bell (191).

Reagents:-

(1) Resorcinol, recrystallised from benzene (0.15% w/v), was

dissolved in absolute alcohol.

(ii) Conc. hydrochloric acid containing ferric chloride (7.5mg/litre).

Procedure:-

The sample (3ml, 10 - 70µg fructose) was mixed with solutions (i) (4ml) and (ii) (5ml) in a narrow pyrex tube. After loosely stoppering the test tube with a marble it was held at a temperature of 77-79° for 30 mins., then cooled and the red colour produced measured with an EEL colorimeter (filter 602, 460mµ) and compared with appropriate standards after blank correction.

(c) Phenol-sulphuric acid method (as developed at the University of Minnesota, 229).

Reagents:-

(i) Phenol (5%, w/v) in water.

(ii) Conc. sulphuric acid (Analar).

Procedure:-

The sugar sample (1ml, 10 - 90µg) and solution (i) (1ml) were mixed and solution (ii) (5ml) added from a wide bore pipette with continual agitation. The same procedure was followed rigorously for all samples. After 10 mins. the tube was cooled by immersion in cold water (5 mins.) and the yellow colour produced measured with an EEL colorimeter (filter 603, 490mµ) and compared with appropriate standards.

(d) Pentose determination by the method of Mejbaum (228).

Reagent:-

1% orcinol solution in 0.1% ferric chloride in conc. HCl. 0.1% ferric chloride in conc. HCl was made up as a stock solution and orcinol was added to portions as required.

Procedure:-

Aliquots in the range 10 - 40µg contained in 4ml of solution were placed in narrow pyrex tubes and 4ml of orcinol reagent added. The contents were heated on a water bath for 30 mins. with glass marbles as loose stoppers. The tubes were cooled and the solutions read using an EEL colorimeter (filter 608, 680mµ).

G.M. 7. The isolation and purification of (1) nucleotides and (2) phosphate esters (general).

(1) Nucleotides.

(a) The charcoal method (186).

Charcoal (Norit A) was prepared for use by refluxing for 6 - 8 hours with hydrochloric acid (6N) after which it was collected by filtering through a Buchner funnel. It was then washed with water until the filtrate was chloride free and dried at 110 - 115° for 15 hours. The pad for adsorption was prepared by pouring an aqueous slurry of a mixture of well washed celite (8g) and the pre-treated charcoal (6g) on to a Buchner funnel (4.25cm diam.) which was allowed

to drain under gravity. A filter paper disc was placed on top of the charcoal-celite pad to prevent drying out. A column of this size was sufficient for 60 - 180μ moles of nucleotide.

Procedure:-

The aqueous extract containing the nucleotides, adjusted to pH 6.8, was allowed to percolate through the charcoal-celite column which was then washed in turn with water (80ml), EDTA (80ml of 0.01M, pH7), water (80ml) and finally with 50% aqueous alcohol, the pH of which was adjusted to 7.5 with dilute ammonia. The fraction eluted with 50% alcohol was collected (100ml) and the solvent distilled off under vacuum to give a salt free nucleotide sample.

(b) The lithium salt method (128).

Procedure:-

The nucleotide containing solution was converted into its free acid form (G.M. 10) and the pH adjusted to 5.5 with lithium hydroxide. The water was removed from the aqueous solution either by freeze drying or by azeotropic distillation with dry methanol at a low bath temperature ($< 40^{\circ}$). This latter procedure proved more effective in the presence of large amounts of inorganic salt.

The dry residue was thoroughly triturated with dry methanol (10ml x 4, G.M. 11) and the supernatants bulked. The insoluble material was discarded. The methanol solution (40ml) was concentrated under vacuum to 10ml and excess dry acetone (12 vols.) added.

After 1 hour at 0° the insoluble nucleotide fraction was collected by centrifugation. Occasionally it was prudent to redissolve the precipitate in methanol in order to repeat this step. The product was finally isolated as a white powder after removal of the remaining solvents in a vacuum desiccator.

(e) The mercury precipitation method (35).

Mercuric acetate solution was prepared by mixing yellow mercuric oxide (13.5g) with glacial acetic acid (9.2ml) and diluting to 100ml with water.

Procedure:-

The nucleotide containing solution was adjusted to pH 3.5 with nitric acid and then diluted with an equal volume of alcohol. Mercuric acetate (30ml/litre) was added and after stirring, the mixture was left at 0° overnight. The precipitate was collected by decantation and centrifugation, then blended with water to give a uniform suspension of volume approximately equal to 1/10th of the original. Hydrogen sulphide was passed through the suspension at 0° for 2 hours, the precipitate filtered off and excess hydrogen sulphide removed by bubbling air for a further 2 hours through the supernatant still kept at 0°. The pH was then adjusted to 7 to prevent hydrolysis of the nucleotide components present and the solution used directly for the next purification step.

(2) Phosphate esters (general).

(a) The barium fractionation method.

Several variations of this method are equally suitable for fractionating phosphate esters (including nucleotides) and have been used interchangeably in this work.

The barium addition was carried out by either (a) converting the aqueous extract into its free acid form (C.M. 10) and then adjusting the pH to 7.5 with barium hydroxide or (b) by adjusting the pH of the solution to 7.5 and adding excess barium ion as its acetate or bromide.

The barium fractionation was carried out by either (a) removing the inorganic phosphate as insoluble barium phosphate and then precipitating out the barium salts of the phosphate esters by the addition of 4 volumes of alcohol, or (b) by precipitating both inorganic and ester phosphates as their barium salts by the addition of 4 volumes of alcohol and then submitting this mixture to further fractionation. This was accomplished by extracting the precipitate obtained with warm (37°) water (4 aliquots) which resulted in solution of barium ester phosphates only. Addition of alcohol to the aqueous solution then resulted in their recovery. In all cases the barium salts of the phosphate esters were collected by centrifugation and washed in turn with alcohol, acetone and ether and finally dried in a vacuum desiccator.

(b) The magnesia mixture method.

Magnesium chloride (5.5g) and ammonium chloride (10g) were weighed into a flask (100ml) and water (50ml) added. Ammonia (10ml of 0.88) was added and the solution diluted to 100ml (1ml solution ≡ 270u moles phosphate).

Procedure:- The sample was neutralised with ammonia (10%) and excess magnesia mixture was added. The solution was required to be 1.5M with respect to ammonium hydroxide. After standing at 0° for 1 hour the insoluble precipitate of magnesium ammonium phosphate was filtered off leaving an inorganic phosphate free solution.

(c) The brucine salt method.

The sample containing the phosphate ester (1 mole) was converted into its free acid form (G.M. 10) and a saturated alcoholic solution of brucine (1 mole, 395) added. The solution was evaporated to dryness and the residue dissolved in the minimum volume of hot water and left to crystallise at 0°. Quantitative crystallisation was encouraged by the dropwise addition of acetone. The crystals were isolated by centrifugation and characterised by melting point etc. Reconversion to an inorganic salt form was brought about by dissolving the brucine salt in the minimum volume of water and adding dilute sodium hydroxide dropwise until the solution was faintly red to phenolphthalein. A white precipitate was produced which was removed by shaking with chloroform (1 vol.) leaving the phosphate ester as its

inorganic salt form and ready for the next purification step.

C.M. 8. Standard solutions and suitable dilutions for analytical studies on nucleoside diphosphate sugars and sugar phosphates.

Solutions of 1mg/ml were made up in all cases as primary standards, suitable dilutions being made as required.

Paper chromatography, paper electrophoresis and thin layer chromatography.

The primary standards were used directly for these techniques.

Spectral studies.

For a direct spectral reading of the nucleotide samples a 1 to 25 dilution of the primary standards was usually adequate.

Inorganic phosphate and reducing power determinations both before and after mild acid hydrolysis.

Aliquots (1ml) of the primary standards were mixed with N/25 hydrochloric acid (1ml) and held at 100° for 10 mins. Samples of the mildly acidic solution were removed for the determination of inorganic phosphate (1ml) and reducing power (0.2ml) and the results expressed in μ moles compared.

G.M. 9. The preparation of chromatographic materials.

(1) Ion exchange resins.

(a) Dowex 1 x 4, chloride (200 - 400 mesh).

The resin was pretreated by suspending in a large volume of water and decanting those fines which failed to settle in 10 mins. The coarse fraction obtained after repetition of this process a few times was then cyclised with acid and alkali. This was done by stirring with hydrochloric acid, 1N (15 mins.), then washed with water, following which it was stirred in sodium hydroxide, 1N (15 mins.). After three cycles concluding with an acid treatment the resin was well washed with water and suitable quantities suspended in water were used for the preparation of columns. Before use the column was eluted with hydrochloric acid 1N (20 bed volumes) and then with distilled water until the pH was approximately 7.

(b) Dowex 1 x 4, borate and Dowex 1 x 4, bicarbonate.

These anionic forms were prepared directly from the chloride form of the resin. Columns of suitable dimensions filled with resin in the chloride form were eluted with 0.5M potassium borate ($K_2B_4O_7\ 10H_2O$) (or 5% sodium bicarbonate) until chloride free (30 bed volumes). The columns were then washed with water (50 bed volumes) until the pH was approximately 7.

Other ion exchange forms were prepared in an analogous manner to the above substituting the appropriate eluting species.

(2) DEAE-cellulose.

(a) For sugar phosphate studies.

DEAE-cellulose when required for sugar phosphate studies was converted into the carbonate form by eluting with a concentrated solution of ammonium carbonate(2M, 50 bed volumes) and then equilibrating the column with the dilute ammonium carbonate (0.01M, 50 bed volumes) to be used.

(b) For lipid studies.

DEAE-cellulose when required for lipid studies was used in either the hydroxyl of acetate forms.

Hydroxyl form:- DEAE-cellulose in the hydroxyl form could, if necessary, be generated from another form with 0.1N sodium hydroxide. After freeing from air bubbles by leaving overnight in a vacuum desiccator, the dry material was added as a slurry in methanol into a column of suitable dimensions. After eluting with methanol (30 bed volumes) the DEAE-cellulose was equilibrated with the chloroform-methanol solution (30 bed volumes) to be used.

Acetate form:- The DEAE-cellulose (hydroxyl) sample was left in contact with glacial acetic acid for 18 hours, then applied to a suitable column as a slurry in the same solvent. The DEAE-cellulose was eluted in turn with glacial acetic acid (20 bed volumes), methanol (30 bed volumes) and finally the appropriate chloroform-methanol solvent (30 bed volumes). The lipid mixture was then applied

directly in this chloroform-methanol solution.

(3) Silicic acid.

Silicic acid (Mallinckrodt Chemical Co.) was washed repeatedly by a batchwise technique with methanol until the supernatant became clear. A slurry of the material was then poured into a suitable column which was eluted with chloroform (10 bed volumes). The lipid dissolved in chloroform was then applied directly.

For a separation of polar from non-polar lipids a short broad column gave the most satisfactory results (7cm long x 2.5cm diam.). The use of longer columns resulted in unacceptably slow flow rates.

G.M. 10. The interconversion of salts of phosphate esters including nucleotides.

The metal salt (Ba, Li, Na) of the sugar phosphate or nucleotide was dissolved in water if necessary with the addition of a small amount of cation exchange resin in the hydrogen form to aid solution. The solution was then applied to a column of the same resin and washed through until the effluent was no longer acid.

The solution, if required in free acid form was used as such, otherwise it was adjusted to the appropriate pH with the required base.

G.P. 11. Anhydrous conditions.

In this work good quality chemicals preferably "Analar" grades were employed where possible. When anhydrous reaction conditions were essential solid compounds, unless stated otherwise in the Experimental, were dried over phosphorus pentoxide at 10^{-1} mm Hg and 50° for 18 hours prior to use.

Solvents were also of "Analar" quality and with the exceptions to be mentioned, were sufficiently dry to permit direct use without further drying. With methanol, ethanol, pyridine and acetone, however, the following procedures for drying these solvents were applied:-

(a) Methanol. Suitably dry methanol samples were prepared by a Grignard reaction for which a small amount of dry methanol (250ml) was required to prime the reaction.

To a reaction flask (5 litres) containing clean dry magnesium turnings (15g) and a trace of resublimed iodine, dry methanol (100ml) was added dropwise liberating heat with the concomitant release of hydrogen gas. After brisk reaction had been established the methanol (1.9 litres) to be dried was added dropwise to the stirred solution. When addition was complete the contents were refluxed for two hours. The dry methanol was collected by distillation and stored until required.

(b) Ethanol. Ethanol (4 litres) was dried by adding sodium (28g)

following which diethylphthalate (110g) was added under anhydrous conditions. After refluxing for 2 hours the ethanol was distilled with the exclusion of moisture, the first 25ml of distillate being discarded.

(c) Pyridine. This solvent was prepared in a suitably dry form by refluxing pyridine (2.5 litres) with barium oxide (250g) under anhydrous conditions until the refluxing temperature rose to 113 - 115°. If this temperature was not obtained after 6 hours further additions of barium oxide (250g) were made and refluxing continued until the temperature was within these limits. The fraction distilling between 113 - 115°, after discarding the first few ml, was collected and stored over sodium hydroxide pellets until required.

(d) Acetone. Good quality acetone samples were shaken continuously for 18 hours with anhydrous sodium sulphate and stored over this dehydrating agent until required.

G.M. 12. The examination of lipid fractions for glycolipid components.

Lipid (5 - 10mg) was placed in a thick-walled glass tube (6mm x 6cm) and sulphuric acid (0.1ml of N) added. The tube was sealed and maintained at 100° for 6 hours. The contents were neutralised with saturated barium hydroxide and the insoluble precipitate centrifuged off. The supernatant, in parallel with an equivalent amount of the unhydrolysed material dissolved in methanol,

was applied to paper for chromatographic analysis (G.M. 1, solvent 3). The resulting chromatogram was sprayed with silver or benzidine reagents (G.M. 4 - 3) and the sugar patterns obtained, prior and subsequent to acid hydrolysis, compared.

PART I. SECTION I.

Expt. 1. Synthesis of isopropylidene uridine.

(a) From uridine obtained by hydrolysis of RNA.

Dry uridine (5g), obtained as an amorphous powder by hydrolysis of yeast RNA, was mixed with anhydrous copper sulphate (10g), prepared by the careful heating of a well ground sample of the pentahydrate. The mixture was suspended in dry acetone (125ml) containing concentrated sulphuric acid (0.125ml) in a sealed flask fitted with a mechanical stirrer. Reaction was allowed to proceed at 37° for 68 hours with the exclusion of moisture, at the end of which time, as judged by paper electrophoresis using a borate buffer (G.M. 2), the copper sulphate was filtered off and the acidity neutralised by shaking with solid calcium hydroxide (5g) for 1 hour. The calcium salts were removed by filtration and the dark brown filtrate warmed with charcoal (2g, BDH, decolourising) for 2 mins. After removal of charcoal by filtration the acetone was distilled under vacuum leaving a pale yellow gum which could be crystallised by dissolving in hot dry acetone (10ml) and adding pet.ether until turbid. The isopropylidene uridine, isolated as a crystalline mass, was obtained in a yield of 25% (1.5g).

(b) From uridine obtained commercially.

Uridine (10g, Light) dried as in G.M. 11, was converted

into its isopropylidene derivative by an entirely analogous process to that described in Expt. 1a. The yield of 65% (7.3g), a considerable improvement on that obtained above was attributable to the higher quality of the uridine here used. The pale yellow gum, obtained in this and subsequent preparations, was assayed for purity by paper electrophoresis using a borate buffer (G.M. 2). If the absence of unblocked uridine was established the gum was used directly in subsequent experiments. The removal of a small amount of unblocked uridine was accomplished by the addition of Dowex 1 x 4 resin in the borate form (40ml, G.M. 9-1) to a suspension of the gum in cold water (40ml). After stirring for 1 hour and after removal of resin the filtrate was evaporated under vacuum to give a clear glass on repeated azeotropic distillation with dry methanol.

Expt. 2. Synthesis of isopropylidene adenosine.

Dry adenosine (2g, G.M. 11) was added to a filtered solution of anhydrous zinc chloride (5.5g) in dry acetone (55ml) and refluxed with exclusion of moisture for 5 hours. The solution, which gradually clarified, was left at room temperature for a further 12 hours and then concentrated under vacuum to a third of the total volume (20ml). The slightly viscous concentrate was poured into a warm (40°) solution of barium hydroxide (15g of octahydrate in 100ml). The mixture was cooled at once to room temperature and carbon dioxide

bubbled through to neutrality. Zinc and barium carbonates were filtered off and washed thoroughly with alternate lots of boiling methanol (100ml) and water (100ml) until the washings contained no more carbohydrate. The clear filtrate was concentrated under vacuum at $< 40^{\circ}$ to a volume of 50ml, whereupon isopropylidene adenosine crystallised. The crystals (0.75g) were obtained after filtration, washing with water and drying at 100°. More product could be obtained by evaporating the mother liquors to dryness at $< 40^{\circ}$, drying at 100° and extracting continuously with dry acetone (5 hours). This fraction (0.80g) was crystallised from boiling 95% methanol (30ml) thus giving a total yield of 1.55g (75%). Paper electrophoresis (G.M. 2) indicated that the combined sample was not contaminated with adenosine.

Expt. 3. Synthesis of isopropylidene guanosine and isolation using (a) barium hydroxide and (b) cation exchange resin.

(a) Barium hydroxide.

Dry guanosine (3.5g, G.M. 11) was refluxed for 5 hours in anhydrous acetone (60ml) containing zinc chloride (8.5g). Solvent was removed under vacuum from the clear solution obtained and the resulting syrup was dissolved in cellosolve (2-ethoxyethanol, 5ml) and shaken with dry ether (150ml). The gummy solid, after washing with two further 100ml quantities of dry ether was dissolved in warm

cellosolve (25ml) and a solution of barium hydroxide (20g of the octahydrate in 70ml) added. The mixture was well shaken, cooled and neutralised by prolonged bubbling of carbon dioxide. The voluminous precipitate was filtered, washed with hot water (200ml) and hot cellosolve (15ml) to remove the sparingly soluble guanosine derivative from the insoluble zinc and barium carbonates. The combined filtrate and washings (300ml) on cooling deposited the crude product which crystallised readily from hot water in a yield of 60% (2.1g). The purity of the product was checked by paper electrophoresis (C.M. 2).

(b) Cation exchange resin.

Dry guanosine (3g, C.M. 11) was added to a solution of anhydrous zinc chloride (8g) in dry acetone (80ml) and the mixture refluxed for 6 hours under anhydrous conditions. The clear solution obtained was evaporated to a thick syrup and ammonium hydroxide (8N) added until the initially formed precipitate redissolved. Amberlite IR 120 resin in the ammonium form (16ml, C.M. 9-1) was added and any precipitate formed was dissolved by adding concentrated ammonium hydroxide. The mixture was stirred vigorously for 20 mins., the resin was then removed and washed twice with dilute ammonia (10ml x 2). This resin treatment was repeated four times (16ml resin x 4) after which the solution (150ml) was boiled in an open vessel until evolution of ammonia had ceased. On cooling, crystalline isopropylidene

guanosine deposited and the yield of 2.3g (70%) was shown to be uncontaminated with guanosine (G.M. 2).

Expt. 4. Synthesis of isopropylidene cytidine.

Dry cytidine (1g, G.M. 11) and a good quality sample of dry p-toluenesulphonic acid (8g) were suspended in dry acetone (150ml). Complete solution, which occurred gradually, was aided by gentle warming (30°). After 2½ hours at this temperature the acetone was distilled under vacuum and a suspension of Dowex 1 x 4 anion exchange resin in the bicarbonate form (30ml, G.M. 9-1) in iced water (100ml) was added to the residue. After agitation for 1 hour the resin was removed and washed with water (100ml). The filtrate and washings were evaporated to a clear syrup by repeated azeotropic distillation with dry methanol. The glass of isopropylidene cytidine, which contained no unblocked material (G.M. 2), was obtained in a yield of 90% (0.95g).

PART I. SECTION II.

Expt. 5. Preparation of diphenyl phosphorochloridate.

Phosphorus oxychloride (250g) and phenol (250g) were refluxed together for 6 hours in a two-necked flask equipped with a reflux condenser and a thermometer, precautions to exclude moisture

being taken. The temperature was gradually raised to an ultimate value of 260° by the end of the reaction after which the product was fractionally distilled under vacuum. The diphenyl phosphorochloridate which distilled at 168° (9mm Hg) was obtained in a yield of 57% (190g) based on phenol.

Expt. 6. Synthesis of uridine-5'-phosphate using diphenyl phosphorochloridate.

To dry isopropylidene uridine (500mg, Expt. 1) dissolved in cold (0°) dry pyridine (10ml), diphenyl phosphorochloridate (600mg, Expt. 5) was added dropwise with stirring. Near the end of the addition white crystals of pyridine hydrochloride appeared. The reaction mixture was left overnight at 4° then poured into iced water (50ml). The oil, which settled out, was extracted three times with chloroform (10ml x 3). The combined chloroform extracts were washed successively with cold solutions of N hydrochloric acid, sodium bicarbonate and finally water. The clear chloroform solution was then dried with anhydrous sodium sulphate and the solvent removed under vacuum. The oil obtained was dissolved in dry ethanol (16ml) and submitted to hydrogenolysis with platinum oxide catalyst (200mg). The uptake of hydrogen was slow and rather erratic and was quite often not complete after 8 hours (theoretical 4 moles). After the reduction was complete the catalyst was filtered off and the solvent removed

under reduced pressure. The residue was dissolved in water (10ml) and the acidic solution refluxed for 1½ hours. After cooling the solution was neutralised with barium hydroxide and a barium fractionation carried out (G.M. 7-2a), the product being isolated in 2% (20mg) yield as impure barium salt.

Expt. 7. Preparation of dibenzyl phosphite.

A solution of phosphorus trichloride (52.2ml) in dry benzene (450ml) was cooled to 0° and placed in a three necked flask (1 litre) fitted with a thermometer, a dropping funnel and a mechanical stirrer, precautions being taken to exclude moisture. To the flask immersed in a freezing mixture of salt and ice a solution (at 0°) of benzyl alcohol (123.2ml) and diethylaniline (192ml) was added slowly so that the temperature did not rise above 5°. After the addition which took 2½ hours the solution was stirred for a further 1½ hours at 0 - 5°. A further dropwise addition of benzyl alcohol (61.5ml) over 1 hour was carried out keeping the temperature below 10°. The stirring was continued for a further 1½ hours, water (200ml) was then added and the phases separated. The organic phase, which looked very dirty, was washed successively with water (200ml x 3), aqueous ammonia (200ml of 3N x 3) and water (200ml x 3) after which it was dried with anhydrous sodium sulphate and evaporated under vacuum using an oil pump. The temperature of the oil bath was raised to 150° and the

residual liquid thoroughly stripped. On cooling a buff coloured mass crystallised out in a yield of 90%(140g). This material was used directly in all subsequent experiments. A further purification step which could be carried out with some hazard was to distil the dibenzyl phosphite at 170° and 10^{-5}mm Hg, a process which was found unnecessary in this work.

Expt. 8. Preparation of dibenzyl phosphorochloridate.

To dibenzyl phosphite (278mg, Expt. 7), dissolved in dry benzene (5ml), an equivalent of N-chloro-succinimide (134mg) was added and reaction allowed to proceed for 1 hour at room temperature. The insoluble succinimide which separated was centrifuged off and the dibenzyl phosphorochloridate either used directly in benzene solution or else the solvent was removed under vacuum and the pale yellow oil obtained dissolved in the dry solvent of choice and used immediately. The yield of product obtained was virtually quantitative.

Expt. 9. Synthesis of uridine-5'-phosphate using dibenzyl phosphorochloridate.

Dry isopropylidene uridine (450mg, Expt. 1) dissolved in dry pyridine (6ml) at - 50°, was treated with dibenzyl phosphoro-chloridate (from 1g of dibenzyl phosphite, Expt. 8). The flask and

its contents were held at -30 - -40° for three hours, a temperature sufficiently high to maintain the mixture in a molten state. After a further overnight period at room temperature, the pyridine was removed and the gum obtained was dissolved in ethanol, precipitated with ether and then redissolved in ethanol. Hydrogenolysis was carried out using a palladium-charcoal catalyst (0.3g of 10%), the uptake of hydrogen being rapid (1 hour). The amount of hydrogen taken up was only 20% of the expected value, indicating loss of material at the previous washing stage. The catalyst was filtered off and the solvent removed under vacuum. Sulphuric acid (25ml of N) was added to the gum and the temperature kept at 75° for 1½ hours. The solution was neutralised with barium hydroxide and a barium fractionation carried out (G.M. 7-2a). The barium salt of uridine-5'-phosphate was obtained in 2 - 3% (15 - 20mg) yield.

Expt. 10. Preparation of polyphosphoric acid.

Orthophosphoric acid (1.3g of 85%, w/v) was warmed in a beaker to 60° and phosphorus pentoxide (1g) slowly added with stirring to avoid the formation of lumps. When solution was complete the mixture was cooled whereupon the reagent was ready for use.

Expt. 11. Synthesis of uridine-5'-phosphate using polyphosphoric acid.

Isopropylidene uridine (200mg, Expt. 1) was thoroughly

mixed with polyphosphoric acid (1ml, Expt. 10) in a stoppered flask and the mixture maintained at 60° for 2 hours. Solution of the solid was not complete after this time and the contents darkened considerably. Water (10ml) was added and the solution warmed on a steam bath for 30 mins. The pH was adjusted to 7 with sodium hydroxide, excess barium acetate (3g) was added and a barium fractionation carried out (G.M. 7-2a). The barium salt of uridine-5'-phosphate was isolated in a yield of 45% (150mg).

Expt. 12. Preparation of cyanoethyl phosphate (CEP).

Phosphorus oxychloride (30.6g, 18.4ml) was mixed with anhydrous ether (200ml) in a three necked flask (500ml) containing a thermometer, a sealed stirrer and a pressure equalising dropping funnel stoppered with a drying tube. The solution was cooled to -13° in an ice-salt bath and a mixture of dry pyridine (15.8g, 16.1ml) and hydracrylonitrile (14.2g) added dropwise with vigorous stirring. (3-hydroxypropanenitrile) The addition took about 1 hour in order to maintain a reaction temperature of -10° or below. A precipitate of pyridine hydrochloride formed during the reaction which was deemed complete after a further 1 hour. The flask contents were poured slowly into a vigorously stirred mixture of water (750ml), pyridine (80ml) and ice (300g). Barium acetate solution (100g in 300ml) was added and the precipitate formed was removed by filtration. The barium salt of

cyanoethyl phosphate was precipitated from the filtrate by the slow addition of alcohol (2.2 litres). This was isolated by centrifugation and washed successively with alcohol, acetone and ether giving a yield of 70% (32g) of air-dried product. The barium salt of cyanoethyl phosphate could be stored indefinitely at room temperature without deterioration.

Expt. 13. Preparation of a standard solution of CEP.

The dried barium salt of CEP (16.1g, Expt. 12) was dissolved in water with the aid of Dowex 50 cation exchange resin in free acid form (G.M. 9-1) and washed through a column (2.5cm x 12cm) of the same resin. The acid eluate was neutralised with pyridine (20ml) and the solution concentrated under vacuum to remove most of the water. The pyridine solution obtained (20ml) was transferred to a graduated flask (50ml) and made up to the mark with pyridine. This clear solution which was stable for 4 - 6 weeks at 0° contained 1m mole of CEP/ml.

Expt. 14. Synthesis of uridine-5'-phosphate (using CEP).

Dry isopropylidene uridine (1.5g, 6m moles, Expt. 1) was dissolved in dry pyridine (15 ml) and standard CEP solution (18ml, Expt. 13) added. The pyridine was removed under vacuum at a bath temperature of $< 40°$. The gum obtained was redissolved in dry

pyridine (15ml) and the solvent again removed. This step was repeated three times. DCC (6g) was added to the gum dissolved in dry pyridine (15ml) and the well-stoppered flask kept at 26° until reaction was complete. Samples (0.1ml) were withdrawn at intervals, diluted with water (0.1ml) and after 15 mins. conc. ammonium hydroxide (0.2ml) was added. After 30 mins. at 60° the ammonia was removed by evaporation under vacuum and the aqueous solution obtained assayed by paper electrophoresis using a phosphate buffer pH 7.5 (G.M. 2). After completion of the reaction (6 hours) water (2ml) was added and after a delay of 30 mins. the solvents were evaporated off under vacuum. Excess lithium hydroxide (100ml of 0.5N) was added and the mixture refluxed for 1 hour after which it was filtered and the clear filtrate passed through a column (1.8cm x 15cm) of Amberlite IR 120 resin in free acid form (G.M. 9-1). The eluate, the pH of which was 2.5, was refluxed for 1½ hours and then neutralised with lithium hydroxide. The volume was reduced (50ml) and a barium fractionation (G.M. 7-2a) performed resulting in the isolation of the barium salt of uridine-5'-phosphate in 70% yield (1.7g). On submitting the sample to a lithium fractionation (G.M. 7-1b) the lithium salt of uridine-5'-phosphate was obtained in an overall yield of 65% (1.4g).

Expt. 15. Synthesis of cytidine-5'-phosphate (using CEP).

Dry isopropylidene cytidine (1.0g, 4m moles, Expt. 4) was

dissolved in dry pyridine (12ml) and standard CEP solution (12ml, Expt. 13) added. The pyridine was removed under vacuum at a bath temperature of $< 40^{\circ}$ and the gum obtained was redissolved in pyridine (15ml). The evaporation and solution in pyridine was repeated three times. DCC (4g) was then added to the gum dissolved in dry pyridine (12ml) and the well-stoppered flask held at 26° until the reaction was complete. The progress of the phosphorylation was followed as for uridine-5'-phosphate (Expt. 14) by removing samples and assaying with paper electrophoresis (G.M. 2) after the water and ammonia treatments. At the completion of the reaction (6 hours), water (2ml) was added and after a further 15 mins. the solvents were removed under vacuum. Excess lithium hydroxide (75ml of 0.5N) was added and the mixture refluxed for 1 hour. On cooling, the insoluble material was filtered off and the alkaline solution passed through a column (1.8cm x 12cm) of Amberlite IR 120 in free acid form (G.M. 9-1). The eluate (150ml, pH 2.5) was refluxed for 1½ hours, the pH adjusted to 7 with lithium hydroxide and the volume reduced under vacuum to 30ml. A barium fractionation (G.M. 7-2a) was carried out giving the barium salt in 105% yield (1.6g) which, on conversion to the lithium salt form (G.M. 7-1b) gave a yield of only 10% (100mg).

The methanol insoluble material (1g) from this attempt to obtain the lithium salt was further hydrolysed with excess hydrochloric acid (25ml of 0.2N) for 30 mins. at 100°. After neutral-

isation with lithium hydroxide and a lithium fractionation (G.M. 7-1b), lithium cytidine-5'-phosphate in 50% (620mg) yield was obtained.

On repeating the experiment with 500mg of isopropylidene cytidine and using identical experimental conditions to those described above with the inclusion of the strong acid hydrolysis treatment (0.2N HCl for 1½ hours at 100°) at the point where resin had been used previously. Cytidine-5'-phosphate was isolated as the barium salt in 67% (650mg) and as the lithium salt in 60% (440mg) yields directly.

Expt. 16. Synthesis of adenosine-5'-phosphate (using CEP).

Dry isopropylidene adenosine (1.2g, 4m moles, Expt. 2) was dissolved in warm pyridine (25ml) containing CEP (12ml, Expt. 13). The pyridine was removed at a bath temperature of less than 40° under vacuum. The solution of the gum in pyridine and its evaporation under vacuum was repeated three times. The dry gum ultimately obtained was dissolved in dry pyridine (25ml) with warming and, after adding DCC (4g), was left at room temperature for 15 hours. The progress of the reaction and the isolation of the product were similar procedures to those followed for uridine-5'-phosphate (Expt. 14), resulting in the isolation of the barium salt (1.3g) and ultimately the lithium salt (0.80g) in 60% yield.

Expt. 17. Synthesis of guanosine-5'-phosphate (using CEP).

Dry isopropylidene guanosine (650mg, 2m moles, Expt. 3) was dissolved (with difficulty) in 50% aqueous pyridine (25ml) after which CEP solution (8ml, Expt. 13) was added. The contents were concentrated by distillation under vacuum at 30°. The solution of the gum obtained in pyridine and its concentration under vacuum were repeated three times. The gum, dissolved in warm dry pyridine (25ml) was treated with DCC (4g) and left for 18 hours by which time reaction was complete as judged by paper electrophoresis using a phosphate buffer (G.M. 2). The product was isolated as for uridine-5'-phosphate (Expt. 14) the only difference being that the barium fractionation step (G.M. 7-2a) was carried out from hot rather than cold solution due to the low water solubility of barium guanosine-5'-phosphate. The barium salt was obtained in 85% (0.90g) yield and on conversion to its lithium salt an overall yield of 65% (510mg) of product, free of inorganic phosphate was obtained.

PART I. SECTION III.

Expt. 18. Preparation of silver diphenyl phosphate.

Diphenyl phosphorochloridate (10ml, 12.7g, Expt. 5) was heated on a steam bath with sodium hydroxide (40ml of 2.5N) until all the oil dissolved and the reaction became homogeneous (1 hour).

The solution was neutralised with conc. nitric acid, after which water (100ml) and silver nitrate (170ml of 10%) were added. The mixture was heated to boiling and filtered through a pre-heated Buchner funnel. The filtrate was left at 0° for 3 hours and the precipitate of silver diphenyl phosphate collected by centrifugation and purified by reprecipitating twice from hot water (80ml). The yield of dried material was 40% (5g).

Expt. 19. Preparation of dibenzyl phosphate (via bromidate).

A mixture of crude dibenzyl phosphite (11.25g, Expt. 7), carbon tetrachloride and aqueous pyridine (10ml in 30ml H_2O) was cooled to 0° and a solution of bromine in carbon tetrachloride (2ml in 7.5ml) added with stirring during 3 hours. The temperature was kept below 10° (in an ice bath). Stirring was continued for a further 1 hour, then conc. HCl (12.5ml) was added and the layers separated (a reddish coloured oil formed between the layers). The aqueous layer was removed and the carbon tetrachloride solution was washed several times with water. The carbon tetrachloride was evaporated off under vacuum to leave a yellow oil which was dissolved in sodium hydroxide solution (50ml of N). On reacidifying (6N HCl) the oil reformed. To this acid solution chloroform (25ml) was added and after shaking, the chloroform layer was isolated, washed with water, dried with anhydrous sodium sulphate and the solvent distilled giving a gum which was

dissolved in dry ether (20ml) and left at 0°. Ill-defined crystals formed which were eventually isolated in an overall yield of 30% (4g).

Expt. 20. Preparation of dibenzyl phosphate (via sodium salt).

Dibenzyl phosphite (50g, Expt. 7) was dissolved in carbon tetrachloride (250ml) and the mixture cooled in ice. A solution of sodium hydroxide (80g in 160ml H_2O) was added slowly with vigorous stirring over 30 mins. (a three-necked 500 ml flask fitted with a stirrer was used). Considerable heat was evolved and a copious white precipitate was produced almost immediately. The stirring was continued for a further 5 hours at room temperature and the reaction completed by refluxing the mixture in a water bath for 2 hours with constant stirring. The mixture was then cooled and the crude sodium salt collected. The salt was washed with carbon tetrachloride (30ml), then with dilute sodium hydroxide (30ml of 2N). The product could be isolated directly, as such, by drying over phosphorus pentoxide at room temperature, but usually the sodium salt (48g) was dissolved in warm water (100ml), acidified with hydrochloric acid (6N), then extracted into chloroform (50ml x 2). The chloroform solution was well washed with dilute bicarbonate (50ml) and water (100ml), and finally dried with anhydrous sodium sulphate. The insoluble salts were filtered off and the chloroform removed under vacuum. The gum obtained was dissolved in dry ether (75ml) and left at 0° for

4 hours, after which the crystalline deposit of dibenzyl phosphate was filtered. The product, obtained in overall yield of 76% (39g) gave a melting point of 79° (in agreement with the published figure).

Expt. 21. The preparation of silver dibenzyl phosphate.

Dibenzyl phosphate (2.8g, Expt. 20) was dissolved in 95% ethanol (15ml) and a 2% solution of ethanolic silver nitrate (100ml) added. After leaving for 2 hours at 0° the insoluble precipitate of silver dibenzyl phosphate was collected and recrystallised from hot water in a yield of 82% (3.3g).

Expt. 22. Preparation of triethylammonium dibenzyl phosphate.

This was prepared as required by dissolving dibenzyl phosphate (280mg, 1m mole, Expt. 20) in dry benzene and adding a solution of triethylamine (113mg, 1m mole, purified by distillation at 89°) dissolved also in dry benzene.

Expt. 23. Preparation of reagent for production of bromo acetylated sugars involving (a) preparation of dry hydrogen bromide and (b) saturation of glacial acetic acid with the dry HBr.

(a) Preparation of dry HBr.

Tetralin (10ml or other suitable quantity depending on amount of HBr required) was placed in a dry flask fitted with a

dropping funnel and a delivery arm. Dry air was passed through the tetralin for 30 mins. to remove any moisture present, otherwise the yield of HBr was reduced. Bromine was added dropwise to the tetralin from the dropping funnel thus producing a constant stream of HBr gas. To prevent escape of any bromine a tetralin trap was included in the system between the generator and the solution being treated with HBr. A 47% yield of HBr, based on bromine, was obtained under dry conditions.

(b) Saturation of glacial acetic acid with the dry HBr.

The dry HBr, prepared as above, was passed directly into glacial acetic acid in a closed system until the solution was saturated (1 hour). This stage was recognised by observing the escape of HBr from the acetic acid outlet tube.

Expt. 24. Preparation of acetyl bromide reagent.

Bromine (60g, 21ml) was added dropwise to a suspension of red phosphorus (10g) in glacial acetic acid (100ml) with cooling (1 hour). After the reaction was complete (an additional 2 hours) the mixture was filtered through glass wool which retained the insoluble red phosphorus. The clear brown fuming liquid was stored at 0° in a well-stoppered flask and aliquots removed as required.

Expt. 25. Preparation of penta-O-acetyl glucose.

Acetic anhydride (50g) and dry pyridine (65ml) were cooled

to 0° and finely powdered anhydrous glucose (10g) added. The suspension was shaken at 0° until all the glucose had dissolved (20 hours), the mixture left at room temperature for an additional 18 hours, then poured with stirring into ice and water (200ml). The gum obtained crystallised gradually upon kneading in the cold with a glass rod. The crude product was recrystallised from 95% ethanol in a yield of 43% (9.0g).

Expt. 26. Preparation of penta-O-acetyl mannose.

Penta-O-acetyl mannose was prepared by an entirely analogous method to that used for penta-O-acetyl glucose (Expt. 25). The yield of product obtained after recrystallising from ethanol was 38% (8g from 10g of mannose).

Expt. 27. Preparation of bromo tetra-O-acetyl glucose.

(a) From penta-O-acetyl glucose.

Penta-O-acetyl glucose (3g, Expt. 25) was dissolved in HBr saturated glacial acetic acid (10ml, Expt. 23) and left at room temperature for 2 hours. Chloroform (10ml) was added to the reaction mixture and the contents poured into iced water (100ml). The chloroform layer was isolated, well washed with cold water (30ml x 2), then poured with stirring into an open beaker containing cold saturated sodium bicarbonate solution (100ml). After effervescence had sub-

sided, the chloroform solution was collected, washed with water and dried with anhydrous sodium sulphate. The resulting gum, obtained after removal of chloroform under vacuum was crystallised from ether-pet.ether (10ml, 1:1, v/v) in a yield of 75% (2.5g) and with a melting-point of 83-4°.

(b) From glucose.

Dry glucose (2.5g) was added portionwise with shaking to acetyl bromide reagent (25ml, Expt. 24) in a well-stoppered flask. External cooling was necessary to keep the temperature below 35°. After 2 hours at room temperature the reaction was diluted with chloroform (25ml) and poured with stirring into iced water (50ml). The chloroform layer was isolated, washed with water, saturated bicarbonate solution and then water once more after which it was dried with anhydrous sodium sulphate. The gum obtained after removal of chloroform was crystallised by dissolving in ether (5ml) and adding pet.ether until turbid. After 2 hours at 0° the crystalline product was isolated in a yield of 45% (2.6g).

Expt. 28. Preparation of bromo tetra-O-acetyl mannose.

(a) From penta-O-acetyl mannose.

The experimental conditions were identical to those described for the preparation of bromo tetra-O-acetyl glucose from its penta-O-acetyl derivative (Expt. 27a). The bromo acetyl mannose

was obtained as a gum, no crystalline material being produced, in a yield of 80% (2.7g from 3g of penta-O-acetyl mannose).

(b) From mannose.

The procedure was again identical to that followed for the equivalent glucose derivative (Expt. 27b). A gum of bromo tetra-O-acetyl mannose was obtained in a yield of 55% (3g of product from 2.5g of mannose).

Expt. 29. Preparation of bromo tetra-O-acetyl galactose.

The experimental procedure was similar to that described for the equivalent glucose containing derivative (Expt. 27b) starting from the free sugar, the bromo tetra-O-acetyl galactose being isolated as a hard oil in a yield of 50% (2.7g product from 2.5g galactose).

Expt. 30. Preparation of bromo tri-O-acetyl xylose.

The experimental procedure was similar to that described for the equivalent glucose containing derivative (Expt. 27b) starting from the free sugar, the bromo tri-O-acetyl xylose being isolated in ill-defined crystalline form in a yield of 40% (2.2g product from 2.5g xylose).

Expt. 31. Preparation of α-glucose-1-phosphate (enzymically).

Soluble potato starch (20g) was stirred with cold water

(50ml) and the suspension added with constant stirring to boiling water (500ml), after which it was set aside to cool (20 - 25°). Two medium sized potatoes (300g) were washed, peeled, sliced, coated with activated charcoal (5g, BDH) and minced using a hand machine. The coarse material was removed by squeezing the macerate through muslin and the juice clarified by centrifugation (10,000 x g). The clear juice was decanted and used directly as enzyme source. A phosphate solution was prepared by dissolving diammonium phosphate (36g) in cold water (250ml).

The phosphate solution (250ml), clear potato juice (150ml) and starch solution (400ml) were mixed, diluted with water to 1 litre and the pH adjusted to 6.7. After adding a trace of toluene to inhibit bacterial activity the digest was left at 26° for 36 hours. After this time the enzymes were denatured by heating rapidly to 95° and when cool the protein precipitate was removed by filtration through a Buchner funnel (9cm) with the aid of a celite pad (10g). α-amylase, prepared either from saliva or by using the clear juice from macerated malt barley, was added to the filtrate (1 litre). After 3 - 4 hours at room temperature the contents were boiled, charcoal (5g) added and the mixture filtered. Aliquots of magnesium acetate, prepared by treating magnesium oxide (20g) with acetic acid (60ml) and diluting with water (100ml), were added to the warm filtrate, the pH of which was adjusted to 8.3 with ammonia,

until no more precipitate formed. After standing for 30 mins. at 0° the precipitate was removed by filtration, potassium acetate (5g) added to the filtrate and the solution reduced under vacuum (60ml) at a bath temperature of less than 40°. Further addition of potassium acetate (12.5g) and pH adjustment to the alkaline range with ammonia were made. After leaving for 4 - 6 hours the precipitate of crude glucose-1-phosphate as its mixed monoammonium monopotassium salt was filtered off and washed with 70% (100ml) and finally 95% alcohol (100ml). The crude product was further purified by solution in water (100ml), passage through an Amberlite IR 120 cation exchange resin in free acid form (G.M. 9-1). After adjustment of the pH to 8.3, the solution was evaporated to a small volume (10ml) under vacuum. Alcohol (15ml) was added to the clear solution to precipitate the dipotassium salt of glucose-1-phosphate. This was centrifuged off after 3 hours at 0°, washed with alcohol and then dried giving 6.2g of dipotassium α-glucose-1-phosphate.

Expt. 32. Preparation of β-glucose-1-phosphate.

(a) Using silver dibenzyl phosphate.

To freshly prepared bromo tetra-O-acetyl glucose (3.0g, Expt. 27) in dry benzene (20ml), dry finely powdered silver dibenzyl phosphate (4.5g, Expt. 21) and drierite (anhydrous calcium sulphate, 1.5g) were added and the mixture warmed to 60° for 30 mins. with

constant shaking in diffuse light and under dry conditions. The mixture was then refluxed for 1½ hours, cooled, treated with decolourising charcoal (1g) and then filtered. The benzene was removed under vacuum and the gum obtained dissolved in dry methanol (50ml) and submitted to hydrogenolysis with palladium-charcoal catalyst (0.5g of 10%). After the uptake of hydrogen (60ml) was complete (1 hour), the solution was filtered and water (10ml) added. The pH was adjusted to 11.3 with sodium hydroxide, kept at this figure for 3 hours, then readjusted to 7.5 with hydrochloric acid. The methanol was removed under vacuum, excess barium acetate (3g) added and the barium salt of β-glucose-1-phosphate isolated (G.M. 7-2a) in a yield of 8% (220mg).

(b) Using triethylammonium dibenzyl phosphate.

Freshly prepared bromo tetra-O-acetyl glucose (4.7g, 10m moles, Expt. 27) was dissolved in dry benzene (15ml) and mixed with a benzene solution of triethylammonium dibenzyl phosphate (10m moles, Expt. 22). The mixture was held at 45° under dry conditions for 8 hours after which the precipitate which formed was centrifuged off and the benzene removed under vacuum. The gum obtained was dissolved in dry methanol (50ml), hydrogenolysed using palladium-charcoal, the uptake being rapid and theoretical (410cc in 30 mins.). After deacetylation and barium fractionation as for Expt. 32a the barium salt of β-glucose-1-phosphate was isolated in 50% yield (2.2g).

The twice reprecipitated barium salt of β-glucose-1-phosphate (400mg) was converted into its sodium form by the addition of sodium sulphate (144mg) and the insoluble barium sulphate which formed was removed by centrifugation. Crude potato juice (1ml), prepared as in Expt. 31, was added to the above solution (1ml) containing a trace of maltotriose (1mg) as primer. Samples (0.2ml) were removed at intervals and added to I_2/KI solution (6ml) and the intensity of colour measured using an EEL colorimeter (filter 608, 660mμ). No production of iodophilic material was observed in any of the experiments tried, thus indicating the absence of α-glucose-1-phosphate in the preparations.

Expt. 33. Preparation of α-mannose-1-phosphate.

(a) Using silver diphenyl phosphate.

Bromo tetra-O-acetyl mannose (1.5g, 3.5m moles, Expt. 28) in the form of a gum, was dissolved in dry benzene (6ml) and silver diphenyl phosphate (1.5g, Expt. 18) added. The mixture was refluxed on a sand bath for 30 mins. with exclusion of moisture. More silver diphenyl phosphate (0.70g) was added and refluxing was continued for an additional 30 mins. On cooling, the insoluble material was centrifuged off, and the benzene removed under vacuum. The gum produced was dried at 40° for 2 - 3 hours by means of an oil pump, then dissolved in dry ethanol (20ml), platinum oxide (300mg) was

added and the solution hydrogenolysed. The uptake of hydrogen was slow (400cc) but complete after 6 hours. The catalyst was filtered off, sodium hydroxide (3ml of 10N) added and the solution refluxed for 3 mins. Water (20ml) was added and the refluxing continued for an additional 2 mins. The barium salt of α-mannose-1-phosphate was isolated by barium fractionation (G.M. 7-2a) in a yield of 30% (450mg).

The product was further purified by converting the barium salt into its brucine salt form (G.M. 7-2c), the melting point of which was 179° (Published value 181°). α-mannose-1-phosphate was recovered from the latter as the barium salt in 75% yield (350mg).

(b) Using triethylammonium dibenzyl phosphate.

The experimental details followed were similar to those described for the preparation of β-glucose-1-phosphate using this reagent (Expt. 32b). Bromo tetra-O-acetyl mannose (1.3g, 3m moles, Expt. 28) was treated with triethylammonium dibenzyl phosphate (3m moles, Expt. 22), the reaction only requiring 2 hours at room temperature for completion. Subsequent hydrogen uptake (125cc) was again rapid (30 mins.), leading to the isolation of the barium salt of α-mannose-1-phosphate in 48% (600mg) yield which was further purified as its brucine salt (Expt. 33a, G.M. 7-2c).

Expt. 34. Preparation of ∝-galactose-1-phosphate.

The details were similar to those followed for the preparation of ∝-mannose-1-phosphate (Expt. 33a), bromo tetra-O-acetyl galactose (1.5g, 3.5m moles, Expt. 29) being treated with silver diphenyl phosphate (Expt. 18). During hydrogenolysis, the 340cc utilised was absorbed over a period of 8 hours. The product was isolated as its barium salt (G.M. 7-2a) in a yield of 23% (360mg). Phosphate (G.M. 6-2) and reducing power (G.M. 6-3a) determinations carried out on a standard solution (G.M. 8), both before and after mild acid hydrolysis, showed a molar ratio of phosphate to sugar of 1.05/1.

Expt. 35. Preparation of ∝-xylose-1-phosphate.

The experimental details were similar to thos followed for the preparation of ∝-mannose-1-phosphate (Expt. 33a), bromo tri-O-acetyl xylose (1.5g, 3.5m moles, Expt. 30) being treated with silver diphenyl phosphate (Expt. 18). The uptake of hydrogen (360cc) required 6 hours. The rather insoluble barium salt of xylose-1-phosphate (G.M. 7-2a) was ultimately isolated in a yield of 25% (380mg). On determining phosphate (G.M. 6-2) and reducing power (G.M. 6-3a) of aliquots of standard solution (G.M. 8), before and after mild acid hydrolysis, the molar ratio of phosphate to sugar was shown to be 1.11/1.

Expt. 36. Preparation of 2-3, 4-5-di-O-isopropylidene fructose.

Dry fructose (5g) was added to a solution of sulphuric acid (2ml) in dry acetone (50ml, 4%). The fructose dissolved over 30 mins. but the reaction was allowed to proceed for an additional 3 hours. At the end of this time the mixture was made alkaline by additions of conc. ammonium hydroxide. The precipitate of ammonium sulphate produced was filtered off. The acetone was removed under vacuum and the gum obtained dissolved in warm 25% aqueous alcohol and allowed to stand at room temperature. The prismatic crystals obtained were collected and dried in a yield of 70% (5.0g). (melting point: 94.5°. published value: 97°).

Expt. 37. Preparation of fructose-1-phosphate.

A benzene solution (10ml) of dibenzyl phosphorochloridate (20m moles, Expt. 8) was added to a solution of 2-3, 4-5-di-O-isopropylidene fructose (5g, 20m moles) in dry benzene (10ml). Triethylamine (3ml, 20m moles) was added dropwise over 10 mins. and the mixture shaken intermittently for 3 hours. The benzene was removed under vacuum and the gum obtained dissolved in dry methanol (100ml) and hydrogenolysed in the presence of palladium-charcoal catalyst (500mg of 10%). The uptake of hydrogen (900cc) was complete in 15 mins. The catalyst was filtered off and the methanol removed under vacuum. The residue was suspended in water (20ml), kept at

a temperature of 77° for 30 mins. and the fructose-1-phosphate isolated as its barium salt (G.M. 7-2a) in a yield of 30% (2.6g). On making up a standard solution (G.M. 8) and determining ester phosphate (G.M. 6-2) and fructose (G.M. 6-3b) the molar ratio of phosphate to fructose was found to be 1.12/1.

Expt. 38. Attempted synthesis of fructose-2-phosphate via the cyclic intermediate (fructose-1-2-phosphate).

(a) Using dicyclohexylcarbodiimide (DCC).

The barium salt of fructose-1-phosphate (200mg, 0.5m moles) was converted into its free acid (G.M. 10), then neutralised with triethylamine (0.07ml, 0.5m moles) and ultimately converted into a syrup by removal of the solvent under vacuum. This residue was dissolved in water (5ml) and tertiary butanol (10ml) containing DCC (515mg, 5m moles) and triethylamine (0.75ml, 5m moles) and the mixture refluxed for 3 hours. The volatile components were evaporated and the residue obtained dissolved in water (5ml) and extracted with ether. Aliquots of the aqueous solution were assayed by paper electrophoresis using a phosphate buffer (G.M. 2). The paper electrophoretogram was sprayed with benzidine-TCA reagent (G.M. 4-3b) in an attempt to detect the fructose containing entities. No material of mobility corresponding to a cyclic phosphate was present. Aliquots from the aqueous solution were treated with mild acid

(0.1N HCl at 100° for 5 mins.) and mild alkali (1N LiOH for 5 mins. at 37°) and the hydrolysates obtained fractionated by the barium method (G.M. 7-2a). No ester phosphate was present, all the phosphate being insoluble in water (inorganic).

(b) Using p-tolylcarbodiimide.

An identical experiment to the above was set up substituting p-tolylcarbodiimide for DCC and excess triethylamine. This modification was also unsuccessful in that no evidence of the formation of a cyclic phosphate by paper electrophoresis (G.M. 2) was obtained. Similarly, barium salt fractionation did not result in the isolation of an ester phosphate fraction (G.M. 7-2a).

Expt. 39. Preparation of 1,3.4,5-tetra-O-acetyl fructopyranoside.

Fructose (66g) was added to a solution of anhydrous zinc chloride (6g) in acetic anhydride (340ml). The contents of the flask, cooled in an ice-salt mixture, were stirred constantly for 16 hours. The fructose gradually disappeared and small needles crystallised from the solution. The contents were stirred for an additional 1 hour at -15°, then filtered. The unwashed crystals were transferred to a desiccator and dried over sodium hydroxide pellets. The mother liquor was treated with solid sodium bicarbonate and extracted with chloroform (100ml). The chloroform layer was then isolated, washed with saturated sodium bicarbonate (100ml) and

three separate volumes of water (100ml x 3). The chloroform was dried with anhydrous sodium sulphate. The sodium sulphate was removed and the solvent distilled off leaving an oil which was co-distilled with toluene. The residue was dissolved in dry ether and left overnight at room temperature. The well-formed crystals were collected and stored over phosphorus pentoxide at room temperature. Yield 50% (58g). Melting point of 129° (published value 131-132°).

Expt. 40. Preparation of 2-bromo 1,3,4,5-tetra-O-acetyl fructopyranoside.

Tetra-O-acetyl fructose (1.4g, 4m moles, Expt. 39) was dissolved in HBr saturated glacial acetic acid (5ml, Expt. 23) and the mixture left for 1 hour at room temperature. The excess reagents were removed by repeated co-distillation with toluene at 30° (25ml x 4).

Expt. 41. Preparation of fructopyranose-2-phosphate.

The syrup of 2-bromo 1,3,4,5-tetra-O-acetyl fructopyranoside (Expt. 40) was dissolved in dry benzene and the pH adjusted to 7 by the careful addition of triethylamine dissolved in benzene. The pH was judged by removing small aliquots and applying them to wet litmus paper. Triethylammonium dibenzyl phosphate (4m moles, Expt. 22) was added and precipitation occurred immediately. After 1 hour at

room temperature, the precipitate was centrifuged off and the benzene was removed under vacuum. The gum obtained was dissolved in dry methanol (50ml) and hydrogenolysed in the presence of palladium-charcoal catalyst (200mg of 10%). The uptake of hydrogen (200cc) was complete in 30 mins., after which water (15ml) was added and the pH adjusted to 11.3. This pH was maintained by addition of further alkali as required, the flask and contents being held meanwhile at room temperature. The pH was then adjusted to 7.5 with acid and after removal of methanol by distillation under vacuum, the barium salt (G.M. 7-2a) of fructopyranose-2-phosphate was isolated in a yield of 20% (320mg).

Expt. 42. Preparation of 1,3,4,6-tetra-O-benzoyl fructofuranoside.

To fructose (18g, 0.1 moles) in anhydrous pyridine (300ml), benzoyl chloride (58ml, 0.5 moles) was added at such a rate that the temperature was maintained at 60-65° without the need to apply external cooling. After the addition was complete (15 mins.) the mixture was left for 15 mins. and then poured into an ice-cold solution of sodium bicarbonate (42g in 1.5 litres). The oil obtained was dissolved in chloroform (250ml) and the solution washed with saturated sodium bicarbonate solution (100ml) and water (100ml x 2). After drying with anhydrous sodium sulphate and removal of solvent (cf. Expt. 39) the gum obtained was crystallised from 95%

ethanol (20ml). After two days the crystals were isolated in a yield of 60% (25g). Melting point 123° (published value 123°).

Expt. 43. Preparation of inulin acetate.

Inulin (25g, BDH) was stirred with dry pyridine (250ml) at 80° for 45 mins. The solution obtained was cooled and the stirring continued while acetic anhydride (45ml) was added dropwise. After an additional 6 hours stirring, the clear solution was poured into water (2.5 litres). The inulin acetate which separated was filtered off and washed copiously with water. The crude product (26g) was purified by crystallisation from methanol (250ml) and the crystals isolated in a yield of 50% (22g).

Expt. 44. Preparation of 1,3,4,6-tetra-O-acetyl fructofuranoside.

Inulin acetate (10g) was dissolved in a mixture of glacial acetic acid (100ml) and acetyl bromide (20ml). HBr in glacial acetic acid (20ml, Expt. 23) was added and the mixture left for 3 hours at room temperature. The contents were poured into an ice-cold solution of sodium acetate (10g in 1.5 litres) and the pH adjusted to 5.5 with sodium bicarbonate (170g). The solution was extracted with chloroform (100ml x 5), the combined extracts dried with anhydrous sodium sulphate and the product was obtained as an oil after distilling off the chloroform under vacuum. Yield 9g (80%).

Expt. 45. Alkaline treatment of 1,3,4,6-tetra-O-benzoyl fructofuranoside and 1,3,4,6-tetra-O-acetyl fructofuranoside.

1,3,4,6-tetra-O-benzoyl fructofuranoside (1.016g, 2m moles, Expt. 42) and 1,3,4,6-tetra-O-acetyl fructofuranoside (0.704g, 2m moles, Expt. 44) were refluxed separately with standard lithium hydroxide solution (25ml of 0.1N). After 30 mins. the mixtures were cooled and the excess alkali titrated with standard acid (0.1N). With the acetyl derivative complete hydrolysis of the acetyl esters had occurred (5ml of acid required, theoretical quantity of alkali was 20ml). However, with the benzoyl derivative only a slight degree of hydrolysis had taken place (22ml of acid was required) under identical conditions.

Expt. 46. Preparation of 2-bromo tetra-O-acetyl fructofuranoside.

1,3,4,6-tetra-O-acetyl fructofuranoside (0.7g, 2m moles, Expt. 44) was dissolved in HBr saturated glacial acetic acid (2.5ml, Expt. 23) and left for 1 hour at room temperature. The excess reagents were removed by co-distillation with dry toluene (25ml x 3) at a bath temperature of 30°.

Expt. 47. Preparation of fructofuranose-2-phosphate.

The gum of bromo tetra-O-acetyl fructofuranoside (2m moles, Expt. 46) was neutralised with triethylamine as in Expt. 41 and

triethylammonium dibenzyl phosphate (2m moles, Expt. 22) added, whereupon precipitation occurred immediately. After 1 hour at room temperature the precipitate was centrifuged off, the benzene removed under vacuum and the gum obtained was dissolved in dry methanol and hydrogenolysed with palladium-charcoal catalyst (100mg of 10%). The uptake of hydrogen (100cc) was complete in 30 mins. The catalyst was filtered off, water (15ml) was added and the pH adjusted to 11.3 and held at this value for 3 hours at room temperature. After pH adjustment to 8.6, the methanol was removed under vacuum and the aqueous solution treated with magnesia mixture (G.M. 7-2b). To the inorganic phosphate-free solution obtained after filtration, 8-hydroxyquinoline (1ml of a 5% solution in alcohol) was added and the insoluble precipitate obtained removed. The aqueous filtrate was diluted with ammonium carbonate (1 litre of 0.01M) and the solution applied to a column (15cm long x 4cm diam.) of DEAE-cellulose in the carbonate form (G.M. 9-2). The eluate was collected (25ml fractions) and analysed for chloride (qualitatively using silver nitrate) and fructose (G.M. 6-3b). 0.5ml samples were used for both analyses. Stronger ammonium carbonate (0.06M) was applied to the column after all the chloride was eluted. The progress of elution is shown (Fig. 11)p109. The tubes containing fructose were bulked and evaporated down under vacuum at a bath temperature of 30° to give 100mg of a white powder (13% yield).

On making up a standard solution (G.M. 8) and determining ester phosphate (G.M. 6-2) and fructose (G.M. 6-3b) the molar ratio of phosphate to fructose was found to be 1.07/1.

PART I. SECTIONS IV and V.

Expts. 48-53. Preparation of the tri-n-octylammonium salts of sugar-1-phosphates.

Expt. 48. α-glucose-1-phosphate. Expt. 49. β-glucose-1-phosphate.

Expt. 50. α-mannose-1-phosphate. Expt. 51. α-xylose-1-phosphate.

Expt. 52. Fructose-1-phosphate. Expt. 53. α-galactose-1-phosphate.

The experimental details followed for the preparation of the tri-n-octylammonium salts of the above sugar-1-phosphates were identical in all cases. The appropriate sugar-1-phosphate (1 mole, Expts. 31 - 37) was converted into its free acid form (G.M. 10) and treated with an ethanolic solution of tri-n-octylamine (1 mole). The solvent was removed under vacuum and the residue converted into a clear glass by repeated azeotropic distillations with dry ethanol. The glass obtained was stored over P_2O_5 at 0° until required.

Expt. 54. Preparation of uridine-5'-phosphoramidate.

The free acid of uridine-5'-phosphate (1m mole, 325mg, Expt. 14), prepared from the lithium salt form (G.M. 10), was

dissolved in formamide (2ml) and ammonia (2.5ml of 2N). DCC (1g, 5m moles) was dissolved in tertiary butanol (7ml) and added to the nucleotide solution. The two phase reaction mixture was maintained at 80° in a stoppered flask. After becoming homogeneous (2 hours) it was held at this temperature for an additional 7 hours and then cooled. Samples were removed and assayed directly by paper electrophoresis using a phosphate buffer pH 7.5 (G.M. 2). The precipitate (DCU) was filtered off and well washed with water. The tertiary butanol was removed under vacuum and the aqueous formamide solution extracted with ether (5ml x 3). The product after drying over P_2O_5 was treated with excess dry acetone (30ml) producing a white powder in virtually theoretical yield.

Expt. 55. Preparation of P_1-uridine P_2-diphenyl pyrophosphate.

Uridine-5'-phosphate free acid (1m mole, 325mg, Expt. 14), prepared from its lithium salt (340mg, G.M. 10), was converted into its tri-n-octylamine salt by the addition of tri-n-octylamine (1m mole, 400mg) in ethanol (20ml). The salt was converted into an anhydrous glass by repeated evaporation under vacuum with dry benzene and dioxan. The glass was dissolved in a dry dioxan-benzene mixture (18ml, 2:1, v/v) and a solution of diphenyl phosphorochloridate (1.2m moles, 325mg) in the same dioxan-benzene mixture (10ml) added. A solution of tri-n-butylamine (3.6m moles, 670mg) in dioxan-benzene

(15ml, 2:1, v/v) was added dropwise over 10 mins. The cloudy solution was left at room temperature for 3 hours. The solvents were removed under vacuum to leave a gum which could be stored indefinitely in this form. When required the gum was shaken with an ether-pet.ether mixture (50ml, 1:1, v/v) which was then allowed to settle for 3 hours. The supernatant was discarded and the gum used directly.

Expts. 56-59. Preparation of nucleoside phosphoromorpholidates.

Expt. 56. U-5'-phosphoromorpholidate.

Expt. 57. A-5'-phosphoromorpholidate.

Expt. 58. G-5'-phosphoromorpholidate.

Expt. 59. C-5'-phosphoromorpholidate.

A solution of DCC (824mg, 4m moles) in tertiary butanol (15ml) was added dropwise to a refluxing solution of the appropriate nucleoside-5'-phosphate (1m mole, Expts. 14 - 17) in free acid form (G.M. 10) which was itself dissolved in a mixture of water (10ml), tertiary butanol (10ml) and morpholine (0.34ml, 4m moles). The addition was carried out over 3 - 4 hours and the mixture refluxed for an additional 3 hours until paper electrophoresis (G.M. 2) using a pH 7.5 phosphate buffer, of samples removed from the reaction flask, showed only one spot with approximately half the mobility of the parent nucleotide. If some monophosphate still remained after this

time, more DCC (2m moles) and morpholine (2m moles) were added and the refluxing continued until reaction was complete. The insoluble precipitate present after cooling, was filtered off, well washed with tertiary butanol (10ml) and the filtrates bulked. After distillation under vacuum (10ml), the largely aqueous solution was extracted with ether (10ml x 3) and any insoluble material filtered off. The aqueous solution was dried azeotropically under vacuum with dry methanol. The gum thus obtained was converted into a white powder by addition of excess dry acetone (40ml) to a methanol solution (5ml) of the material. The powder was centrifuged and stored over P_2O_5 at 0° until required. The yields obtained were virtually quantitative in all cases.

Expts. 60 - 74. Preparation, isolation and analyses of nucleoside diphosphate sugar compounds.

Expt. 60. UDP-α-glucose.

Expt. 61. UDP-α-glucose.

Expt. 62. UDP-α-glucose.

Expt. 63. UDP-β-glucose.

Expt. 64. UDP-α-mannose.

Expt. 65. UDP-α-xylose.

Expt. 66. UDP-l-fructose.

Expt. 67. UDP-α-galactose.

Expt. 68. ADP-α-glucose.

Expt. 69. ADP-α-mannose.

Expt. 70. ADP-α-xylose.

Expt. 71. ADP-l-fructose.

Expt. 72. GDP-α-glucose.

Expt. 73. GDP-α-mannose.

Expt. 74. CDP-α-glucose.

The experimental details followed for preparing, isolating and analysing the above nucleoside diphosphate sugar compounds were identical in all cases, the only variable being the gradient used for ion exchange chromatography. The yields of individual products obtained and their purity are noted in Tables VI p.151 and VII p.153 respectively.

(a) Preparation.

The activated nucleotide (1m mole, Expts. 54 - 59) was dissolved in dry pyridine (15ml) and the mixture evaporated to dryness under vacuum to ensure anhydrous conditions. A pyridine solution of the tri-n-octylammonium salt of the selected sugar-1-phosphate (3m moles, Expts. 48 - 53) was added to the above activated nucleotide and the clear solution obtained was left at room temperature for 4 days with exclusion of moisture. Samples were removed at intervals and studied by paper chromatography (G.M. 1) using the Leloir solvents.

(b) Isolation.

On completion of the reaction, the pyridine was distilled off at a bath temperature of $<40^{\circ}$ and aqueous sodium acetate (1g in 25ml) added. The aqueous solution was extracted with ether (25ml x 4) and diluted to 500ml. This neutral solution was applied to a Dowex 1 x 4 resin in the chloride form (G.M. 9 -1) of dimensions 12cm long x 1.8cm diam. After washing with water (500ml), followed by 0.003N HCl (1 litre), a lithium chloride gradient at pH 2.7

(0.003N HCl) was applied. The eluting system adopted in all cases utilised two parallel-walled flasks joined together by means of a siphon (Fig. 16)p147. Both vessels contained 1.5 litres of solution initially. The reservoir concentration with respect to lithium chloride was 0.05M for the cytidine and adenosine nucleotides while a reservoir salt concentration of 0.12M lithium chloride was employed for the guanosine and uridine derivatives. The column eluate was collected in fractions (50ml), the OD_{260} readings of which were measured to locate the nucleotide containing entities (Figs. 17_Ap148 and 18_Np149). The appropriate fractions were bulked, the pH of the solution was then adjusted to 5.5 with lithium hydroxide and the volume reduced using a rotary evaporator at a bath temperature of $<35^{\circ}$. The dry powder obtained by azeotropic distillation with dry methanol was triturated with more methanol (5ml) and any insoluble material discarded. Excess dry acetone (40ml) was added to the clear supernatant and the precipitate produced collected after 1 hour at 0° and the white powder obtained stored in a vacuum desiccator at 0° until required. The yields obtained are recorded in Table VIp151.

(c) Analysis.

The purity of the samples were checked by making up standard solutions (G.M. 8). These were used directly for paper chromatography (G.M. 1) and paper electrophoresis (G.M. 2) studies.

Suitably diluted aliquots were employed for measuring reducing power (G.M. 6-3a) and for phosphate analysis (G.M. 6-2) both before and after mild acid hydrolysis (0.02N HCl for 10 mins. at 100°). The results obtained, expressed in μ moles per ml, are noted in Table VII, p.153.

PART II.

Expt. 75. Preparation of protein extracts from wheat scutella.

Wheat grains (600, var. Atle), after treatment with a dilute hypochlorite solution (20 mins.) and water washing, were germinated on wet blotting paper in stainless steel trays at 26° in the dark. After 60 hours, when the seedlings were 2cm long they were harvested, the scutella removed with a scalpel and collected in cold water (2°). As an alternative to wet blotting paper, the porous inert mica, vermiculite, was used in several experiments. The mica was soaked in nutrient solution and the grains germinated as described above. Complications such as anaerobic germination were minimised by this alternative. The isolated and well washed scutella (2.5g) were ground with glucose solution (0.1M, 4ml) in a chilled glass homogeniser with the aid of a pinch of acid washed sand. The extract was decanted slowly with continual stirring into a measuring cylinder containing acetone (80ml) kept at a temperature

of -15°. After leaving at this temperature for 30 mins. the acetone was decanted off and the remainder removed quickly using a vacuum desiccator. The powder was stored at 2° under vacuum, in which form it was reported to remain active with respect to several enzymes for months (195).

For enzyme studies, the acetone powder (30mg) was weighed out and ground up in a glass homogeniser with 0.05M phosphate buffer pH 7.3 (1.5ml). The grinder was washed with the same buffer (0.5ml) and the homogenate and washings (2ml) combined. After 30 mins. at 2° the insoluble material was removed by centrifugation (6,000 x g for 5 mins.). The supernatant (2ml) was collected and 4 volumes of saturated ammonium sulphate added. After 30 mins. at 2° the precipitate was collected by centrifugation (12,000 x g for 15 mins.) and the supernatant discarded. The precipitate was dissolved in 0.05M phosphate buffer pH 7.3 (1ml) and dialysed overnight against a 0.02M concentration of the same buffer at 2° (2 litres). The volume of the impermeate was adjusted to 1.5ml and this was then used directly as enzyme source (2mg acetone powder/0.1ml).

Expt. 76. Preparation of protein extracts from dried peas.

The powder, obtained by hammer milling dried peas (500g, var. Maple) was stirred for 30 mins. at room temperature with sodium bicarbonate (0.1M, 430ml). Magnesium sulphate (2M, 12ml) was added

and the precipitate, which was allowed to form overnight at 2°, was removed by centrifugation (9,000 x g for 10 mins.). The clear supernatant (380ml) was adjusted to pH 6.5 with monopotassium phosphate (2M) and solid ammonium sulphate (114g) was added. After leaving for 3 hours at 0° the precipitate was collected by centrifugation (10,000 x g for 15 mins.) and the supernatant discarded. The precipitate was dissolved in water (100ml), the pH adjusted to 7.2 with dipotassium phosphate and the solution dialysed overnight at 2° against two changes of distilled water (3 litres x 2). The slightly opaque solution (150ml) was then either used directly as enzyme source or else freeze dried, in which form it remained active for a limited period (6 weeks). When required, freeze dried material was weighed out, dissolved in water and used directly as enzyme source (3mg/0.1ml).

Expt. 77. Preparation of crude potato juice.

(a) Using charcoal.

Washed, peeled and thinly sliced potatoes (500g) were lightly covered with activated charcoal (15g, BDH) and minced using a hand machine. The macerate was squeezed through muslin and the juice clarified by centrifugation (12,000 x g for 20 mins.) yielding a light brown coloured liquid (250ml).

(b) Using reducing agent.

Potato slices (925g), prepared as above, were soaked in water (1 litre) containing sodium dithionite (7g). After 15 mins. they were minced and the macerate squeezed through muslin and clarified by centrifugation, as for the charcoal treatment, to give a light yellow juice (340ml).

Expt. 78. Fractionation of crude potato juice.

(a) Using ammonium sulphate.

To the clarified juice (340ml, Expt. 77) an equal volume of saturated ammonium sulphate was added. The heavy precipitate produced was left for 1 hour at 2° and then collected by centrifugation (12,000 x g for 15 mins.). The supernatant was discarded. The precipitate was dissolved in water (110ml), ammonium sulphate was added to 0.3 saturation (19.3g) and after leaving for 30 mins. at 2° the precipitate was centrifuged off (9,000 x g for 15 mins.) and discarded. Ammonium sulphate (14g) was added to the supernatant to 0.5 saturation and after 1 hour at 2° the precipitate was collected by centrifugation (12,000 x g for 15 mins.). After removal of the supernatant the precipitate was dissolved immediately in water (50ml) and used directly as enzyme source or else freeze dried (4.6g). The freeze dried samples were dissolved in water and used as required for enzyme studies (3mg/0.1ml).

(b) Using alcohol.

Clarified potato juice (200ml, Expt. 77) was placed in a tall thin-walled beaker, arranged to fit into a freezing unit of acetone/Drikold (-20°). Chilled absolute alcohol, from a large capacity burette, was added down the side of the beaker to ensure pre-cooling before coming into contact with the continuously stirred solution. The rate of addition was not allowed to exceed 10ml/min. When the required amount of alcohol was added, the contents were held at -15° for 10 mins. before centrifuging off the precipitate. During the initial stages of the alcohol additions (up to 5%) the temperature of the aqueous solution was held at 0°, or just above this, to prevent the predominantly aqueous solution from freezing. Ethanol (25ml) was added to the clarified potato juice (200ml) giving an alcohol concentration of 12%. The precipitate formed was centrifuged off (9,000 x g for 10 mins.) and discarded. Further alcohol additions (75ml) were carried out giving an ultimate alcohol concentration of 32%. The precipitate was centrifuged (9,000 x g for 10 mins.) and retained. After removal of the aqueous alcohol the insoluble fraction was dissolved up as quickly as possible in water (20ml) and either used directly as enzyme source or else freeze dried (3g). As with the ammonium sulphate fractionation the freeze dried samples were dissolved in water and used as required for enzyme studies (2mg/0.1ml).

Expt. 79. Preparation of starch grain enzyme systems.

(a) Using the method of Leloir.

Fresh firm potatoes (50g) were washed, peeled, sliced and then ground in a mortar with water (50ml) containing cysteine (10mg). The macerate obtained was squeezed through muslin to remove the cell debris. The cloudy suspension was centrifuged (750 x g for 5 mins.) and the supernatant discarded. The white sediment was re-suspended in water (50ml) and centrifuged as before. This procedure for washing the starch grains was repeated three times (50ml x 3). The starch grain preparation ultimately obtained was treated with 4 volumes of acetone (6ml) at -15° and the grains collected by centrifugation. This acetone treatment was repeated three times. After removal of acetone the grains (1.2g) were stored in a vacuum desiccator at 2° until required.

(b) Using the method of Pottinger and Oliver.

Young firm potatoes (50g) were washed, peeled, sliced and then homogenised for 30 secs. in a Waring Blender with sucrose-citrate solution (30ml, 0.5M sucrose, 0.05M citrate pH 7) and cysteine (1ml, 0.5M hydrochloride neutralised immediately before use). After filtering through muslin the starch grains were isolated by centrifugation (750 x g for 5 mins.) and the supernatant discarded. The starch grain precipitate was re-suspended in sucrose-citrate solution (30ml) and centrifuged as before. This

step was repeated three times (30ml x 3) and the starch grain preparation obtained used directly as enzyme source.

Expt. 80. The action of scutella extracts on nucleotide-substrates.

Identical experimental conditions were employed for all the digests carried out using scutella extracts.

The nucleotide-substrates (6mg), prepared in Part I, were dissolved in water (0.1ml) and added to scutella extracts (0.3ml, =6mg of acetone powder, Expt. 75). Magnesium sulphate (0.05ml of 0.05M) was added and the digests made up to 0.5ml either by adding water (0.05ml) or possible acceptor (2mg). The digests were maintained at room temperature and samples (0.1ml) removed at intervals (0,5,20 and 60 mins.) and deproteinised mainly be adding to alcohol (0.2ml). After 30 mins. at 0° the protein precipitates were centrifuged off and the supernatants used directly for paper chromatographic analysis (G.M. 1). Occasionally the extracts (0.1ml) were deproteinised by adding zinc sulphate (0.05ml), followed by the equivalent of barium hydroxide (0.11ml) and the precipitates centrifuged. The resulting clear supernatants were used directly for analysis. Suitable controls (enzyme blanks etc.) were included in all cases. The clarified samples were applied to paper chromatograms (G.M. 1, solvents 3,4, and 5) and after development the papers were treated with benzidine-TCA or silver reagents (G.M. 4-3). Occasionall

supplementary tests were included, such as assaying for amylose with I_2/KI.

The actual digests attempted, the primers or activators added, if any, and the results obtained are noted in Table IX p.169.

Expt. 81. The action of dried pea extracts on nucleotide-substrates.

The enzyme experiments carried out were identical to those described above for the scutella preparation (Expt. 80). All the nucleotide-substrates prepared in Part I were treated with the pea enzyme (9mg freeze dried powder, Expt. 76). The actual digests carried out, the primers or activators added and the results obtained are summarised in Table X p.170.

Expt. 82. The action of fractionated potato juice on nucleotide-substrates.

The experimental conditions followed were identical to those described for the scutella enzymes (Expt. 80). Crude potato juice was not used directly due to the high content of free sugars present which interfered with the analysis. 6 - 8mg of fractionated potato juice protein (Expt. 78) were used in each digest.

The digests carried out, the primers or activators added and the results obtained are summarised in Table XI p.171.

Expt. 83. The action of starch grain preparations on glucose containing nucleotides.

(a) Using the Leloir system.

Identical digests were set up using, in turn, UDP-α-glucose, UDP-β-glucose, ADP-α-glucose, GDP-α-glucose and CDP-α-glucose.

Nucleoside diphosphate glucose (6mg in 0.1ml), starch grain enzyme preparation (6mg, Expt. 79a) and maltose or maltotriose (2mg in 0.1ml) were mixed and maintained at room temperature, separate tubes containing these quantities being set up for each of the intervals 0, 5, 30 and 60 mins. Reaction was terminated by adding alcohol (0.5ml) and after shaking vigorously for 2 mins. the supernatant was decanted. This extraction step was repeated and the supernatants bulked and analysed by paper chromatography (G.M. 1, solvent 3).

With the glucose containing nucleotides, in no case could a sugar spot of R_f less than the added maltose or maltotriose be detected either with benzidine-TCA or with silver reagents (G.M. 4-3).

(b) Using the Pottinger and Oliver system.

Digests were set up containing starter starch (150μg, 0.1ml), glucose-6-phosphate (3μ moles, 0.1ml), nucleoside diphosphate glucose (6mg, 0.1ml), tris buffer pH 7.8 (0.1ml) and starch grain enzyme (0.01ml) in a total volume of 0.5ml.

Progress curves were obtained by setting up individual digests for varying time intervals (0, 5, 15, 30 and 60 mins.).

The enzyme(s) was inactivated by boiling (2 mins. at 100°). The zero point was obtained by adding the enzyme solution directly to a digest tube containing the other ingredients at 100°. The starch grains were added from a dilute suspension in as uniform a manner as was practicable. The digests were incubated at 26° with continual agitation. The starch present was estimated by a micro adaptation of the method of Pucher, Leavenworth and Vickery (232) as follows:-

Reagents - 20% aqueous sodium chloride.

I_2/KI. Iodine (7.5g) and potassium iodide (7.5g) were ground with water (50ml) and diluted to 250ml.

Alcoholic sodium chloride. Ethanol (350ml), water (80ml) and 20% aqueous sodium chloride were diluted to 500ml with water.

Alcoholic sodium hydroxide (0.25N). Ethanol (350ml), water (100ml) and sodium hydroxide (5N, 25ml) were diluted to 500ml with water.

Procedure.

To the solubilised starch in a digest tube, sodium chloride (0.5ml of 20%) and I_2/KI (0.2ml) were added and the contents mixed. After 20 mins. the precipitate was centrifuged and the supernatant discarded. The precipitate was suspended in alcoholic sodium chloride (0.5ml) and after centrifugation the supernatant was again discarded. To the dark brown iodine complex, alcoholic sodium

hydroxide (0.2ml) was added and the tube shaken until all the colour was discharged. The precipitate was centrifuged, washed with alcoholic sodium hydroxide (0.5ml) and the supernatant discarded. The starch precipitate was suspended in water (0.5ml), dilute sulphuric acid (0.5ml of N) added and the tube immersed in a boiling water bath for 5 mins. The volume was made up to 5ml and the contents boiled for an additional 3 mins. and then allowed to cool. Samples (0.1ml) were removed, diluted with water (0.9ml) and the total sugar measured by the phenol-sulphuric acid method (G.M. 6-3c).

Typical results obtained with UDP-glucose and ADP-glucose are noted in Fig. 19p176.The influence of glucose-6-phosphate and the results obtained when starch grains are incubated on their own are also illustrated in this figure. The difficulty involved in pipetting uniform samples of starch grain preparations must, however, be born in mind when comparing the above results.

PART III.

Expt. 84. The extraction of nucleotides from (a) a commercial sample of potato starch and (b) a freshly prepared sample, by eluting with 50% aqueous methanol.

(a) A commercial sample.

Commercial potato starch (200g, H and W) was placed in a

Buchner funnel (9cm diam.) and washed continuously without suction with 50% aqueous methanol. The eluate was collected in fractions (30ml) and the OD_{260} of each recorded (Table XII)p181.

A typical UV spectrum of one of the fractions is shown in Fig. 20p181.

(b) A freshly prepared sample.

An identical experiment to the above was performed using 200g of fresh potato starch prepared from 6 lbs. potatoes by the method of Leloir (Expt. 79a) but excluding the acetone treatment.

The elution pattern and spectrum directly comparable with the commercial potato starch sample are recorded in Table XIII and Fig. 20 respectivelyp181.

Expt. 85. The extraction of nucleotides from potato starch by a batchwise treatment with 50% aqueous methanol.

Commercial potato starch (3 kilos, Hopkin and Williams) was mixed with 50% aqueous methanol (6 litres) and shaken mechanically for 18 hours at room temperature. The starch was allowed to settle and the supernatant decanted off. If cloudy, the solution was filtered. The total volume was measured and the OD_{260} reading recorded. Two further treatments with 50% aqueous methanol were carried out as described above and the clear supernatants combined.

The results for two separate starch grain extractions are

recorded in Table XIV p.183.

Expt. 86. The fractionation of the nucleotide components extracted from potato starch.

(a) A 50% methanol extract from commercial potato starch (4.5 kilos, Expt. 85) containing 9,600 OD units was concentrated under vacuum to 3 litres and the neutral solution applied to a column (12cm long x 1.8cm diam.) of Amberlite IR 120 resin in the hydrogen form (G.M. 9-1). 3,300 OD units were retained by the resin and could not be completely removed by elution with N ammonium hydroxide. The solution containing the 6,300 OD units that were not retained was adjusted to pH 7.5 with dilute sodium hydroxide and applied to a column (12cm long x 1.8cm diam.) of Dowex 1 x 4 resin in the chloride form. The column was washed with water (500ml) until the OD reading was zero. Th UV material not retained by the resin was discarded (1,400 OD units).

A linear acid and salt gradient was set up over 6 litres. 0.0002N hydrochloric acid was the initial concentration in the mixing flask whereas the composition of the reservoir solution was 0.01N w. r. t. hydrochloric acid and 0.15M w. r. t. lithium chloride. The progress of the elution was followed (Fig. 21a, p.187) by measuring the UV_{260} reading of each fraction (50ml). The two flat peaks obtained were bulked separately, and freed from lithium chloride by a charcoal column treatment (G.M. 7-1a). A lithium fractionation was not

attempted due to the high concentrations of lithium chloride present. The UV spectra and paper electrophoretic results did not correspond with known nucleotide materials. The presence of heavy metals in the samples, presumably extracted from the charcoal, were thought to account for the anomalous results obtained. As these contaminants could not be removed completely by lithium, barium or mercury precipitation techniques (G.M. 7), the experiment was discontinued at this stage.

(b) A 50% aqueous methanol extract (36 litres) containing 23,000 OD units (from 10 kilos of starch, Expt. 85) was evaporated down under vacuum to 4 litres. This aqueous solution was diluted with ethanol (4 litres) and the pH adjusted to 3.7 with nitric acid. A mercury precipitation step (G.M. 7-1c) was carried out. The resulting nucleotide solution (2 litres), free of mercury salts and excess hydrogen sulphide, was adjusted to pH 7.5 and applied to a column (12cm long by 1.8cm diam.) of Dowex 1 x 4 resin in the chloride form. The applied solution contained 8,504.5 OD_{260} units, 186mg inorganic phosphate and 140mg ester phosphate (G.M. 6-2). The resin was washed with water (1 litre) until the UV reading of the effluent dropped to zero (6,680 OD_{260} units were retained). The 1,825 OD_{260} units not held were discarded.

The gradient employed and the analysis of the effluent were similar to those described in (a) above. The elution pattern

obtained is illustrated in Fig. 21b p187.

(c) A modification of the above experiment (Expt. 86b) was carried out using 25,400 OD_{260} units derived from 11 kilos of starch (Expt. 85). The concentrated extract was submitted to two mercury precipitation steps (G.M. 7-1c) in an attempt to purify further the nucleotides present.

The nucleotide solution from this which contained 7,220 OD units was applied to a Dowex 1 x 4 (chloride) column (18cm long by 1.8cm diam., G.M. 9-1). No UV material was eluted from the column even after copious washing with water (1 litre). A linear gradient was set up over 6 litres. The mixing flask contained 0.0002N hydrochloric acid and the reservoir 0.01N hydrochloric acid in 0.06M lithium chloride. A solution of 0.01N hydrochloric acid in 0.15M lithium chloride was applied directly to the column after the linear gradient was complete to remove the remaining nucleotides.

The results obtained are illustrated in Fig. 22a p188.

(d) A further confirmatory experiment was attempted using an extract containing 18,300 OD_{260} units (from 8 kilos of starch, Expt. 85). After a single mercury precipitation step the nucleotide-containing solution (8,550 OD_{260} units) was applied to a column (15cm long by 1.8cm diam.) of Dowex 1 x 4 resin in the chloride form (G.M. 9-1). The resin was washed with water (1 litre) until the UV reading of the effluent dropped to zero (3,410 OD units were

not retained).

The column was washed with three separate eluting systems:-

(1) 0.003N HCl,

(2) A linear salt gradient over 4 litres from 0 to 0.06M LiCl, at a constant pH of 2.7 (0.003N HCl), and

(3) A linear acid and salt gradient over 4 litres from 0.003 to 0.01N w.r.t. HCl and 0.06 to 0.15M w.r.t. lithium chloride.

The elution pattern obtained is illustrated in Fig. 22b p188

Expt. 87. <u>The characterisation of the fractionated starch nucleotides</u> (Peaks 1 - 7).

Peaks 1 - 7, illustrated in Figs. 21_A p187 and 22_B p188 were bulked separately and isolated as their lithium salts (G.M. 6-1b).

Standard solutions of each peak (1mg/ml) were made up initially and suitable dilutions made as required.

For paper chromatography, paper electrophoresis and thin layer chromatography the primary standards were used directly as in G.M. 8. For a direct reading of the nucleotide samples for concentration and spectroscopic base determinations a 1 to 25 dilution of the primary standards was adequate. For phosphate (inorganic, acid labile and total, G.M. 6-2) and pentose (G.M. 6-3d) analyses, dilutions of 1 to 1 and 1 to 10 respectively proved suitable. For base determinations by perchloric acid digestion, separate samples

of original lithium salt nucleotides were used.

A summary of the analyses are recorded in Table XV, p191

Peak 1. This symmetrical peak equivalent to 6% (350 OD units, 33mg lithium salt) of the total UV retained by the anion exchange resin (Fig. 21a) was not present in every starch grain nucleotide extract investigated. The material was apparently pure judging by paper chromatography. From the analytical data (1 mole of adenosine, 2 moles of phosphate and 2 moles of pentose) and from the position of elution from the ion exchange resin the compound was almost definitely nicotinamide-adenine dinucleotide (NAD). This was confirmed by the method of Colowick, Kaplan and Ciotti (205):-

Potassium cyanide solution (4M, 1ml) was added to a sample (3ml) of eluate and the UV absorption of the mixture at 325mμ was read against a mixture of the same sample (3ml) and sodium carbonate solution (2M, 1ml).

The sample with cyanide had a 260mμ reading of 0.95 and a 325mμ value of 0.37 while the blank measured 1.05 at 260mμ and zero at 325mμ. This behaviour is characteristic of genuine NAD.

Peak 2. This symmetrical peak, obtained from all extracts, consistently accounted for, at minimum, 22% of the total UV held by the resin.

Chromatographic analysis indicated the presence of only

one UV absorbing component, the mobility of which in the two Leloir solvents (G.M. 1, solvents 1 and 2) indicated identity with adenosine monophosphate. Phosphate analysis had on this occasion to be partially discounted due to the very high inorganic phosphate blanks. AMP and inorganic phosphate are known to be eluted together at a pH of 2.7 (192). The fact that the compound was eluted with 0.003N HCl and that the base was adenine was indicative of AMP.

Peak 3. This small peak, eluted after AMP and obtained in all cases, accounted for 3% of the absorption held by the column. The base present was identified as uracil and the phosphate content suggested UMP. The pentose value was low which was further proof of a pyrimidine base as pyrimidine-ribose bonds are not completely hydrolysed under the conditions of the determination(G.M. 6-3d).

Peak 4. This peak was found by paper chromatography to be composed of three UV absorbing components, the total OD_{260} accounting for 12 - 15% of the nucleotide present. The sample was banded on to Whatman No. 3 MM thick paper and developed in the Leloir pH 7.5 solvent. Three UV absorbing bands of approximately equal intensity were observed, eluted with water and studied individually.

The band of highest R_f (0.42) which upon analysis revealed a base-pentose-phosphate molar ratio of 1-2-2, suggested that the compound was ADP-ribose, the breakdown product from NAD. The

concentrations of NAD and ADP-ribose, in comparable extracts, were approximately the same. No ADP-ribose was identified in the NAD containing extract (Fig. 21b)p187. As the base present was adenine and the breakthrough point from the column just preceded that of ADP this virtually confirmed the opinion that the compound was ADP-ribose.

The band of intermediate R_f (0.34) contained the base adenine. Mild acid hydrolysis (G.M. 8, 0.02N HCl) released phosphate and reducing power both of which were absent in the original. The R_f values for the sample and authentic chemically synthesised ADP-∝-glucose (Expt. 68) were identical in solvents 1 and 2 (G.M. 1). Paper chromatographic analysis of the material hydrolysed under mildly acidic conditions (0.02N HCl for 10 mins. at 100°) revealed the presence of trace amounts of glucose (G.M. 1, solvent 3).

The position of elution and the analytical data confirmed that this band was ADP-glucose. These results were consistently obtained with potato starch extracts (Fig. 22)p.188.

The band of lowest R_f (0.13) had a base-ribose-phosphate molar ratio of 1-1-1. As the base was identified as guanine this sample was almost certainly guanosine monophosphate (GMP).

Peak 5. This large fraction consistently accounted for more than 52% of the nucleotide material present. The material from this peak, considered pure on the basis of paper chromatographic analysis, had a base-ribose-phosphate molar ratio of 1-1-2. The base was

identified as adenine (G.M. 6-1), hence the compound was tentatively identified as ADP. Confirmation of this identity was obtained by noting that 50% of the phosphate was acid labile (G.M. 6-2); the paper chromatographic behaviour of the compound was characteristic of ADP and resolution by ion exchange chromatography of a mixture of the sample with authentic ADP failed.

Peaks 6 and 7. These two ill-defined peaks accounting for 9% of the OD held, were not analysed rigorously at this stage. However they did contain substantial amounts of organic phosphate. When submitted to paper chromatography (G.M. 1, solvents 1 and 2) they remained at the origin even after 36 hours development which would suggest that they were possibly polymeric phosphate derivatives.

PART IV.

Expt. 88. The extraction of lipids from potato starch by a batch-wise treatment with hot 85% aqueous methanol.

Commercial potato starch grains (2 kilos, Hopkin and Williams, 20% moisture content) were refluxed with aqueous methanol (5 litres of 90%) for 3 hours. The effective concentration of the aqueous methanol was 83%. The warm suspension was filtered and the clear solution collected. Two further extractions were carried out in a similar manner and the supernatants bulked. The solvents were

removed under vacuum yielding 0.85g of oily residue (0.425g/kilo).

Expt. 89. The extraction of lipids from potato starch by eluting with hot 90% aqueous methanol.

A 9" diam. visible flow pipe line, fitted with a reduction joint and tap at the lower end, was used to contain the starch for the elution. The constricted part of the column was filled with a cotton wool pad and the commercial potato starch grains (8 kilos, Hopkin and Williams) were carefully poured on to this, as a slurry in methanol. The starch was washed continuously with hot aqueous methanol (90% at 60°) and the eluate recovered, reheated and applied once more to the column to economise on the amount of methanol required. This recycling of the eluate was continued until the effluent had attained a temperature of 45°. This was encouraged by jacketing the pipe line with heating tape (Electrothermal Ltd.), the temperature of which was held at 60°. The elution was completed by washing the starch with a fresh solution of hot aqueous methanol (3 litres of 90%). The clear eluate (15 litres) was collected and evaporated down under vacuum yielding 4.5g of oily residue (0.56g/kilo).

Expt. 90. <u>The direct analysis of crude lipid extracts of potato starch for glycolipids after the removal of free sugars by a Folch technique.</u>

Crude lipid extract (0.56g from 1 kilo, Expt. 89) was triturated with chloroform-methanol (2:1, v/v, 50ml) and the insoluble material centrifuged off and discarded. This step was repeated three times. The lipid extract (30ml) was then sealed in cellophane tubing and dialysed for 60 hours against four changes of constantly agitated distilled water (4 litres x 4). The aqueous layer of the opaque two phase impermeate was discarded. The chloroform solution was washed with water, dried with anhydrous sodium sulphate and the solvents removed under vacuum yielding an oily residue (0.47g).

This lipid fraction was acid hydrolysed and analysed for sugars (G.M. 12). Paper chromatography (G.M. 1, solvent 3) confirmed the presence of substantial quantities of glucose, galactose and glycerol, all of which were absent in the unhydrolysed sample. This latter result was in marked contrast to the paper chromatographic analysis of the original crude lipid fraction prior to dialysis, when it had been shown that substantial amounts of several sugars, including glucose, fructose and sucrose were present.

Expt. 91. The purification of the glycolipids from potato starch using (a) DEAE-cellulose and (b) silicic acid chromatography.

(a) DEAE-cellulose.

Crude potato starch lipid (4.5g, Expt. 89) was triturated with chloroform-methanol (2:1, v/v, 50ml x 3) and the insoluble material (1.2g) discarded.

The chloroform-methanol solution (150ml, containing 3.3g lipid) was applied to a DEAE-cellulose column (12cm long by 2.5cm diam., 15g) in hydroxyl form (G.M. 9-2) which had been pre-equilibrated with chloroform-methanol (2:1, v/v).

The material which passed directly through the column without retention (DEAE Fraction I, Fig. 26) plus the washings (500ml) were collected. The solvent was removed under vacuum producing an oil equivalent to 84% (2.8g) of the material applied. This fraction contained substantial amounts of galactose and smaller quantities of glucose and glycerol, after acid hydrolysis and paper chromatography (G.M. 1, solvent 3) as revealed by silver and benzidine-TCA reagents (G.M. 4-3). All of these were absent in the unhydrolysed sample.

The DEAE-cellulose column was now eluted with chloroform-methanol (2:1, v/v) made 5% with respect to glacial acetic acid (300ml). The solvents were evaporated off under vacuum, yielding a gum in 8% (260mg) overall yield (referred to as DEAE Fraction II). The acetic acid was completely removed by co-distillation with

toluene. The presence of glucose, fructose, galactose and glycerol in this fraction was established by means of acid hydrolysis and paper chromatography (G.M. 12). However, the original sample also contained glucose, fructose and possibly sucrose, thus reducing the significance of this result.

The DEAE-cellulose column was finally eluted with chloroform-methanol (2:1, v/v) containing aqueous lithium chloride (5ml of 0.5M LiCl/100ml solution, 300ml). The eluate was collected (300ml), shaken with water (30ml) and after 30 mins. the organic layer was isolated, washed with water (30ml), dried with anhydrous sodium sulphate and the solvents removed under vacuum. No residue was obtained at this stage indicating the absence of charged entities such as sulpholipids.

These results are summarised in Fig. 26p.217.

(b) Silicic acid.

DEAE Fraction I (2.5g, Expt. 91a) was dissolved in chloroform (100ml) and applied directly to a silicic acid column (7cm long by 2.5cm diam.) prepared as described in G.M. 9-3. The column was then washed with excess chloroform (200ml) and the total eluate was collected and the solvent removed under vacuum to give an oil which accounted for 88% (2.2g) of the material applied.

Acid hydrolysis and paper chromatography of this fraction (G.M. 12) revealed a complete absence of hexose sugars and only trace

amounts of glycerol were present. The inference from this result, bearing in mind also the acidic nature of the material, was that the predominant components were fatty acids.

The column was then washed with 10% methanolic chloroform (150ml) and the solvents distilled off as before. The yield of brown oil (200mg) represented 8% of the total lipid applied. This fraction was shown to contain, on acid hydrolysis and paper chromatography (G.M. 12), large quantities of galactose and somewhat smaller amounts of glucose and glycerol. The sugar containing fractions were thus purified considerably by this step.

Elution of the silicic acid column with higher methanol concentrations yielded virtually no material, all the lipids being removed in the first two fractions.

DEAE Fraction II (230mg, Expt. 91a), dissolved in chloroform (50ml) was applied directly to a silicic acid column (7cm long by 1.8cm diam., G.M. 9-3). The eluate together with chloroform washings (120ml) were collected and the solvent distilled off yielding virtually no residue. Triglycerides, fatty acids and other non-polar lipids were therefore absent from this fraction.

The silicic acid was eluted with 10% methanolic chloroform (120ml). The eluate was collected and the solvents removed under vacuum yielding a brown oil equivalent to 87% (200mg) of the material

applied. This fraction on acid hydrolysis (G.M. 12) was shown to contain glucose together with less than the equivalent amounts of galactose and glycerol. Sugars were completely absent in the original fraction.

Higher concentrations of methanol applied to the column removed trace amounts of dark brown sugar containing lipids from the silicic acid. However the presence of sugars in the unhydrolysed samples reduced the significance of these findings. This result is in agreement with reports which stress that free sugars are eluted from silicic acid columns with methanol concentrations greater than 15%.

The programme outlined in this experiment, together with results and yields at the various stages, are summarised in Fig. 26 p.217

Expt. 92. <u>The characterisation of the potato starch glycolipids.</u>

Although DEAE Fractions I and II vary somewhat in the proportions of individual sugars present, evidence obtained from analytical studies indicate that at this stage of the work both fractions can be considered together. When, as in Expt. 93, silicic acid chromatography by itself, was used for purification of the lipids no resolution was obtained and both fractions were eluted together, with 10% methanolic chloroform. Further analyses tended to reinforce this conclusion as summarised below.

(a) Phosphate analysis.

The fact that the glycolipid material was eluted with 10% methanolic chloroform solutions from silicic acid columns would suggest the absence of phosphate containing lipids (Fig. 25)p212. This was confirmed by analysing for ester phosphate (G.M. 6-2). The excessive charring of the lipid samples, submitted to perchloric acid oxidation for this purpose, was troublesome but results which satisfactorily established the absence of phosphate in these samples was obtained.

These results were in accord with the solubility behaviour of the samples in acetone. With rare exceptions phospholipids are insoluble in acetone. It was here found that all samples were in fact freely soluble in this solvent.

(b) Saponification.

Lipid fractions (20-50mg) were mixed with aqueous potassium hydroxide (N, 2-5ml) and maintained at 37° for 24 hours. Each suspension was neutralised with sulphuric acid (0.5N) and the aqueous solution extracted with chloroform-methanol (2:1, v/v, 2ml). The chloroform layer was collected, washed with water, dried with anhydrous sodium sulphate and the chloroform removed under vacuum to give an oil (Non-saponifiable fraction). Acid hydrolysis of this fraction (G.M. 12) liberated glucose as the sole sugar entity and control experiments showed its absence in the unhydrolysed

material (non-saponifiable).

The aqueous solution (Saponifiable fraction) was poured through a column (6cm long by 1cm diam.) of mixed bed resin (Biodemineralite) and a sample of the eluate applied directly to a paper chromatogram. The remainder was hydrolysed with sulphuric acid (G.M. 12) and the paper chromatographic results compared. The saponified fraction, prior to acid hydrolysis, was free of reducing sugar, the only positive findings being with the silver reagent (G.M. 4-3a) when trace amounts of glycerol and rather indefinite slow moving non-reducing spots were recognised. The acid hydrolysed fraction contained substantial amounts of galactose, glycerol and trace amounts of glucose (saponifiable). These latter results suggested that the galactose at least was attached to glycerol in the form of galactodiglycerides. The presence of trace amounts of glucose could have been due to low concentrations of analogous glucodiglycerides but the possibility of cross contamination could not be completely excluded at this stage.

Expt. 93. <u>The analysis of fresh potato starch for glycolipids.</u>

Starch grains (500g), obtained from fresh potatoes (10 lbs) by the method of Leloir (Expt. 79a) but omitting the acetone treatments, were shaken with acetone (2 litres) at room temperature for 2 hours and the acetone supernatant collected. This acetone

extraction was repeated twice and the supernatants bulked (6 litres). The solvent was removed under vacuum to give a white emulsion (10ml). Water (20ml) was added and the milky solution was extracted with chloroform-methanol (2:1, v/v, 20ml). The chloroform layer was separated and the aqueous phase re-extracted. The combined chloroform solutions were washed with water (30ml x 2), dried with anhydrous sodium sulphate and the solvents removed under vacuum yielding an oil (0.60g).

This fraction (0.60g) was dissolved in chloroform and applied to a silicic acid column (7cm long by 1.8cm diam., B.D.H. 9-3). The chloroform eluate and washings (100ml) yielded a yellow oil on removal of solvent equivalent to 80% (0.48g) of the material applied. No hexose sugars and only a trace of glycerol were liberated on acid hydrolysis, the inference being that this fraction is mainly composed of free fatty acids.

Elution of the silicic acid with 10% methanolic chloroform (100ml) displaced the remainder (17%, 0.10g) of the applied lipid which contained lipid-bound glucose with only trace amounts of what appeared to be galactose and glycerol.

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