STUDIES ON SYNTHETIC AND NATURALLY OCCURRING ENZYME METABOLITES

THESIS

presented for the degree of

Doctor of Philosophy

in the

University of Glasgow

by

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June, 1968.

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SUMMARY

In the General Introduction there is included a brief description of the biology and the chemical structure of starch followed by an account of contemporary opinions regarding the metabolism of starch. Carbohydrate is translocated in higher plants as sucrose which must be regarded as both the initial and the end product of starch metabolism. The process whereby it is converted to amylose and amylopectin and then in due season the carbohydrate remobilized as sucrose are examined in detail. It is shown that all of the enzymic activities invoked are not only possible but have actually been demonstrated either in the higher plants or else closely associated with starch metabolism in other organisms.

In the remainder of the Thesis an examination of the free sugars and the sugar phosphates which can be extracted from potato tubers in association with starch granules is described in detail. Potato starch granules were leached with aqueous methanol and the extract concentrated by vacuum distillation. The neutral sugars and the sugar phosphates present in this extract were then separated from each other using an anion exchange resin. The free sugars were identified by a variety of techniques but principally paper chromatography, and a further separation of the mono- and

disaccharides was effected by charcoal column chromatography. The concentrations of the various sugars were determined colorimetrically, especial care being taken to adopt methods which gave a specific result for the sugar being examined and avoided interference from the other sugars present. Of the sugars identified, sucrose, glucose, fructose, maltose and ribose, sucrose was by far the most abundant (208 mμmoles per g. of starch extracted). Glucose and fructose were present in almost equal amounts (66 mμmoles per g. of starch) whilst there was less ribose (26 mμmoles/g.) and maltose (12.3 mμmoles/g.). In a separate analysis myo-inositol was also identified (2.6 mμmoles/g.).

The sugar phosphates proved to be more difficult to isolate and analyse. After considerable preliminary exploration it was decided merely to separate by anion exchange chromatography those esters which form a complex with borate from those which do not do so. The phosphate groups were then removed by enzymatic hydrolysis following which the carbohydrate moieties were identified and quantified. Extensive use was made of gas liquid chromatography at this stage.

In a typical experiment only 3.4% of the phosphate associated with starch granules was extracted. Of this extractable phosphate 78% was inorganic whilst of the organic phosphate (120

mμmoles/g. of starch) only 9.8% was ascribable to identified carbohydrate phosphates. Of these glycerophosphate was the most abundant (3.25 mμmoles/g.) followed by glucose-6-phosphate (2.77 mμmoles/g.), myo-inositol monophosphate (2.51 mμmoles/g.) and sucrose phosphate (2.13 mμmoles/g.). Glucose-1-phosphate and fructose-6-phosphate were also present (0.54 and 0.52 mμmoles/g. of starch respectively).

In a final section is described the application of a selection of these techniques to starch granules freshly extracted from potatoes of known history. Qualitatively there appeared to be little difference between this starch and starch prepared and purchased commercially except that the presence of free ribose could not be confirmed. However with the exception of inorganic phosphate all of the identified metabolites were extracted in higher concentrations from fresh starch that from commercial starch.

PREFACE

The experimental work described in this Thesis was carried out in the Agricultural Chemistry Section of the Chemistry Department, University of Glasgow, from September, 1964, to February, 1968, under the supervision of Dr. W. R. Rees.

To Dr. Rees I should like to express my grateful thanks for his advice and encouragement through all the phases of this work.

Thanks to the generosity of Professor J. M. Robertson, F.R.S., I have been able to avail myself of the facilities of the Chemistry Department in addition to those in the Agricultural Chemistry Section.

Also it gives me pleasure to acknowledge the debt of gratitude I owe to my wife for her patience and help during this time.

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Abbreviations used.

All of the sugars referred to are the dextrorotatory isomers.

m.eq.	mille equivalent
µg.	microgram
m.mole	millemole
µmole	micromole
mµmole	millemicromole
O.D.$_{(260\ m\mu)}$	optical density at a wavelength of 260 millemicrons.
O.D.U.	optical density units.
T.L.C.	thin layer chromatography
G.L.C.	gas liquid chromatography
GADH	glucose aerodehydrogenase
TEAB	di-triethyl ammonium tetraborate
R_G	paper chromatographic mobility relative to glucose.
TMS-ether	trimethyl silyl ether
Pi	inorganic phosphate
Porg.	organic phosphate
P á labile	acid labile phosphate
PPi	inorganic pyrophosphate

G-1-P	α-D-glucose-1-phosphate
G-6-P	D-glucose-6-phosphate
F-6-P	D-fructose-6-phosphate
R-5-P	D-ribose-5-phosphate
F-1:6-diP	D-fructose-1:6-diphosphate
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ADPG	adenosine diphosphate glucose
A(U)DPG	adenosine or uridine diphosphate glucose
$NADP^+$	nicotine adenine dinucleotide phosphate

Abbreviations for the uridine nucleotides follow the same pattern as those for the adenosine nucleotides.

GENERAL INTRODUCTION: being primarily concerned with the metabolism of α-1:4-glucans in plants, but not excluding reference to animal and bacterial organisms.

By permitting greater local concentrations of population, the production of starch by primitive agriculturalists probably initiated the development of civilization. It is natural therefore that the use of starch and starch products developed at a very early stage in the history of mankind. It was systematically used by the Egyptians for the production of papyrus in 4,000 B.C. (1). Somewhat later (170 B.C.), Cato described a method for the preparation of starch which, it is interesting to note, was not substantially different from the methods presently in use. The Arabians, about 975 A.D., knew that starch could be converted to an artificial honey by the action of saliva (α-amylase). Kirchoff discovered in 1811 that acid hydrolysis of starch produced sugar, and it is probably from this that our present understanding of the nature of starch developed. However, it was not until comparatively recently, in 1940, that definite evidence for its chemical heterogeneity was presented (2, 3). Progress since then has been more rapid and the chemical structure of starch as an α-1:4-glucan with occasional α-1:6- linkages has now been described

in great detail.

Many of the higher plants synthesize starch at some stage of their life cycle. In some plants it can be formed by all parts of the plant and not merely in specialized storage tissues. In the potato plant, for example, there is starch in the tubers, in the leaves (4), and in the berry of the mature plant. Structurally the starch of the berry does not appear to differ profoundly from that of the tuber (5).

The starch of leaves, particularly young leaves, is interesting because of its transient nature. It is most abundant on warm sunny afternoons and disappears very rapidly as the leaves wilt (6). The generally accepted although apparently unproven explanation for this is that the rate of production of carbohydrate by photosynthesis exceeds the capacity of the translocation system to remove it from the leaf. To prevent osmotic damage to the chloroplasts either photosynthesis must slow down or the carbohydrate be polymerised to a higher molecular weight compound.

Although principally associated with the higher plants, starch has been detected in a wide variety of other organisms. It is present in both red and green algae, bacteria, fungi, and lichens (7). There is even a single report of the production of starch by human wound tissue (8).

The chemical structure of starch.

The chemical heterogeneity of starch mentioned previously referred to amylose and amylopectin. Amylose is basically a single chain of up to about 6,000 α-1:4- linked glucose units, having one reducing end and one non-reducing end. Amylopectin is a multi-branched structure of α-1:4- linked chains about 25 glucose units long. The chains are joined together by α-1:6- linkages to form a polymer of ten thousand or more glucose units. Thus amylopectin has one reducing end and about 4% of the glucose units are at non-reducing ends. These, however, are only the basic structures and there are a few anomalous linkages, which will be discussed later.

There is a third glucan component of plants which, since it is very similar to animal glycogen, is known as phytoglycogen. The only difference between this and amylopectin is that phytoglycogen has a higher degree of branching (9, 10). A few of the properties of these polymers are compared in Table 1.

Starches are fairly uniform in relation to their proportionate content of amylose and amylopectin, this being about 20-25% and 75-80% respectively, whilst phytoglycogen, if it is present, may constitute as much as 5% of the total starch. There are of course well known exceptions to this, such as waxy maize, which is almost 100% amylopectin, or in some varieties of sweet corn there may

TABLE 1.

Comparing the chemical and physical parameters of the glucose polymers known as starch. This table is compounded from various references mentioned in the text.

	AMYLOSE	AMYLOPECTIN	PHYTOGLYCOGEN	GLYCOGEN
Molecular Weight	$<10^6$	$\sim 10^7$	$\sim 10^7$	$\sim 10^7$
Average chain length	1,500-6,000	20-25	7-15	10-14
% 1:6 linkages	a few	4-5		8-10
β-limit	70-80%	50-60%	40-50%	40-50%
External chain length	large	12-17	8-9	6-9
Internal chain length	large	7-8	5-6	3-4
Colour of iodine complex	deep blue-black	purple red	brown	brown
λ max. I_2 complex	650 mμ	540 mμ	460 mμ	460 mμ

be more phytoglycogen than other types of starch (11). Other mutations of sweet corn have larger amounts of amylose than usual and through continued genetic development this has been gradually raised until the present amylose content is in the range of 80-85% (1).

Total acid hydrolysis of starch yields D-glucose, whilst partial acid or enzymic hydrolysis yields maltose and higher homologues of the maltose series. Since maltose is α-1:4-glucosyl glucoside, this shows that the principal linkage of starch is α-1:4. Further confirmation of this is provided by methylation studies. Exhaustive methylation of starch followed by acid hydrolysis yields 2,3,6-trimethyl glucose as the principal product, again indicating an α-1:4- linkage.

If the starting material is amylose, then after methylation and hydrolysis there is also present about 0.5% 2,3,4,6-tetramethyl glucose arising from the non-reducing end of the chains. Methylation studies by Meyer, Brentano and Bernfeld suggested a chain length of about 300 units for amylose (2). Potter and Hassid determined the chain length of various preparations of amylose by periodate oxidation, which suggested lengths of around 1,000 units (12). By the careful application of a variety of very mild techniques and the rigorous exclusion of oxygen, Greenwood was able to isolate from several plants amylose having apparent chain lengths of 1,300 to 4,400 glucose units (13).

Exhaustive methylation of amylopectin followed by hydrolysis yields 91% 2,3,6-trimethyl glucose, about 4% 2,3,4,6-tetramethyl glucose from the non-reducing ends, and also about 5% 2,3-dimethyl glucose. This arises from the branching points and indicates that positions 1, 4, and 6 are blocked, so that the linkage between the chains must be through positions 1 and 6 (14). The isolation of isomaltose, α-1:6-glucosyl glucose from acid and enzymic hydrolysates of amylopectin confirms this contention (15, 16, 17).

There are also, however, a few anomalous linkages in both amylose and amylopectin. Attention was drawn to these by unexpected hindrances to the hydrolytic action of α- and β-amylase, and also by some equally unexpected hydrolysis products.

β-Amylase (E.C. 3-2-1-2; α-1:4-glucan maltohydrolase) is an exo acting hydrolase which attacks the non-reducing end of an α-1:4-glucan to liberate β-maltose (18). Under normal conditions the smallest homologue which it will hydrolyse is maltotetraose. With amylopectin β-amylase can hydrolyse only the outer chains of the molecule as far as a branch point, whereupon action ceases. Normally only 50-60% of the molecule is hydrolysed in this manner, the remaining fragment being the β-limit dextrin.

α-Amylase (E.C. 3-2-1-1; α-1:4-glucan-4-glucan hydrolase) attacks the interior glycosidic linkages in a random fashion so that maltose appears only slowly in the hydrolysate. As before the end products are maltose and maltotriose, although in this case the proportion of maltotriose will be higher than with β-amylolysis. At high enzyme concentrations maltotriose can be slowly cleaved to glucose and maltose (19).

β-Amylase should hydrolyse amylose completely and the crude preparations such as were used to define the enzyme's activity do indeed do this. However, with rigorously purified preparations of β-amylase the conversion of amylose to maltose falls to 68-84% depending upon the source of the starch (13). Various suggestions have been made about the nature of blockages to β-amylolysis and about the rôle of Z-enzyme in contributing to the action of β-amylase to result in 100% hydrolysis of amylose.

Peat et al. found that the β-limit dextrin from several samples of amylose was attacked by Z-enzyme (20) and that the activity of the enzyme could be stimulated by addition of β-glucosidase from almonds. Z-enzyme itself proved to be inactive upon the α-1:6-linkages of amylopectin. Thus they concluded that it was a β-glucosidase and that the β-limiting anomalies of amylose consisted of occasional β-1:4-glucosidic linkages (21).

However further study showed that Z-enzyme has no action upon α-1:3-, α-1:4-, α-1:6- or β-linked disaccharides (22). Banks et al. were able to show that its action was that of random hydrolysis of both amylose and the β-limit dextrin of amylose, and was thus indistinguishable from that of α-amylase (23). Later workers confirmed this and demonstrated that almond emulsin preparations also contain traces of α-amylase activity (24). Understanding the nature of Z-enzyme unfortunately yields no information about the nature of the anomalies in amylose, since its action merely serves to bypass them.

Hamilton and Smith oxidised amylose and amylopectin exhaustively with periodate, using incubation periods as long as five months to ensure complete oxidation (25). From the products they recovered unchanged 0.2-0.5% of the original glucose. Periodate will cleave two carbon atoms bearing vicinal hydroxyl groups such as positions 2 and 3 of an α-1:4-glucan. Thus these results suggested that a small proportion of the glucose units are either linked α-1:3- or have side chains attached through an α-1:3- or an α-1:2- linkage (26). Nigerose (α-1:3-glucosyl glucoside) has been identified several times amongst the disaccharides produced by hydrolysis of starch and glycogen, particularly from amylopectin (27, 28). The α-1:3- link is not rare since it comprises half the linkages of

nigeran (29) and it has been formed *in vitro* by a disproportionation of glucose and maltose using an enzyme preparation from *Aspergillus oryzae* (30). Thus it would appear that a few of the glucose units of amylopectin and possibly amylose may be joined by an α-1:3-linkage.

However the α-1:3- linkage probably does not constitute a barrier to β-amylolysis. The presence of small quantities of nigerose in β-amylolytic digests of Floridean starch suggests that the enzyme may be able to cope with this type of linkage (31). Also Hamilton and Smith found that there is no difference in the proportion of non-periodate-oxidisable glucose in amylopectin and its β-limit dextrin (26). Clearly this is not the reason for the formation of a β-limit dextrin from amylose.

The amylose β-limits observed by early workers may be spurious for even the least rigorous of the conditions experienced by the amylose during its extraction, such as the presence of oxygen, can introduce anomalous structures and so have the effect of decreasing the β-limit. (32) Banks and Greenwood have reviewed this phenomenon recently and they have also shown that the starch fraction known as "amylose" is by no means homogeneous (33). The β-limit of the amylose they isolated decreased as the temperature of the water used to leach it from the granule was increased (34). Furthermore,

they were able to separate samples of amylose having low β-limits into two sedimenting peaks by ultracentrifugation (35).

In 1962 Kjölberg and Manners characterised an "isoamylase" from yeast which specifically cleaved α-1:6- linkages (36). With this enzyme they were able to raise the β-limits of two samples of amylose from 97% to 100% and from 76% to 97%. Accordingly they concluded that the barriers in amylose are α-1:6- linkages and nothing else. Using a similar α-1:6-hydrolase activity, Banks and Greenwood confirmed these results after taking precautions to ensure that their preparations contained no α-amylase activity (37). On the basis of a great deal of careful study these authors have concluded that the total amylose obtained from any one botanical source will contain both linear chains and branched chains, wherein the branches are of considerable length.

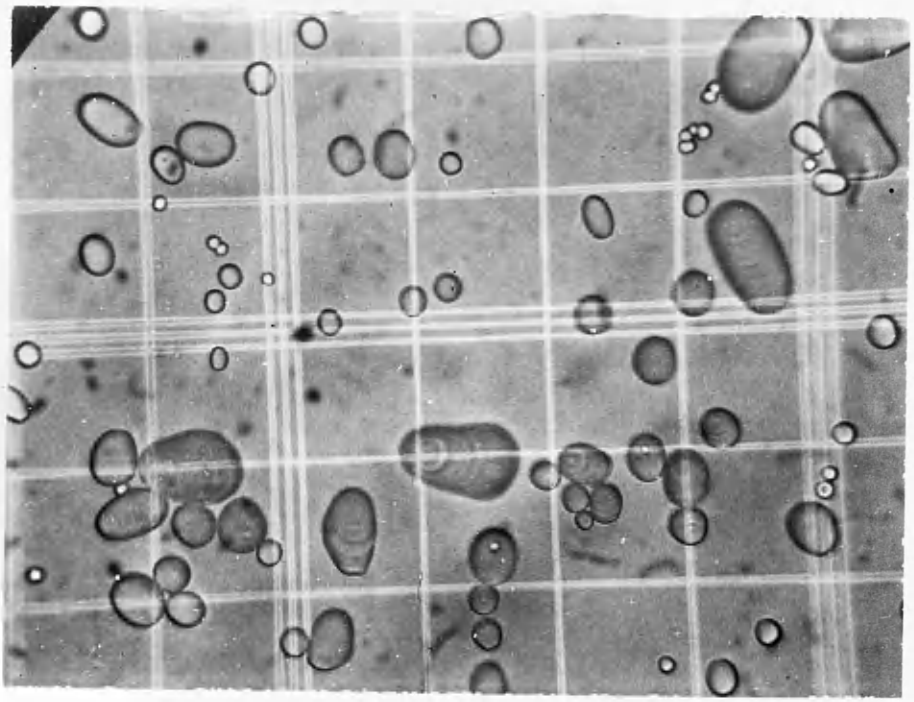
The early conception of starch as two fractions, a linear amylose and a multibranched amylopectin has become more complicated. Amylose contains both the linear and branched structures just described whilst the amylopectin fraction has been reported to contain glucans intermediate between amylose and amylopectin (1) and between amylopectin and glycogen (10). Finally, there is the highly branched, short chain structure, phytoglycogen.

The starch granule.

Starch occurs as discrete granules of varying size and shape from 3-100μ. These granules are normally found in the plastids of higher plants, in the chloroplasts of green tissues and in the leucoplasts in storage tissues, which may become greatly enlarged to accommodate the reserve starch.

Little is known of the fine structure of the starch granule and the various methods used to study it have often yielded conflicting results. Perhaps the first systematic investigation was that of Leeuwenhoek, who published some quite accurate observations in 1719 (1).

Microscopically the granules appear as a series of concentric layers arranged around the hilum, Fig. 1 (39). Meyer suggested in 1895 that the layers were a result of diurnal growth of the starch granule by apposition (40). This theory has been criticised for two reasons: the assumption that the layers are indeed a diurnal feature, and also that the granule does not necessarily grow by apposition. It has been suggested that starch granules may be formed by intussusception or by rapid crystallization of starch from a coacervate. However, both these possibilities were eliminated by the elegant experiments of Badenhuizen and Dutton, who isolated radioactive starch granules from potato tubers following



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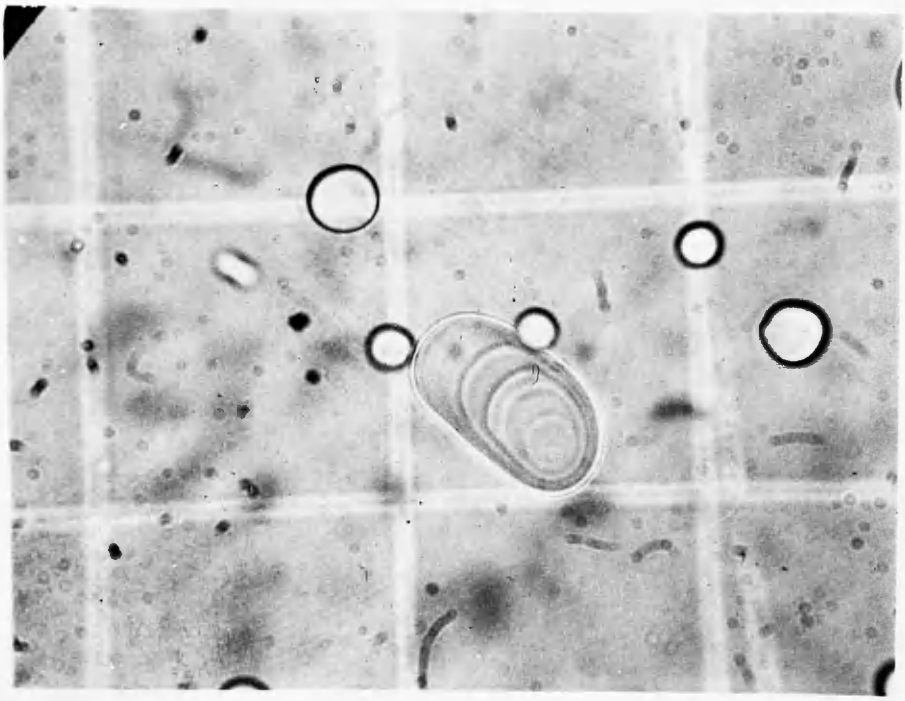


x 800

Fig. 1: Potato starch granules. The sides of the small squares are 50 microns long.

exposure of the plant to labelled carbon dioxide (41). Radio-autography of these granules revealed that growth was a gradual process over a period of days and also that the activity was confined to the peripheral layers from which it could be completely removed by β-amylase, leaving the granule almost intact.

The aetiology of the concentric layers is less certain. Various workers have advanced evidence in support of Meyer's hypothesis (42, 43, 44, 45). By correlating the number of observable layers with age of granule it is found that approximately one layer is formed per day. Cereals grown under constant illumination developed starch grains with no discernible layers, but rings could be imposed at will by the introduction of a dark period (45). On the other hand, when potatoes are grown in a constant environment they have normally developed rings (45, 46). It could be argued that potato tubers always develop in a constant environment some distance removed from the site of carbohydrate synthesis. It would be interesting to know the effect of constant illumination upon the starch granules of the potato berry.

Thus the layers of the granule may indeed be a diurnal feature, although not entirely dependent upon the availability of carbohydrate.

It has been suggested several times that there is an

outer membrane surrounding the granules, particularly potato granules, although its existence has not been convincingly demonstrated and its supposed nature is still more uncertain (47). Suggestions have been a layer of protein or a different physical phase of starch than that pervading the granule, such as a layer of pure amylopectin. Many workers have shown in the past few years that intact starch granules are in themselves complex enzyme systems having many of the enzymes of starch metabolism more or less firmly bound to the granule (e.g. 48, 49). Clearly the presence of enzymes associated with the surface of the granule does not constitute a protein membrane, although they could be detected as such. Similarly, there are reports that the proportion of amylose in the granule decreases with maturity (e.g. 50, 51). It is perhaps conceivable that in some circumstances this could be interpreted to mean a special surface layer of amylopectin. However, the possibility that the surface of the granule is exclusively amylopectin was ruled out by an elegant experiment of Nordin's, who isolated both radioactive amylose and amylopectin after irradiating the surface of some starch granules with low energy tritium atoms. (52).

Phosphoric acid and starch.

Isolated and purified potato starch contains a small percentage of phosphoric acid. The majority of this is firmly bound

to the polymer and cannot even be removed by electrodialysis of an autoclaved starch paste (53). Soxhlet extraction of potato starch with 80% dioxan reduced the phosphorus content by only 9% after 48 hours and 11% after 120 hours. Similar results were obtained by the author, although a higher phosphorus content was observed and a smaller percentage of it removed by the considerably milder method that was used. Incidentally, a major part of the work to be described in this Thesis is an analysis of the sugar phosphates that can be extracted from starch by mild aqueous leaching.

Most of the phosphoric acid associated with potato starch is to be found in the β-limit dextrin of amylopectin (54, 55, 56), to which it is covalently bound as a glucose-6-phosphate ester (54, 57, 58). This esterified phosphate probably constitutes part of the barrier to complete β-amylolysis (59).

Amylose, on the other hand, contains very little phosphate, most of which can be removed (60, 61, 62). Peat et al. have calculated that there can be no more than one bound phosphate group for every 2,400 glucose units (21). Since the β-limit of amylose is not increased by previous incubation with phosphatase, this esterified phosphate cannot be the previously discussed barrier to complete β-amylolysis, which suggests that it may be attached at or near the potential aldehyde group (33).

There is apparently no definite information about the origin or the function of the phosphoric acid which is bound to starch, except to note that it has a profound effect upon some of the physical properties such as swelling of the granule upon hydration. However it should definitely be considered as an integral feature of the biology of starch (63).

THE METABOLISM OF STARCH - GENERAL ASPECTS.

The chemistry and biology of starch have been discussed in detail for it is only by an insight into the true nature of starch that its metabolism may be understood. Any comprehensive description of the metabolism of starch should encompass the following processes:-

(a) The arrival of sucrose at the region of synthesis and its conversion to starch precursor.

(b) The polymerisation of this precursor to a linear α-1:4-glucan.

(c) The conversion of the linear glucan to a branched chain glucan.

(d) The eventual mobilization of starch.

(e) The conversion of the mobilization products to sucrose.

As a convenient basis for discussion, it will be held that the immediate precursor of starch is ADPG, which incorporates glucose, the reaction being catalysed by the enzyme starch synthetase (E.C. 2-4-1-21, A(U)DPG; α-1:4-glucan α-4 -glucosyl transferase).

The conversion of amylose to amylopectin is catalysed by Q-enzyme (E.C. 2-4-1-18, α-1:4-glucan; α-1:4-glucan 6-glycosyl transferase).

Phosphorylase (E.C. 2-4-1-1, α-1:4-glucan orthophosphate glucosyl transferase) initiates the degradation of starch by removing a terminal non-reducing glucose unit and by converting it to α-D-glucose-1-phosphate.

The current theories of starch metabolism are not well established for there have been two major upheavals in the last decade, both originating from Leloir and his colleagues. First of all he reported that glycogen and amylose could be synthesized in vitro from UDPG (64, 65). Then a few years later he suggested that the natural precursor of starch is ADPG, but that the natural precursor of glycogen is UDPG (66, 67). This firmly and finally divorced glycogen from starch; although structurally it is very similar to phytoglycogen (precursor exclusively ADPG (68)), the enzymatic resemblances are merely superficial.

A third and equally important upheaval may be impending, for there have been several indications in the literature lately that amylose is not necessarily the precursor of amylopectin and that their biosynthetic pathways may differ (69). It has even been suggested that phytoglycogen is the precursor of both amylopectin and amylose (70).

One of the modern concepts of biochemistry is that anabolism and catabolism usually follow different pathways. This

means that both processes can be thermodynamically favourable and that they can be controlled by mechanisms other than mass action. The theory demands that decisions be taken as to the likely metabolic rôle of various enzymes and because of this it is a concept peculiarly applicable to the metabolism of starch. Thus many of the amylose synthesizing enzymes, such as amylosucrase, are no longer thought to have a significant function in starch metabolism.

The readily reversible synthesis and degradation of glucans catalysed by phosphorylase were described by Cori and by Hanes between 1937 and 1940 (71, 72). At equilibrium the ratio of Pi to G-1-P is low enough to suggest a fully reversible function for the enzyme (73). This provided for the first time a coherent theory for both the synthesis and the degradation of glucans, which received ready and wide acceptance.

However in 1954 there were reported two separate studies on the ratios Pi : G-1-P in yeast and in the bark of the locust tree, both of which showed that because of a large excess of inorganic phosphate equilibrium conditions did not obtain (74, 75). From this the latter authors concluded, somewhat courageously, that phosphorylase was not involved in starch synthesis. This view did not gain general acceptance until the work of Leloir suggested a credible alternative to phosphorylase. Even now, ten years after

the discovery of glycogen synthetase, the rôle of phosphorylase as an exclusively degradative enzyme is not wholly accepted. There is some justification for this. The reports that waxy maize contains no A(U)DPG-starch synthetase have not been satisfactorily explained (69, 79). Frydman and Cardini have described a soluble polysaccharide from potatoes which they think is concerned with initiating starch granule formation, but which only accepts glucose in reactions catalysed by phosphorylase and not starch synthetase (76). Similarly there is reported a glycogen storage disease in humans wherein UDPG-glycogen synthetase is completely absent and yet liver biopsy shows the presence of a low but by no means negligible level of glycogen (77). Badenhuizen and Chandorkar have published a series of papers apparently aimed at discrediting the synthetic role of nucleoside diphosphate glucose transferase activities (78).

One of the objects of the work to be described in this Thesis was to measure the concentrations of Pi and G-1-P in the starch granule in response to the suggestion that there may be localised regions within the cell where the relative concentrations are reversed (74).

(a) The conversion of sucrose to ADPG.

Analyses of sieve tube exudates covering some 45 species show that the major material transported in higher plants is sucrose.

These reports stress the complete absence of hexose and of hexose phosphates in the sieve tube sap (80).

It is now generally held that sucrose is the form in which carbohydrate nutrition is sustained in the intact plant and that hexoses only arise by hydrolysis. Since sucrose is the only sugar translocated and starch synthesis does not necessarily occur in the photosynthetic zones, it follows that sucrose must be regarded as the initial precursor of starch, however many intermediates may be involved. That they are closely connected has been shown, for starch can be synthesized from sucrose (81, 82, 92), sucrose and starch are freely interconvertible (75), and if starch synthesis is inhibited then sucrose tends to accumulate (83).

The free energy of hydrolysis of the glycosidic bond in sucrose is relatively high, higher in fact than the α-1:4- linkage of starch.

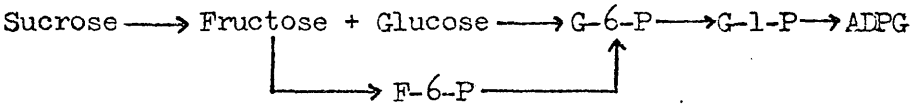
Table 2: Free energy of hydrolysis of various glycosides (84)

GLYCOSIDE	ΔG^{o}
Sucrose	- 6.6 K.cals.
UDPG	- 7.6 "
Maltose	- 4.0 "
Glycogen α-1:4-	- 4.3 "
Dextran α-1:6-	-2.0 "
α-G-1-P	-4.8 "

Because of this it is tempting to suggest that sucrose is converted directly to starch without any intermediate steps. Reactions of this nature are known to occur in bacteria but they require highly artificial conditions and enzymes which are essentially hydrolytic in function (85).

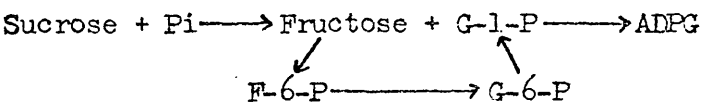
Several routes have been suggested to explain the conversion of sucrose to ADPG:-

(a) By hydrolysis followed by phosphorylation.



However since the free hexose pools of the plant cell do not become equilibrated with radioactive sucrose, this cannot be the pathway involved (86).

(b) By the intervention of sucrose phosphorylase.



This pathway can be eliminated because the essential enzyme, sucrose phosphorylase, has never been detected in higher plants, only in bacteria.

The remaining possibilities are closely related and upon the basis of the evidence presently available it would be quite reasonable to decide that all three are significantly involved.

Briefly they are:-

(c) Sucrose + UDP $\rightleftharpoons$ UDPG + F $\longrightarrow$ starch.

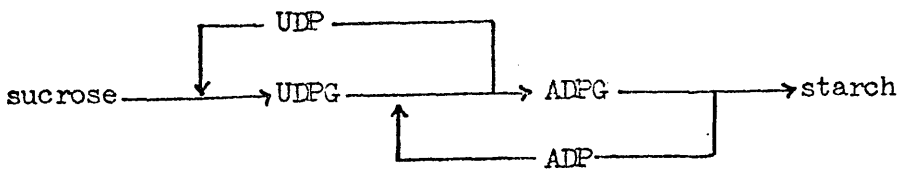
(d) Sucrose + ADP $\rightleftharpoons$ ADPG + F $\longrightarrow$ starch.

(e) Sucrose + UDP $\rightleftharpoons$ UDPG $\rightleftharpoons$ ADPG $\longrightarrow$ starch.

Reference to Table 2 will show that ΔG° for the reaction UDPG + Fructose $\rightleftharpoons$ UDP + Sucrose is only about - 1 K.calorie, which means that it should be freely reversible. Various equilibrial values have been suggested which, although favouring the synthesis of sucrose, are not so large as to preclude the reverse reaction, especially if the product is removed by the advancing synthesis of starch (87, 88, 89). If starch is synthesized from UDPG, as is possible in the absence of ADPG, then it is probably route (c) which is involved (83).

When Recondo and Leloir reported that ADPG is a much better substrate for starch synthetase than UDPG, they also said that it could not be substituted for UDPG in synthesising sucrose (66). However the following year Cardini and Recondo corrected this statement when they demonstrated that ADPG could be as much as 90% as efficient as UDPG in sucrose synthesis (90). The objection to route (d) above is that the synthesis of sucrose from ADPG is very strongly inhibited by the uridine nucleotides (89, 90) which are abundant in plant tissues (91).

What then about the last possibility, the formation of UDPG from sucrose and the transfer of glucose from it to ADPG? It was de Fekete and Cardini who first postulated that UDPG has a primary rôle in sucrose-starch interconversions, whilst starch synthesis is exclusively catalysed by ADPG-starch synthetase (81). This hypothesis avoids the necessity of synthesizing starch from UDPG and of synthesizing ADPG directly from sucrose, both of which reactions are enzymically unfavourable. It is well supported by a considerable body of experimental evidence. Thus, for example, ADP mediates the transformation of sucrose to starch far more effectively than does UDP, but the addition of UDP to an ADP-mediated system has an inhibitory effect (89). ADPG and UDPG both become labelled when the starting material is radioactive sucrose. A direct transfer reaction is probably not involved, viz:-



There is as yet little information available concerning the middle steps of the interreaction. Several routes can be envisaged, all of which have been described, although not necessarily in the higher plants and not necessarily involving uridine as the nucleoside.

$$\text{Sucrose} + \text{UDP} \rightleftharpoons \text{UDPG} + \text{Fructose} \quad (87)$$

$$\begin{cases} (1)\ \text{UDPG} \longrightarrow \text{U} + \text{Pi} + \text{G-1-P} & (93) \\ (2)\ \text{UDPG} \longrightarrow \text{UMP} + \text{G-1-P} & (94) \\ (3)\ \text{UDPG} + \text{Pi} \longrightarrow \text{UDP} + \text{G-1-P} & (95,\ 96) \\ (4)\ \text{UDPG} + \text{PPi} \longrightarrow \text{UTP} + \text{G-1-P} & (81) \end{cases}$$

$$\text{G-1-P} + \text{ATP} \longrightarrow \text{ADPG} + \text{PPi} \quad (97)$$

An advantage of these routes is that the fructose moiety can be incorporated in ADPG by the same mechanism, following its conversion to G-1-P by fructokinase, phosphohexose isomerase and phosphoglucomutase.

$$\text{F} + \text{ATP} \longrightarrow \text{F-6-P} \longrightarrow \text{G-6-P} \longrightarrow \text{G-1-P}$$

It has been reported that the necessary enzymes for this sequence are all closely associated with tapioca starch granules (98).

Obviously the third of the above alternatives is the most feasible, but a decision cannot be made upon the basis of the information presently available.

There are indications that ADPG-pyrophosphorylase occupies a key position in the production of starch and that significant metabolic controls regulate the synthesis of ADPG.

Tsai and Nelson have described two separate mutants of maize in which the capacity to synthesize starch is greatly reduced and sucrose accumulates (83). All the enzymes usually associated with starch metabolism were present except for ADPG-pyrophosphorylase

which was completely lacking. They suggested that what starch there was present, about 25-30% of the normal levels, arose from ADPG or UDPG produced by sucrose synthetase. It would be interesting to know the relative levels of G-1-P and Pi to see if phosphorylase could have been involved.

A series of experiments by Preiss et al. have shown that in spinach chloroplasts ADPG-pyrophosphorylase activity is stimulated by some of the intermediates of the photosynthetic carbon reduction cycle, particularly 3-phosphoglyceric acid (99, 100). Similar controls occur in the synthesis of bacterial glycogen, the sole precursor of which is ADPG. In this case the activators were F-6-P, pyruvate, R-5-P or F-1:6-diP, whilst AMP, ADP, Pi, $SO_4^=$ and phosphoenol pyruvate exerted an inhibitory effect (101, 102, 103). Recent work has indicated that a complex series of complementary inhibitions and counteractivations may operate in Chlorella (104).

To summarise the foregoing paragraphs briefly, the evidence presently available suggests that the production of ADPG from sucrose involves a reversal of sucrose synthetase action and a pyrophosphorolysis of G-1-P. Significant metabolic controls may occur at the latter stage.

(b) The polymerisation of glucose to a linear α-1:4-glucan.

The position of UDPG as a biosynthetic agent was well

established when in 1957 Leloir and Cardini announced that they had succeeded in synthesizing glycogen from UDPG (64). Somewhat surprisingly, it was three years before the parallel synthesis of starch was reported (65). To some extent this delay may have been due to the nature of the enzyme system, whole starch granules, which at that time would have been regarded as a little unconventional. More recently, it has been shown that intact starch granules are compact multienzyme units (98). However, since then the possibility of starch synthesis from UDPG has become well established (91).

Then came the report in 1961 that the rate of transfer of glucose to starch was ten times faster from ADPG than from UDPG. This in itself does not necessarily mean that ADPG is the natural substrate for starch synthetase, for, as Ginsburg pointed out, ortho-nitrophenyl galactoside is a better substrate for β-galactosidase than is lactose (105). There are however several factors which support the dominance of ADPG. The incorporation of glucose from UDPG into starch is inhibited by several of the adenosine nucleotides whereas the uridine nucleotides do not affect incorporation from ADPG (106). Also ADPG has a much greater affinity for starch synthetase than has UDPG (81). Perhaps the most conclusive evidence of all is the discovery of several systems which cannot utilize UDPG. Examples of these are the transient leaf starch of Phaseolus aureus (107),

soluble glucan transferases of spinach chloroplasts (108), tobacco leaves (117), and sweet corn (109), the enzymes synthesizing phytoglycogen in sweetcorn (68), and whole starch grain preparations from soya bean and geranium (110). Another corroborative factor is that following mechanical disruption of starch granules, glucose can no longer be transferred from UDPG whereas transference from ADPG is enhanced.

The relative activities of ADPG and UDPG in other biosynthetic pathways have naturally enough been investigated, but in none of them was ADPG favoured as it is in starch synthesis. Thus in starch ADPG was 1,000% more active than UDPG, whereas in sucrose synthesis it was only 90% as effective (90), in glycogen synthesis 50%, and arbutin synthesis in wheat germ 150%, but it showed no activity at all in locusts (111).

This does not altogether preclude the synthesis of starch from UDPG. As Leloir has pointed out, there can be as much as ten times more UDPG than ADPG in plant tissues (91). Other workers, however, have found relatively higher levels of ADPG (112). Within potato starch granules or adsorbed onto their surface, there is no detectable UDPG, only ADPG (113), whilst of the other nucleotides, AMP and ADP predominate (114). This is particularly significant because starch synthesis is not simply a matter of

competition between substrates. As mentioned before, the incorporation of glucose from UDPG is inhibited by the adenosine nucleotides. In view of these findings it seems likely that synthesis from UDPG will prove to be a very minor feature.

What then is the nature of this reaction catalysed by starch synthetase? Glucose is transferred from ADPG to the non-reducing end of an α-1:4-glucan. The free energy of the reaction UDPG + $G_n \rightleftharpoons G_{n+1}$ + UDP has been calculated to be about ΔG^{o} 3.3 K.cals. (115 and Table 2). There is no reason why the same reaction with ADPG should give a different result. (Apparent differences in the equilibrial values of ADPG and UDPG for sucrose synthesis have been reported, but it must be assumed that full equilibrium was not attained (89)). As might be expected, reversal of this reaction is difficult to demonstrate and the equilibrium is very much in favour of glucan synthesis. A primer molecule is necessary to start the reaction, maltose being the smallest so far demonstrated (116), whilst there is no apparent upper limit to the size of the acceptor, although the suggestion has been made that a multibranched glycogen molecule may be self-limiting by steric hindrance of the end groups (115).

It has become apparent that more than one enzymic activity is described under the general name of starch synthetase.

Apart from the coexistence of particulate and soluble enzymes in the same tissue (117), there is a wide diversity of acceptor specificities. Some enzymes will transfer glucose only to whole starch granules (117) or to whole granules and maltooligosaccharides (118), whilst other activities find that intact granules are poor primers and must necessarily use disrupted ones (89). One particularly interesting system would only transfer glucose to phytoglycogen and amylopectin, whilst the addition of amylose to the digest resulted in a 25% inhibition of incorporation onto other acceptors (109). On the other hand, in intact granules the glucose incorporated appears to be divided equally between amylose and amylopectin. Since only about 2% of the accepting end groups are amylose as opposed to amylopectin, some systems must transfer selectively to amylose (119).

Thus there are starch synthetase activities which use both UDPG and ADPG and others which use only ADPG; there are particulate and soluble enzymes; and there is a wide variety of acceptor specificities. Some of these may be different forms of the same enzyme, but clearly several different enzymes are involved (110).

De novo synthesis of starch and glycogen is an area which is still largely unexplored. Does it in fact occur? The possibility that cell division transfers some of the cell's glucans which serve as a basis for further synthesis has been discussed. (38)

Certainly seeds are a rich source of starch, but what about the newly fertilized ovum in animals? Is it possible that from unicellular origins the polysaccharide could spread throughout the whole organism? This is what happens with DNA, so there is no certain reason to suppose that it could not happen with polysaccharides. Some glucans are freely soluble in cold water and synthesis onto these could initiate formation of new starch granules. Frydman and Cardini have described just such a mechanism based upon phosphorylase (76). Illingworth et al. achieved the de novo synthesis of glycogen by rabbit muscle phosphorylase using highly artificial conditions (120). With radioactive G-1-P and a very large amount of highly purified enzyme, they found that after a fairly long lag period an iodine staining polysaccharide appeared in the digest. They isolated labelled maltose and maltotriose from the digest, which proved that de novo synthesis had indeed occurred. There was no α-amylase activity present. Perhaps if starch synthetase could be studied under similar conditions the same result would be obtained.

Pottinger and Oliver have claimed that starch synthetase of potatoes is activated by G-6-P (121). Other workers were not able to repeat this observation (112), and it remains the only report of the activation of starch synthetase in higher plants by a normal cell metabolite. Similarly, apart from two isolated and

rather specialised cases, there do not appear to be any naturally occurring inhibitors of starch synthetase (109, 118).

This is in marked contrast to UDPG-glycogen synthetase, which, it is currently believed, occurs in two forms, dependent upon and independent of activation by G-6-P, and which is activated by insulin, inhibited by cyclic 3' 5' AMP and epinephrine and so also by compounds such as benzedrine which inhibit the breakdown of epinephrine (122 to 126). It is by this means that glycogen metabolism is controlled, which serves to illustrate once again the fundamental difference between glycogen and starch, for it will be recalled that control of starch synthesis appears to operate at the stage of ADPG formation.

The study of glycogen storage diseases has made an important contribution to our understanding of glycogen metabolism. Similar investigations of plant systems have tended to lag a little; there is no automatic screening of plants similar to that occurring in human populations. (A good example of this is described in ref.77). However, this deficiency is being remedied to some extent by the work of Nelson and his colleagues who are systematically examining various mutant strains of maize.

One of these mutants, waxy maize, contains as much starch as normal maize but it is composed entirely of amylopectin

with no amylose present. This strain also lacks the ability to transfer glucose from UDPG, the enzyme starch synthetase being absent, although the relative proportions of phosphorylase and Q-enzyme are the same as in normal maize (127). Whelan, amongst others, interpreted this result to mean that amylose was synthesized by UDPG-starch synthetase whilst amylopectin was not, ergo it was synthesized by phosphorylase (119).

These workers also reported that they could observe no transfer of glucose from starch to either UDP or ADP with the enzyme systems of waxy maize. However, because of the free energy barrier, this was an unreliable method for demonstrating an inactivity. In the following year Frydman contradicted this when she found that waxy maize contains a specific ADPG-starch synthetase, albeit with only 20% of the activity of normal strains of maize (118).

A year later, Nelson promulgated the claim that ADPG-starch synthetase activity is confined to a limited number of highly active granules situated exclusively in the embryonic and maternal tissues, whilst the endosperm which is the site of most starch synthesis contains no starch synthetase at all (69). Later he confirmed this and showed that in normal strains two different enzymes are involved, one of which is lost in the waxy mutation (128).

On this basis it would seem clear that amylose is normally

synthesized by A(U)DPG-starch synthetase. Whether this work also justifies the conclusion that starch synthetase is not involved in amylopectin synthesis is doubtful. The crucial role of A(U)DPG has been demonstrated conclusively several times, not least by some concurrent work of Nelson's upon another mutant strain of maize. This strain is unable to synthesize ADPG, as a result of which it only forms 25-30% as much starch as ordinary maize (83). It would be interesting to know the relative levels of various metabolites in waxy maize, particularly the ratio of G-1-P to Pi and whether or not ADPG accumulates.

Briefly to summarise the foregoing paragraphs: starch is synthesized in plants from ADPG and to a lesser extent UDPG by several different enzyme activities. There is no natural activation or inhibition of this reaction, but it does require a preformed α-1:4-glucan as an acceptor. No conclusions can be drawn as yet about the de novo synthesis of starch.

(c) The formation of branched chain glucans.

The name Q-enzyme is used to describe an enzyme activity which causes the rupture of a non-terminal α-1:4- linkage and its transglycosylic attachment to an adjacent chain by an α-1:6-linkage. In other words Q-enzyme converts amylose to amylopectin.

Potato Q-enzyme requires an amylose chain at least 40 glucose units long before the transferase activity can operate (129). The reaction is a random one and it is possible to isolate intermediates between amylose and amylopectin (130). With amylopectin the specificity is different, for the outer chain length need exceed only 14 D-glucose units (131), which has been interpreted to mean that Q-enzyme contains more than one active site (132). An alternative explanation would be that more than one protein is involved. Some tenuous support for this hypothesis was provided by Lavintman when she found that the various branching enzymes responsible for phytoglycogen formation in sweet corn show markedly different behaviour with amylose and with amylopectin (133).

The action of Q-enzyme is independent of and unaffected by either inorganic phosphate or G-1-P. It can stimulate the synthesis of starch by phosphorylase, but this is simply because its action increases the number of available end groups (134).

It is generally believed that the transfer of dextrins occurs between adjacent chains. Thus the smaller homologues of the malto-oligosaccharide series will act as acceptors (135). This suggests that the Schardinger dextrins should also be acceptors, but in fact they inhibit Q-enzyme, which could mean that the enzyme needs a proximal non-reducing end group in the acceptor dextrin (135). In

corroboration of this it appears that the transferred chain is attached quite close to the non-reducing end of the accepting glucan (136).

This more or less summarises what is known about Q-enzyme for it has received less attention than the synthetic and degradative enzymes. What else is known merely serves to elaborate problems, which suggests that its characteristics are different from those of any other enzyme. Consider for example three aspects of this:-

Firstly, its action is apparently irreversible. Other "irreversible" reactions are known, but these either entail large free energy changes or immediate removal of one of the products of the reaction, for example by hydrolysis of pyrophosphate. The free energy of glucan transference from α-1:4- to α-1:6- in glycogen is probably in the range - 0.7 to - 1.3 K.cals. (115); (cf. Table 2, the discrepancy could be due to steric hindrance). Similarly, the product of the reaction is not immediately removed; this means that the reaction should be freely reversible. A possible explanation of this can be adduced. The normal action of Q-enzyme is confined to the interior of a molecule; a central α-1:4- linkage is cleaved and the dextrin transferred to another central glucose unit. However, to reverse the process an entirely different mode of action is required, for after cleaving an α-1:6- linkage, the enzyme would

have to locate the non-reducing end of a chain on which to affix the detached portion of the polymer.

Secondly, it is a reaction which apparently does not attain equilibrium. With a free energy change of this magnitude an equilibrium mixture should contain 70-90% α-1:6- linkages (115). The formation of polysaccharides of this nature has been demonstrated (137, 138), but in amylopectin and glycogen only 5% and 8% of the linkages are α-1:6. Since the limitation to further branching cannot be a thermodynamic one, it must arise from the steric specificity of the enzyme.

This specificity appears to be quite well defined. Q-enzyme of potatoes will act upon amylose to produce amylopectin having 5% α-1:6- linkages, and so it has no action upon natural amylopectin with a chain length of 20 units (139). However, Q-enzymes of bacterial and animal sources will act upon amylopectin to introduce a further 3% of α-1:6- linkages, giving rise to glycogen. Similar results have been reported with Q-enzymes from plants containing phytoglycogen (133). Manners has very precisely defined the specificity of a Q-enzyme preparation from yeast (140). It was inactive with horse muscle glycogen of mean chain length 17 glucose units, but it acted very slowly upon malted barley amylopectin, mean chain length 18 glucose units. Clearly the specificity of

Q-enzyme is of an unusual nature amongst enzymes.

The third aspect, and perhaps the most puzzling one of all, is the coexistence of amylose and amylopectin in starch granules. They may possibly be synthesized by different mechanisms and there may be Q-enzymes which are inactive upon amylose, but it is an indisputable fact that amylose exists in the presence of enzymes capable of converting it to amylopectin. It has been suggested that amylose is synthesized within a protective membrane which is permeable to all enzymes and metabolites except Q-enzyme. However, such membranes have not been detected within the amyloplast (141). A more plausible explanation was suggested by the Japanese school when they described the firm and specific association between starch synthetase and amylose, in vitro (142) and in starch granules (143). There is a possibility, they say, that complex formation between the amylose synthetase and amylose type molecules sterically inhibits the functioning of Q-enzyme, thus allowing the preferential synthesis of long chain amylose molecules. On the other hand, glucan molecules of intermediate size which are unable to form complexes with the enzyme might be more available for amylopectin formation. They say that experiments to test this hypothesis are currently being carried out. Nelson's work on waxy maize could be taken to support this suggestion, since the mutant contains no amylose and also no starch

synthetase (78).

Perhaps it would be easier to understand why Q-enzyme is occasionally inactive if it were known why plants find it advantageous to synthesize both amylose and amylopectin. This, however, is yet another of those areas of starch metabolism which remain largely uninvestigated. A possibility that does not appear to have been fully considered is that amylose forms a more stable carbohydrate reserve than does amylopectin. Amylopectin contains about fifty times more non-reducing ends than amylose. Since there does not appear to be a report extant that phosphorylase has a greater affinity for amylose than for amylopectin, their relative rates of degradation should be roughly proportional to the number of end groups. There does not appear to be any definite evidence to substantiate the hypothesis. Glycogen metabolism seems to be arranged so that a very rapid production of glucose is possible and of course animal tissues do not produce amylose. It would also require the transient assimilation starch of leaves to contain little or no amylose and young shoots to contain a higher proportion of amylose. Meyer compared various tissues of the potato plant (4).

Tissue	% Amylose	% Amylopectin
Leaf starch	21.5	78.5
Tubers	27	73
Shoots	73	27

The proportions of amylose in the leaves and tubers cannot be regarded as being sufficiently divergent to substantiate this theory.

To summarise briefly the foregoing paragraphs, Q-enzyme converts some of the α-1:4- linkages of amylose to α-1:6- linkages without the intervention of phosphate in an apparently irreversible manner and without ever attaining a thermodynamic equilibrium. The steric specificity of the enzyme may be closely defined but neither this nor its occasional inactivation are understood.

(d) The degradation of starch in plants.

The metabolic role of α- and β-amylase in plants is uncertain. Until the discovery of phosphorylase they were believed to be responsible for the degradation of polysaccharides, but now they are no longer credited with this function. Only traces of α-amylase are present in the resting seed until germination, whereupon it becomes preponderant (144). The distribution of β-amylase is haphazard and not apparently closely related to the distribution of its substrate. Thus it is absent in most starch-bearing leguminous seeds, but present in the soya bean, which does not accumulate starch. Similarly, ungerminated barley and wheat are good sources, but oats, maize, sorghum and rice contain none. Functionally the results of β-amylolysis and phosphorolysis are similar except that β-amylolysis

is an energetically wasteful reaction. On the other hand, the action of α-amylase and also that of R-enzyme are supplementary to that of phosphorylase; they enable it to bypass the branching points and so serve to remove all the limitations to complete phosphorolysis.

In 1937 Cori and his colleagues showed that the scission of glycosidic bonds is dependent upon inorganic phosphate and that G-1-P is the product (71). This reversible reaction can be represented:

$$(\alpha\text{-1:4-glucan})_n + Pi \rightleftharpoons (\alpha\text{-1:4-glucan})_{n-1} + \alpha\text{-D-G-1-P}$$

Inorganic ortho-arsenate, $HAsO_4^{=}$, can replace $HPO_4^{=}$ so that the reaction is followed by spontaneous hydrolysis of glucose-1-arsenate to form glucose and ortho-arsenic acid. As with starch synthetase a primer is necessary for normal synthetic action. Potato phosphorylase can be primed with maltotriose or maltotetraose, although amylopectin is a more efficient primer and amylose slightly less so. However, the efficiency of a polysaccharide as a primer is directly proportional to the number of non-reducing end groups - with one limitation, i.e. very long chains will only act as donors and β-limit dextrins can only act as acceptors (145). In the absence of an acceptor, phosphorylase will not bring about any exchange of label between G-1-P and Pi^{32}, neither will it catalyse the arsenolysis of G-1-P (148).

Because of the pKa values of G-1-P and Pi, pK2 = 6.51 and 7.19 respectively at 30°, the equilibrium of the phosphorylase reaction is very dependent upon pH (149, 154). However, if the relative concentrations of only the divalent ions are considered, a constant equilibrium of 2.2 is obtained between pH 5 and 7. It must be remembered that the concentration of non-reducing end groups is unchanged during the course of the reaction, so that the equilibrium is dependent only upon Pi and G-1-P concentrations. Thus in the presence of an excess of Pi, phosphorylase will degrade an unbranched α-1:4-glucan completely.

Phosphorylase requires as prosthetic groups two molecules of pyridoxal phosphate per molecule of enzyme, without which it is inactive (150, 151). These are not firmly bound and can be removed simply by precipitation of the enzyme with ammonium sulphate. The function of these groups is partly concerned with the conformation of the protein; however pyridoxal and 5-deoxypyridoxal will re-aggregate the protein without restoring its catalytic activity. At acid pH's the pyridoxal phosphate is bound to the protein as a Schiff's base, which can be reduced with borohydride without destroying the activity of the enzyme. At present, with this single exception, the rôle of pyridoxal phosphate is confined to amino acid metabolism, where its requirements for activity are the reverse of those just

described. Thus pyridoxal can replace pyridoxal phosphate whilst reduction of the Schiff's base will permanently remove the enzyme's activity.

The essential difference between plant and animal glucan metabolism is very well illustrated by a comparison of the factors which were used when deciding that phosphorylase is a degradative enzyme. In animal systems an overwhelming case for this can be presented, but there is far less evidence supporting the same conclusion in plant systems.

The first factor, which has already been discussed, is that the relative concentrations of Pi and G-1-P in plant and bacterial systems would prohibit the synthesis of polysaccharides by phosphorylase. Secondly, the discovery of nucleoside diphosphate sugar-mediated synthesis has provided an eminently feasible alternative. Little else can be said for the degradative rôle of phosphorylase in plant systems, against which has to be set the work of Nelson et al. showing that the endosperm of waxy maize contains no starch synthetase.

In the case of glycogen metabolism, on the other hand, there is further evidence to support this conclusion. For example, rabbit muscle phosphorylase is inhibited by UDPG, so that when the substrate for glycogen synthesis is available the degradative enzyme

becomes deactivated (152). Muscle phosphorylase exists in two forms, one with an absolute dependence upon AMP and another which will show 60-70% of its maximal activity without AMP. The conversion of the inactive form to the active form is controlled by various hormones, such as epinephrine, and it has been shown that those agents which increase phosphorylase activity always lead to increased glycogenolysis. Even more convincing has been the observation that in addition to increasing phosphorylase activity epinephrine also decreases the activity of glycogen synthetase (126). Further support has been provided by the study of two glycogen storage diseases. Patients suffering from McArdles Disease have all the necessary enzymes for glycogen metabolism except phosphorylase, the result being that glycogen tends to accumulate in their muscle tissue to a level some 30% higher than usual (153). On the other hand, sufferers of a disease described by Spencer-Peet have no UDPG-glycogen synthetase and the liver of these people may only contain about 20% of the normal levels of glycogen (77).

To summarise; so far no regulation of the phosphorylase reaction has been described in plants comparable to the elaborate system operating in animals. There is available conclusive proof that animal phosphorylase is a degradative and not a synthetic enzyme. By comparison, it seems probable that it has a similar rôle

in plants, although it would be dangerous to take this analogy too far.

(e) The utilization of glucose-1-phosphate by plants.

When glycogen is mobilised in animals, the G-1-P which is produced is either immediately oxidised, or it is hydrolysed to form glucose, which is the principal sugar transported in blood. In plants, on the other hand, the regions wherein carbohydrate is stored frequently do not have high energy requirements, so that most of it will have to be transported to other parts. Thus it is necessary to consider the possible mechanisms whereby G-1-P could be converted into sucrose, which is the only sugar involved in translocation.

The conversion of starch to sucrose has been reported several times (75, 155), and it has been established that potato tubers are a rich source of sucrose-synthesizing enzymes (156).

In an earlier section the conversion of sucrose to ADPG via UDPG and G-1-P was examined in detail, and it was shown that the metabolic rôle of UDPG : D-fructose-2-glycosyl transferase (sucrose synthetase) is probably the formation of UDPG from sucrose prior to starch synthesis (in starch-bearing tissues only; elsewhere the enzyme may well be primarily concerned with sucrose synthesis). If this is the case, then the same enzyme and mechanism cannot be

involved in the synthesis of sucrose. An alternative route has been established, once again by Leloir and his colleagues. At the same time as their original description of sucrose synthesis, they reported a synthesis of sucrose phosphate by the reaction UDPG + F-6-P $\rightleftharpoons$ sucrose phosphate + UDP (157). This reaction was studied in detail by Mendicino, who established that the structure of sucrose phosphate was the one to be expected and that the enzyme involved was distinct from sucrose synthetase (158). He also measured the equilibrium constant for the reaction at various pH values with somewhat surprising results. He found the constant to be 3250 at pH 7.5 - in other words, formation of sucrose phosphate was almost 100%. At this same pH the equilibrium constant for sucrose synthesis is only about 5. On this basis it seems quite reasonable to assume that the principal rôle of sucrose synthetase is UDPG formation and that of sucrose phosphate synthetase is sucrose formation.

However, various authors have doubted Mendicino's results (159). The free energy change of sucrose synthesis is about 1 K.cal. so that the free energy of hydrolysis of the glycosidic bond in sucrose would be about -6.6 K.cals. (Table 2, page 21). On the other hand sucrose phosphate synthesis appears to have a free energy of about -4.9 K.cals., which means that the free energy of hydrolysis

of the glycosidic bond would be only - 2.7 K.cals. In other words, phosphorylating carbon 6 of the fructose moiety of sucrose apparently lowers the free energy of hydrolysis of the glycosidic bond by 3.9 K.cals. Only 0.8 K.cals. of this can be ascribed to the non-formation of fructopyranose. Leloir has pointed out that glycosidic linkages are generally stabilised by bulky substituents upon carbon 6. These retard the formation of an intermediate oxonium ion which is the rate-determining step of a hydrolysis. Thus α-D-xylopyranose-1-phosphate hydrolyses 1.45 times faster than α-D-glucopyranose-1-phosphate (160). Whether this effect can account for a discrepancy of about 2 K.cals. is, however, uncertain.

Despite the dubiety of the actual results, it remains clear that sucrose phosphate is synthesized by a very favourable pathway. The extent of this reaction will be directed even further towards synthesis by the concomitant removal of the phosphate group. So, in the absence of any evidence to the contrary, it is convenient to assume that, following the phosphorolysis of starch, sucrose is synthesized via sucrose phosphate.

Incidentally, if Mendicino's results are correct, the synthesis of sucrose phosphate by a phosphorolysis reaction should be quite feasible, far more so than is the synthesis of sucrose.

$$\text{G-1-P} + \text{F-6-P} \rightleftharpoons \text{sucrose phosphate} + \text{Pi} \qquad \Delta G^{\circ} \ -2.1$$

This reaction does not occur in sugar beet (161), but perhaps it would be worthwhile repeating some of the earlier work upon sucrose phosphorylase with this possibility in mind.

It is relatively easy to envisage a sequence of reactions leading up to sucrose phosphate synthesis:-

$$(\alpha\text{-}1{:}4\text{-}G)_n + Pi \xrightleftharpoons{\text{phosphorylase}} (\alpha\text{-}1{:}4\text{-}G)_{n-1} + G\text{-}1\text{-}P$$

$$G\text{-}1\text{-}P \xrightleftharpoons{\text{phosphoglucomutase}} G\text{-}6\text{-}P \xrightleftharpoons{\text{phosphohexose isomerase}} F\text{-}6\text{-}P$$

$$G\text{-}1\text{-}P + U(A)TP \xrightleftharpoons{U(A)TP\ \text{pyrophosphorylase}} U(A)DPG + PPi$$

$$U(A)DPG + F\text{-}6\text{-}P \xrightleftharpoons{\text{sucrose phosphate synthetase}} \text{sucrose phosphate} + U(A)DP$$

This sequence has been suggested several times and there is some quite good evidence available to support it. The individual reactions and the enzymes catalysing them are, of course, all well established. Viswanathan and Krishnan state that all the necessary enzymes, except for phosphorylase, can be extracted from tapioca tubers bound to the starch granule (98). Phosphorylase was present in the tuber homogenate and it is possible that the medium they used to isolate the granules may have actively promoted the desorption of this enzyme (162).

The work of Rees and Duncan (113) and that to be described in this Thesis has shown that many of the necessary

intermediates are present in isolated starch granules, a notable exception to this being UDPG. It is, of course, unjustifiable to assume that these metabolites are present in starch granules because of their association with this particular pathway rather than any other mechanism by which they might arise. On the other hand, it is difficult to envisage any other process which could explain the presence of sucrose phosphate in starch granules.

The objections to sucrose phosphate synthesis from ADPG are the same as those for sucrose. UDPG is a better precursor in both cases, whilst the uridine nucleotides are potent inhibitors of the transfer reaction (90). Although there is no UDPG in starch granules and the adenosine nucleotides predominate, the converse to this obtains in the cell fluid. It is perhaps premature at this stage to make a decision about the nucleotide precursor of sucrose phosphate.

Studies on sucrose phosphate synthesis in sugar beet have shown that the label from radioactive G-1-P is incorporated into both the glucose and fructose moieties (163). Unfortunately, the experiments necessary to determine the distribution of radioactivity following the degradation of labelled starch do not appear to have been done.

Briefly to summarise the preceding paragraphs, carbohydrate

is translocated from the reserve areas as sucrose, which is probably synthesized via U(A)DPG : 2,fructose-6-phosphate glucosyl transferase. Most of the necessary enzymes and the metabolites leading up to this reaction are present in the starch granule, but there is no definite evidence available to support the pathway.

Conclusions.

Carbohydrate is translocated in the higher plants as sucrose, which must be regarded as both the initial and the end product of starch metabolism. The processes whereby it is converted to amylose and amylopectin, and then in due season the carbohydrate remobilized as sucrose, have been examined in detail. It has been shown that all of the enzymic activities invoked are not only possible but have actually been demonstrated either in the higher plants or else closely associated with starch metabolism in other organisms.

The overall process can probably be best represented schematically in a cyclic fashion, Fig. 2. The broken line has been used to indicate a reaction where several alternatives are possible and there is no reason for choosing the one shown except that it appears to be the most feasible. From Fig. 2 it can be seen that to convert sucrose to starch requires 1.5 molecules of ATP per molecule of glucose, whilst the reverse process uses only 0.5

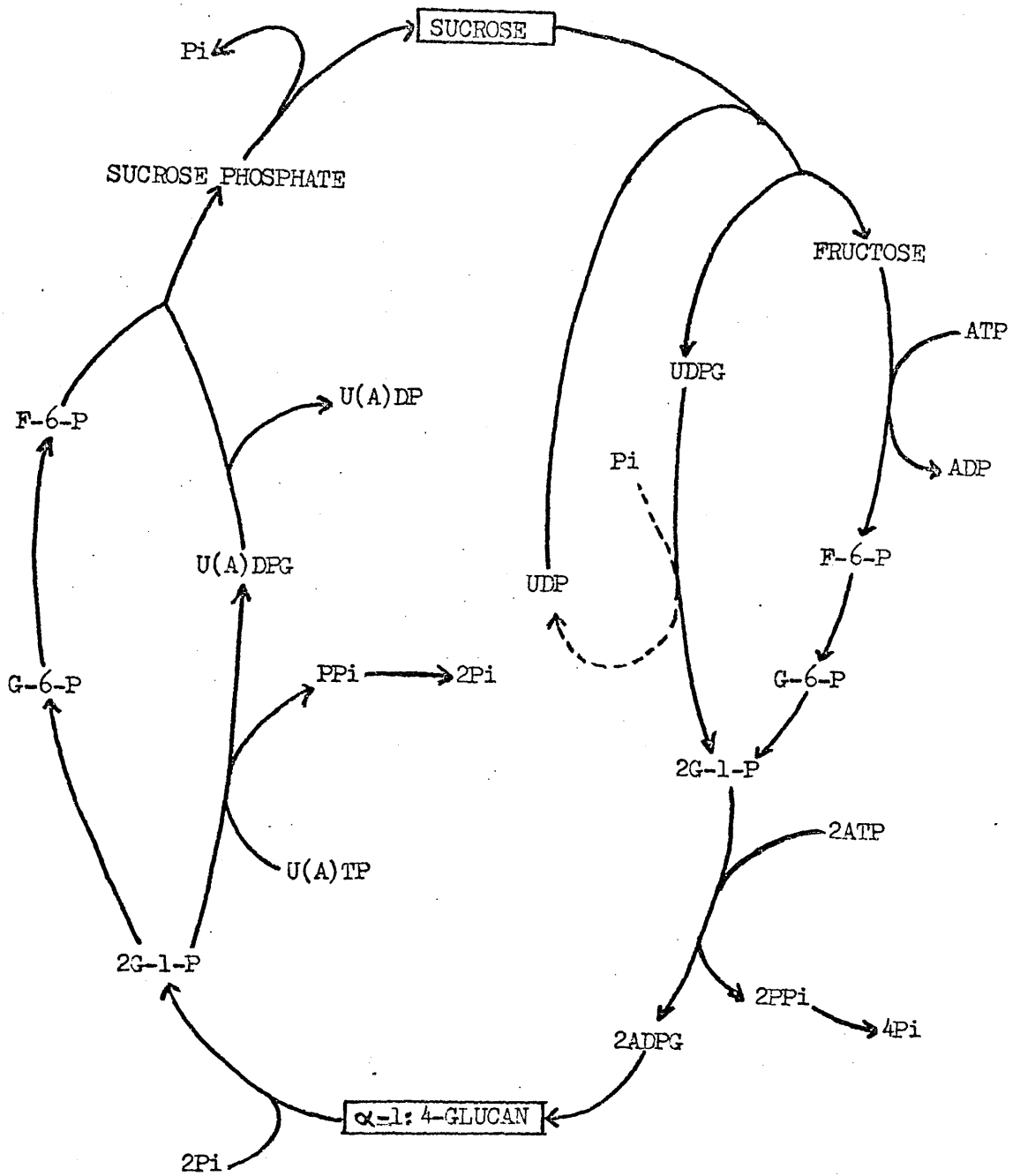


Fig. 2. The interconversion of starch and sucrose.

molecules of ATP.

A very important aspect of starch metabolism which has been entirely omitted from this description is the overall control of the process. It is possible to imagine that the arrival of sucrose in the potato tuber is sufficient to stimulate the deposition of starch. But what is the signal that prompts phosphorylase into activity when the time arrives for growth to resume? That it is under hormonal control seems certain which makes the report that giberellic acid can stimulate phosphorylase activity particularly interesting (164). Equally interesting is the possibility that it is closely connected with the metabolism of phytic acid (165). The problems of dormancy in plants have received a great deal of attention which is outside the scope of this Thesis.

Facets of the work to be described in the remainder of this Thesis have already been mentioned. An investigation of potato starch granules was undertaken to identify and estimate the concentrations of various associated mono- and disaccharides and sugar phosphates. It was thought that metabolites that could be isolated from the potato tuber still associated with the starch granules would be particularly significant in the metabolism of starch.

Discussion: Studies upon the free sugars and the sugar phosphates which can be extracted from potato tubers in association with starch granules.

In the following sections an examination of potato starch grains is described in detail. The account falls naturally into two main sections, viz. that relating to (a) the free sugars, and (b) the sugar phosphates. The analysis of the sugars and the sugar phosphates progressed concurrently, but for convenience of presentation each description is carried forward to its conclusion with little reference to the other section. The first three sections describe work done upon commercially produced starch - these are mainly concerned with the development and refinement of various techniques. Then in a final section is described the application of these techniques to potato starch freshly isolated in the laboratory, together with some discussion of the results obtained. At the risk of repetition a brief summary is presented here to give a continuity which could not be maintained throughout a more detailed description.

Potato starch grains were leached with aqueous methanol and the extract concentrated by vacuum distillation. The neutral sugars and the sugar phosphates present in this extract were then separated from each other using an anion exchange resin. The free sugars were identified by a variety of techniques, but principally

paper chromatography, and a further separation of the mono- and disaccharides was effected by charcoal column chromatography. The concentrations of the various sugars were determined colorimetrically, especial care being taken to adopt or adapt methods which gave a specific result for the sugar being examined and avoided interference from the other sugars present.

The sugar phosphates proved to be more difficult to isolate and analyse. After considerable preliminary exploration, it was decided merely to separate those esters which form a complex with borate from those which do not do so. The phosphate groups were then removed by enzymatic hydrolysis, following which the free sugars were identified and quantified. Extensive use was made of gas liquid chromatography at this stage. Finally, a selection of these techniques was applied to fresh potato starch.

Section I: The extraction of sugars and sugar phosphates from starch grains.

Because of the obvious desirability of maintaining a continuity with the work of Rees and Duncan upon the nucleotides from starch grains, there was little freedom in the choice of an extracting solvent (166). Starch grains tend to swell when suspended in water, which leads to a very slow rate of percolation when a column technique is used for extracting them, as for example is preferable in large scale experiments. An additional hazard in the use of water as an extractant is that the slow rate of desorption of the metabolites from starch grains, continuing as it does for several days, makes bacterial contamination almost a certainty. After testing a variety of aqueous mixtures of organic solvents, they eventually decided that 50% aqueous methanol was the most suitable.

An alternative method of recovering the free carbohydrates from starch grains was briefly considered. This was to cause a rapid disruption of the granules with boiling water, followed by precipitation of the dissolved starch with methanol, leaving the metabolites in solution (Expt. 1). However, because of the viscosity of starch solutions, very large volumes of methanol and water were involved. The recovery of nucleotides by this method

was less than 30% of what had been recovered from the same sample of starch by cold extraction techniques (Table 3). Because of this there was no hesitation in confirming that leaching the grains with aqueous methanol at room temperature was the most suitable method of extracting the free carbohydrates.

The choice of a batchwise or a continuous flow column technique was largely dictated by the amount of starch being extracted; the greater the quantity the more tendency there was to use a column (Expts. 2 and 3). Since it could be assumed that the elution of the sugars and the sugar phosphates would closely follow the nucleotides, the elution of metabolites from the starch was monitored by following the gradual decline of the u/v (260 mμ) absorption of the eluate from an initial level between 1 and 2 down to 0.03-0.05.

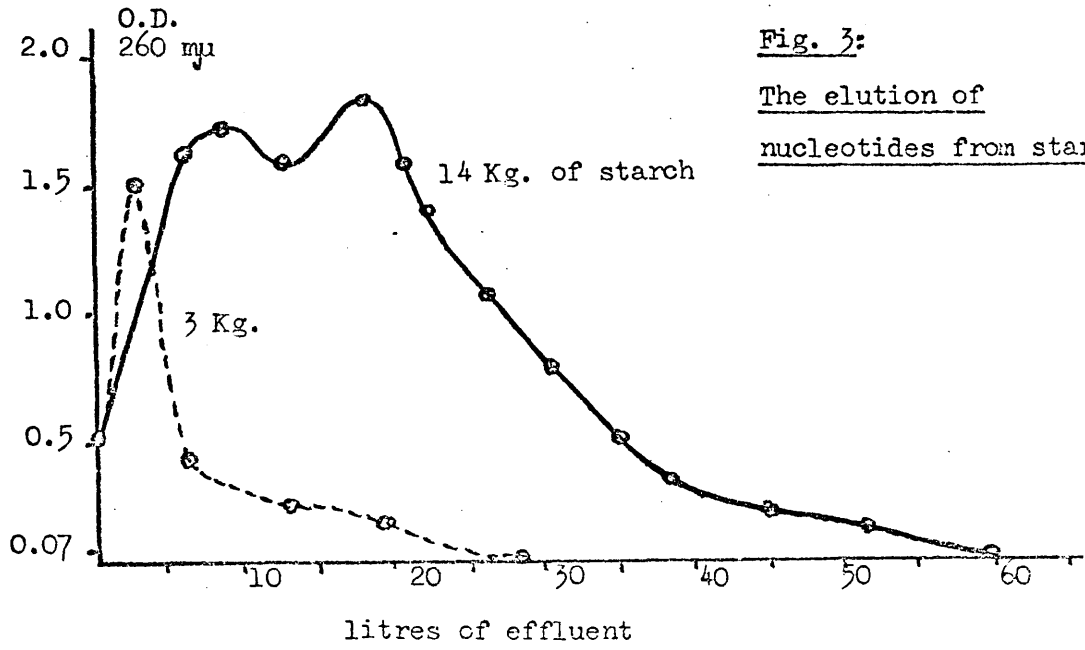


Fig. 3:
The elution of nucleotides from starch.

Greater weights of starch are extracted more efficiently than smaller ones. Thus a column containing 3 Kg. of starch required 8.1 litres of aqueous methanol to reduce the optical density of the eluate (260 mμ) to 0.07, whereas a similar column containing 14 Kg. of starch required only 4.2 litres of aqueous methanol per Kg. to produce the same diminution of the optical density (Expt. 4). The results of several extractions are summarised in Table 3.

Table 3: The extraction of starch with aqueous methanol.

Expt. Number	Weight of starch extracted Kg.	Technique	O.D.U. extracted per gram of starch	Pi	Porg.	% Pi of P total extracted	Total P in starch	% extracted
1	0.14	Boiling water	0.94	0.40	0.23	37%		
4	48.5	C	3.4	0.425	0.12	22%	15.8	3.4
5	1	C	2.6	0.625	0.175	20%	16.1	5.0
8	13.5	C	3.3	0.63	0.17	21%	20.6	3.9
21	5.2	B	3.9	1.04	0.44	30.5%		
25	10	B	3.6	0.471	0.126	27%		
27	6	B	4.15					

Concentrations expressed as μmoles per g. of extracted starch.

B = batchwise extraction.

C = column extraction.

For quantitative studies of the metabolites from starch grains to be meaningful it is necessary to be reasonably certain that they are completely extracted from the grain. Unfortunately this is to some extent an impossible ideal, for very low levels of u/v absorbing material can be leached from the grain continuously as long as elution proceeds. Thus after 1 Kg. of starch had been extracted with 15 litres of 50% aqueous methanol, the $O.D._{(260\ m\mu)}$ of the eluate was 0.035; 10 litres later it had only fallen to 0.02. Since phosphate esters are highly polar compounds it was conjectured that increasing the polarity of the extracting solvent would promote the more rapid desorption of these metabolites. This indeed proved to be the case. When the 1 Kg. of starch which had already been exhaustively extracted by 25 litres of 50% aqueous methanol was extracted with a further 10 litres of 10% aqueous methanol, the low rate of elution of the nucleotide was doubled and further significant amounts of organic phosphate were also recovered (Expt. 5). This experiment is summarised in Table 4. Reducing the methanol content of the extracting solvent did not noticeably decrease its rate of percolation through the column of starch. Equally there would still have been enough methanol present to inhibit bacterial growth.

A question which may profitably be asked at this stage is: how intimately are these metabolites associated with starch

Class of metabolite	Total extracted by aqueous methanol		% of 50% extract
	25 litres of 50%	10 litres of 10%	
$u/v_{(260\ m\mu)}$	2,600 O.D.U.	400 O.D.U.	13.3%
Pi	625 µmoles	34 µmoles	5.1%
Porg.	175 µmoles	22.5 µmoles	11.4%
Sucrose	54 mgs.	0.004 mgs.	
Glucose	10.4 mgs.	0.01 mg.	

Table 4: The extraction of starch with 50% and 10% aqueous methanol.

grains? The fact that they were incompletely removed by very large volumes of extractant would seem to suggest that at least a proportion of them are eluted from well below the surface of the grain. An experiment was performed to show that there is no difference between the metabolites leached during the earlier and middle stages of an extraction (Expt. 27). When 6 Kg. of starch were shaken with 15 litres of 50% aqueous methanol for 6 hours, the supernatant solution contained 11,152 O.D.U. of nucleotide. Following

this, the same batch of starch was shaken with three successive batches of 50% aqueous methanol, which altogether extracted 13,784 O.D.U. Further individual analyses of these two solutions showed that the same sugar phosphates were present in each. Thus it would appear that at least some of the substances extracted from starch come from deep within the granule. Any metabolite so tenaciously associated with the starch that it persists throughout the isolation of the granules and then requires many litres of solvent for its removal must surely have some significance in the metabolism of the grain.

Section II: The analysis of the free sugars which were extracted from potato starch.

1. Chromatographic identification of the free sugars.

Rapid advances have been made in carbohydrate chemistry since the introduction of partition chromatography. Before this, identification of a sugar was largely dependent upon fractional crystallization coupled with measurement of the specific rotation or the melting point of the crystals. Besides being many times more sensitive, chromatography yields a much more definite identification since by comparison with the appropriate standard compounds interference by contaminating carbohydrates is eliminated.

Separation by paper chromatography depends essentially upon differences in the partition coefficients. Four generalizations can be formulated to predict the mobilities and separations which may be obtained. Firstly, that mobility is inversely proportional to the molecular weight of the carbohydrate. Secondly, that ketoses tend to migrate slightly faster than aldoses. Thirdly, within a polymeric series there is a logarithmic relationship between the mobility of the sugar and the chain length. Figure 4 shows this relationship for the series from glucose to maltohexaose; it is probably only coincidence that glycerol with only half a unit also falls on the same line. French has commented on this relationship for

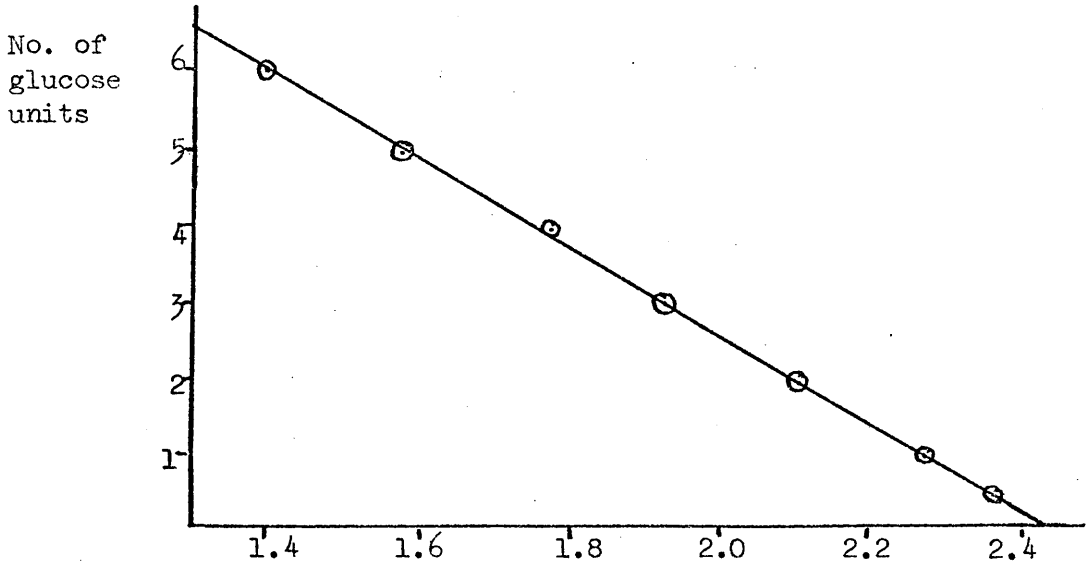


Fig. 4; The relationship between chain length and paper chromatographic mobility of the malto-oligosaccharides.

several series of carbohydrates(167). Fourthly it appears that equatorial hydroxyl groups cause a stronger adsorption of carbohydrates by the stationary phase than do axial groups. Posternak provides a good exposition of this theory in 'Les Cyclitols' wherein he points out that the inositols follow the same generalization (168).

For the rigorous identification of a carbohydrate chromatography in more than one solvent is essential and so a great many have been promulgated. Generally these fall into four classes, acid, neutral, basic and miscellaneous or solvents with a special feature such as aqueous phenol. In the present work solvents from

at least three of these classes were used to give a definite identification.

One of the advantages of paper chromatography is that many of the specific colour reactions used to identify sugars or classes of carbohydrates can be utilized on a very much smaller scale. Perhaps the most useful reagent of all is alkaline silver nitrate which appears to react with almost any compound containing vicinal hydroxyl groups (171). It will give a visible reaction with as little as 0.05 µg. of glucose although its useful application requires probably not less than 0.2 µg. It is largely because this reagent is not easily applicable to thin layer chromatography that that technique was not used for the present work.

The sugars extracted from starch were identified by chromatography in six different solvents. Table 5 shows the mobilities of various sugars relative to D-glucose and, for comparison, those of the sugars from starch alongside the appropriate standard sugar. The results were entirely consistent with the presence of sucrose, glucose, fructose, maltose and ribose (Expt.6). The presence of sucrose and fructose was further confirmed by spraying a chromatogram with α-naphthol dissolved in a mixture of ethanol and phosphoric acid which reacts visibly only with ketohexoses (172).

In the course of subsequent fractionations of the free

SOLVENT	A		B		C		D		E		F	
	C	S.E.	C	S.E.	C	S.E.	C	S.E.	C	S.E.	C	S.E.
Raffinose	31		34				40				0	
Maltose	60	62	57	55	38	34	65		84	84	0	
Sucrose	77	76	77	74	64	61	76	78	95	95	0	
Galactose	84		91		86		89		115		94	
Glucose	100	100	100	99	100	100	100	100	100	100	100	100
Fructose	118	120	119	119	130	130	100	-	143	142	245	245
Arabinose	114		122		143		113		149		470	
Mannose	118		112		122		119		100		162	
Xylose	147		129		152		117		131		555	
Ribose	183	181	159	152	187	190	121	121	176	178	1310	1400
Glycerol	195		214		284		152		223		∞	

Table 5: Mobilities exoressed relative to glucose.of standard sugars.

C = control sugars.

S.E. = starch extract.

The solvents are described in G.M.1.

sugars a solution was separated which apparently contained equal proportions of the carbohydrates identified as maltose and sucrose. These identifications were further confirmed by enzymic hydrolysis using maltase and invertase which completely hydrolysed the maltose to glucose and the sucrose to glucose and fructose. (Expt. 12)

2. The separation of the neutral sugars from the sugar phosphates and the nucleotides.

This separation was desirable because the presence of sugar phosphates would have interfered with the determination of the free sugars and also because it permitted the analysis of both classes of compound from the same sample of starch.

Anion exchange resins such as Dowex-1 will under certain circumstances quantitatively absorb phosphate esters whilst allowing the free sugars an unhindered passage. This offered a simple method of separating them and a pilot experiment was performed to demonstrate the utility of such a method (Expt. 7). The complete separation of glucose and G-1-P which this achieved is illustrated in Figure 5.

In Figure 6 is shown the separation of the free sugars and the phosphate esters in a solution from 5.4 kg. of extracted starch. It is worth noting that peak A which should have been exclusively the free sugars and other non-anionic compounds contained no phosphate at all. Similarly all the carbohydrate of peaks B and C was retained

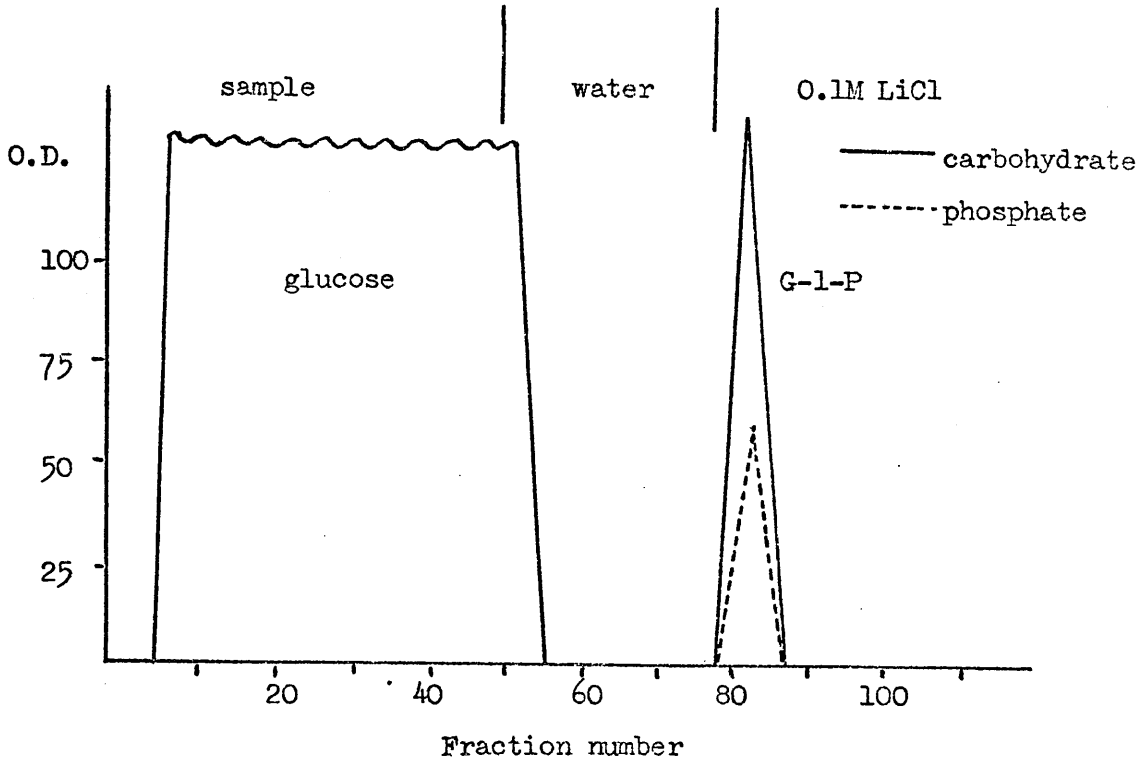


Fig. 5. A model separation of glucose and G-1-P.

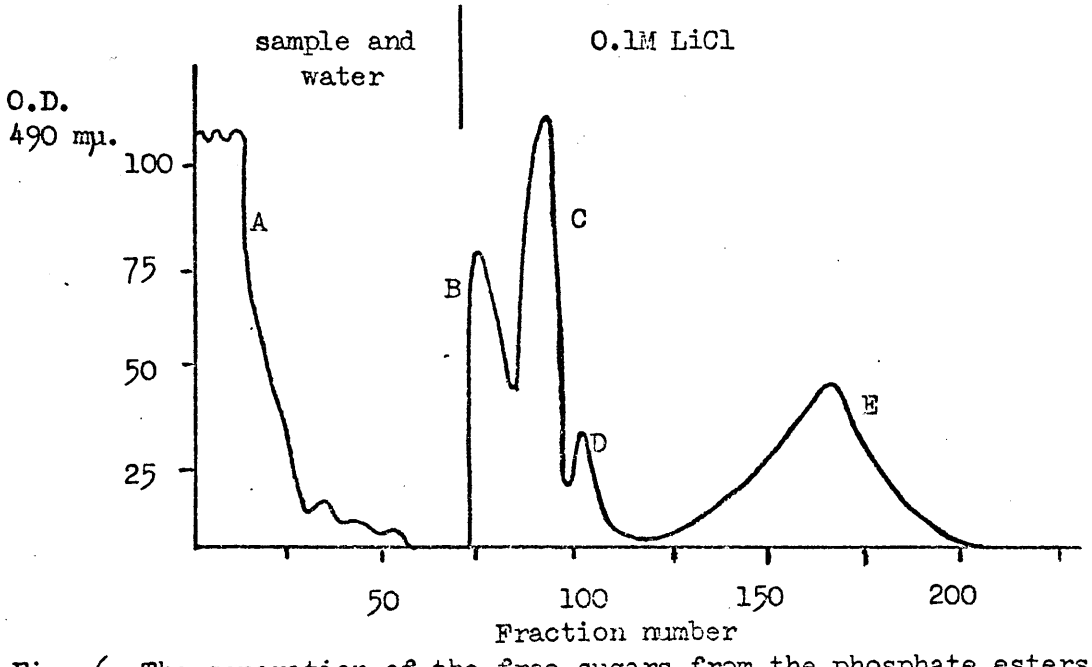


Fig. 6. The separation of the free sugars from the phosphate esters extractable from starch.

by a mixed bed resin suggesting that it was all bound to a phosphate ester and there was no free sugar present (Expt.8). There will be a further discussion of this experiment in Section III.

3. The separation of the mono- and disaccharides.

It was realised that because of their chemical similarity estimation of the mono- and disaccharides would be much simpler if they could be separated from each other. Of the various methods available for achieving this separation charcoal chromatography such as was described by Bacon appeared to be the most suitable (173).

In order to assess the potential of this method a mixture of standard sugars; fructose, sucrose, maltose and raffinose, was applied to a small charcoal-celite column (equal parts Ultrasorb S.C. 120/240 and Celite No.535) Expt.9. The column was then washed with

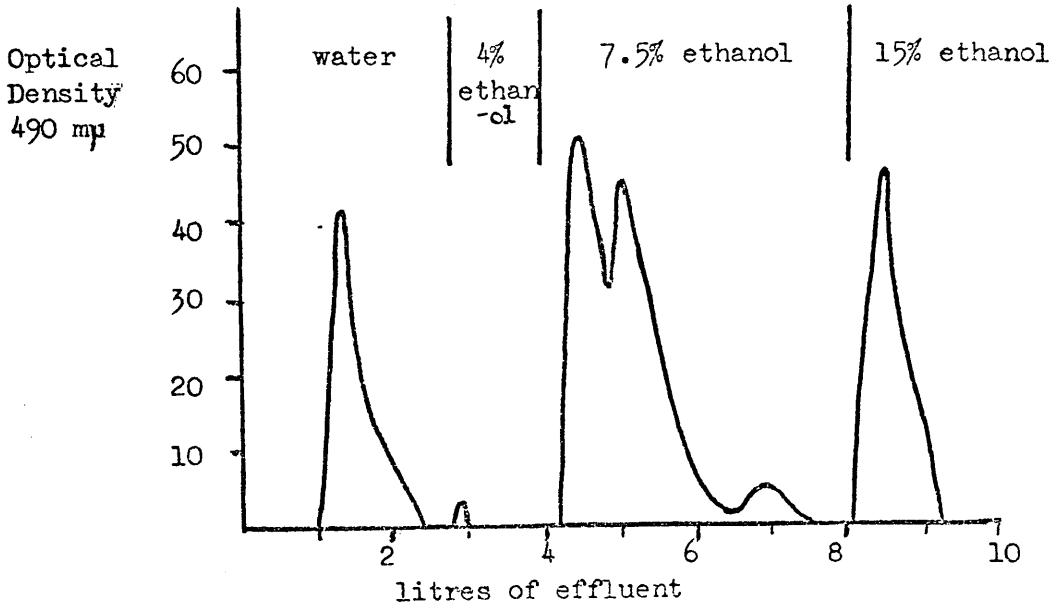
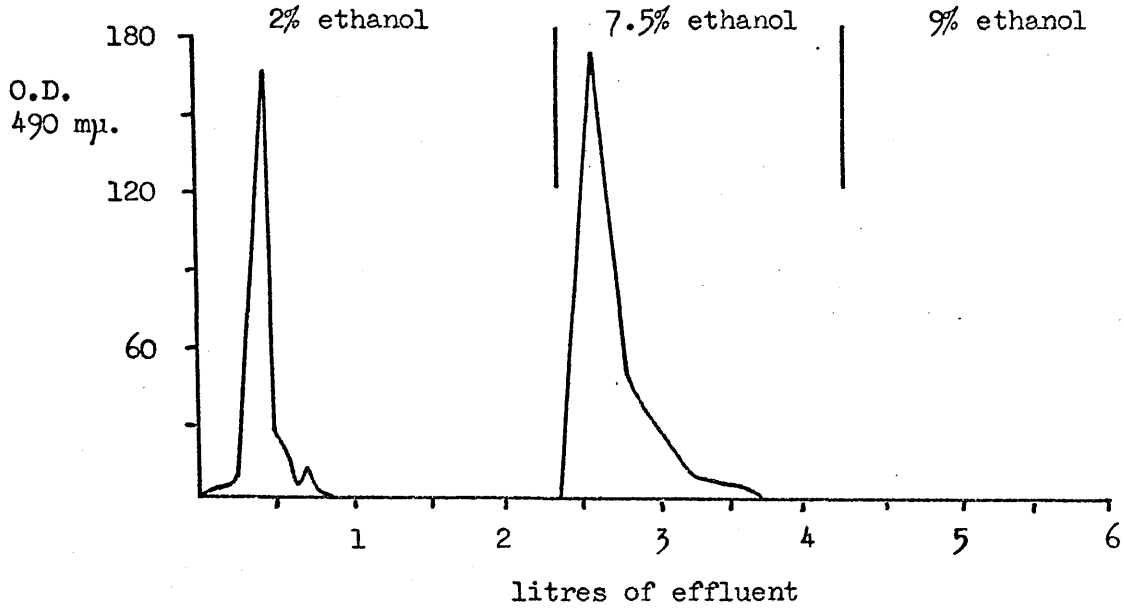


Fig.7 A model separation of the mono- and disaccharides.

water which after some delay caused the elution of fructose (Fig. 7). This was somewhat unexpected because Bacon had found that the grade of charcoal which was being used showed considerable retention of monosaccharides so that he had to use 4% ethanol to elute them. The remaining sugars were eluted as he described, sucrose and maltose with 7.5% ethanol and raffinose with 15% ethanol. The small peak following maltose may have been mellibiose arising from raffinose although this was not checked chromatographically.

With slight modifications this method was used to separate the mono- and disaccharides from 960 g. of extracted starch (Expt. 10). A histogram of the elution pattern can be seen in Figure 8 together with a summary of the mode in which the eluate was divided up for further examination. From this figure it can readily be seen that the monosaccharides were retained by the charcoal only to a small extent and that the main bulk of these was eluted within a volume of 450 ml. The trailing edge of this peak (Fraction III) contained traces of glucose, fructose and ribose as was to be expected but, judging from the spot produced upon paper chromatograms, the major component was a slow-running carbohydrate (solvent (a) Rg = 26, cf. maltotriose 29). Various tests were performed attempting to ascertain the nature of this carbohydrate but because of the limited amount remaining after the concentrations of glucose, fructose and ribose had been measured



Fraction number	Position of elution(ml)	Sugars present in fraction	Volume of fraction	Weight of starch/ml. of fraction
I	0-250	no detectable carbohydrate	-	-
II	250-700	glucose, fructose and ribose	500 ml.	1.88 g.
III	700-1500	traces of glucose, fructose and ribose, also unknown carbohydrate.	25 ml.	38.3 g.
IV	1500-2450	no detectable carbohydrate	-	-
V	2450-2750	sucrose	500 ml.	1.88 g.
VI	2750-4000	sucrose and maltose	100 ml.	9.6 g.
VII	4000-6000	barely discernible traces of sucrose.	-	-

Fig. 8: The separation of the mono- and disaccharides of potato starch.

a complete identification was not possible (Expt.11). However the following information was garnered which suggests that it was unlikely to have interfered with the concentration studies.

1. It was not retained by anion exchange resin and so it is unlikely to have been a uronic acid, a phosphorylated or a sulphated sugar.
2. It was retained by Ultrasorb charcoal to a greater extent than the monosaccharides but considerably less than sucrose, generally the most mobile of the disaccharides.
3. From its paper chromatographic mobility it was probably not a simple monosaccharide.
4. It gave a visible reaction with both silver nitrate and periodate-benzidine reagents which suggests that it contained at least two vicinal hydroxyl groups.
5. It gave no visible reaction with either aniline-phthalate or benzidine-TCA reagents suggesting that it contained neither a reducing group nor an acid labile linkage which could give rise to a reducing group.
6. It gave no visible reaction with α-naphthol in ethanol-phosphoric acid solution, thus eliminating ketoses.

These qualifications tend to suggest a polyhydroxylated low molecular weight compound such as a cyclitol (myo-inositol, Rg=41). However

since it remained unidentified there could be some uncertainty about the measured concentrations of Fraction III. It will be seen later that because of the low concentrations in this Fraction compared with Fraction II any such uncertainty has very little effect upon the final results.

4. The measurement of the concentrations of the partially isolated sugars.

This phase of the work was concerned with determining the concentrations of glucose, fructose and ribose in the presence of each other and similarly maltose and sucrose. Individually these sugars would have presented no problems since each one could have been determined by a general carbohydrate reagent such as phenol sulphuric acid (169). This was clearly not possible with a mixture of sugars. Thus it was necessary to select from amongst the many that have been promulgated, techniques which were capable of the required specificity.

The practices of colorimetry need no description. Generally the arbitrary values from the galvanometer of the colorimeter are related to concentration by means of a previously determined standard curve. However this introduces several unnecessary variables. Firstly, the absorption of light by many chromophores changes with time, often rising to a maximum and then

decaying away. Using rigorously defined conditions and accurate measurement of time this difficulty may perhaps be circumvented but it is frequently impossible to achieve this ideal, particularly since a finite time is required to measure the intensities. Secondly, there is deterioration of the reagents used, although this might be avoided by preparing fresh reagents each time. Thirdly, there is the possibility of changes in the response of the instrument used, which in a busy teaching laboratory is more likely than might be imagined. These difficulties were all eliminated in the present work by preparing a fresh standard curve each time an accurate estimation was required. At least two estimations of each sugar were made with several samples of solution being subjected to each estimation.

Fructose and the fructose moiety of sucrose were determined using the resorcinol technique (170). So far as is known this reaction is specific for ketohexoses. Glucose and ribose, the two monosaccharides present with fructose in the solutions being examined gave no visible reaction with as much as 400 µg. of sugar and, equally, varying amounts of glucose or ribose did not cause any variation in the response to fructose (Expt. 13). The estimations of Fractions II, III, V and VI are described in Expt. 14.

There is apparently only one reagent extant which is satisfactorily specific for the determination of glucose, that of

Salomon and Johnson (174). This is a complex reagent which links the oxidation of glucose with the reduction of ortho-tolidine hydrochloride, producing a blue chromophore; as the commercial preparation "Clinistix" it is widely used in hospitals for testing urine. Unfortunately the author was not aware of its existence when this work was carried out although it was employed several times in the later stages.

Attempts were made to measure glucose concentration by the cysteine-sulphuric acid reaction wherein a solution of carboyhydrate is heated with sulphuric acid and then L-cysteine hydrochloride added to produce a yellow colour (175). Pentoses form a chromophore with maximal absorption at 390 mμ, which contributes to the absorption of glucose at 414 mμ. Assuming that the absorbance peak is symmetrical as was suggested by Dische, the absorption of pentoses should be equal at 366 mμ and 414 mμ. Thus any extra absorbance of a solution at 414 mμ should be attributable solely to the glucose present. This was not in fact found to be the case. Isochromatic wavelengths for pentose at 414 mμ were observed initially at 358 mμ, only rising to the expected 366 mμ after one hour, whilst after several hours the isochromatic wavelength approached 380 mμ. (Expt. 15).

However, despite this expedient, the effect of the pentose is not entirely eliminated for it also inhibits the development of the hexose chromophore. Two standard curves were drawn for concentrations

of fructose between 10 µg. and 100 µg., one with no ribose and the other containing decreasing amounts of ribose as the fructose concentration increased. Convergent lines were produced; in other words the less pentose there was present the more closely did the absorbance due to fructose approach the theoretical value.

Another artifice which was briefly considered was the addition of a large excess of ribose with the reagent so that the small amount present in the solution being examined would make little difference. However this was impossible because the intensity of the colour so produced would have been too great to be accurately measured.

The best technique seemed to be to measure the ribose present in each solution being examined and then to adjust it so that they all contained the same concentration of ribose. Having done this, standard curves of hexose concentration can be prepared under the same conditions by the addition of a fixed amount of ribose to each sample.

Fructose is reported to give 112% of the response of glucose and this was borne out by experimental observation.

In order to estimate glucose therefore the following protocol was drawn up.

1. Estimate the pentose concentration of each solution and adjust it to the same value for each one.
2. Estimate the fructose concentration of each solution.
3. Using the cysteine-sulphuric acid reaction draw up standard curves for glucose plus ribose and for fructose; estimating the unknown solutions at the same time.
4. Calculate the interference of the fructose and subtract it from the total optical density. Then compare the residual value with the standard curve of glucose plus ribose.

When this protocol was followed with a sucrose solution it proved impossible to correlate the concentrations of glucose and fructose. It was realised that this technique could not yield reliable results and that an alternative would have to be devised.

The enzyme D-glucose aerodehydrogenase (GADH, E.C.1-1-3-4) specifically oxidises β-D-glucopyranose to form D-glucono-1:5-lactone (176). Used in conjunction with the Somogyi-Nelson technique for measuring reducing power it offered a specific and relatively sensitive method for estimating glucose.

In order to establish the conditions necessary for complete oxidation a progress curve was plotted for a typical reaction (Fig.9). As can be seen there was a very rapid initial fall in the reducing power which then continued to diminish slowly for several hours.

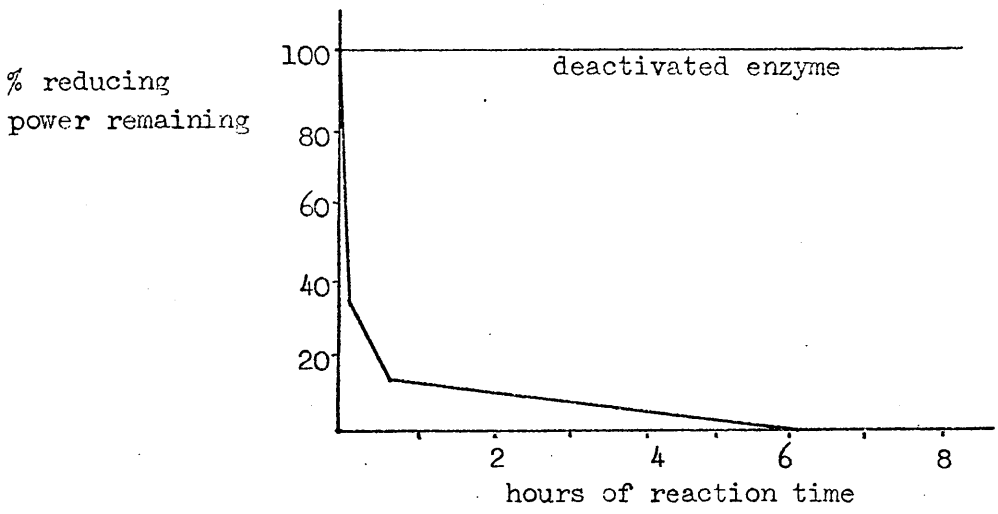


Fig. 9: Progress curve of GADH action.

As an incubation period of 15 hours was contemplated these conditions were satisfactory and no attempt was made to improve them (Expt. 16).

It was crucial for this estimation that GADH should show no activity with fructose or ribose. This was shown conclusively by digests set up containing various sugars, the reducing power of which was compared with similar solutions wherein the enzyme was replaced by water (Table 6). The GADH preparation also contained maltase and invertase activities which accounts for the decreasing reducing power of the maltose solution and the increasing reducing power of the sucrose solution.

The Somogyi-Nelson method for estimating reducing power is one of the most unsatisfactory techniques of those available to the biochemist and yet potentially one of the most useful (177, 178). Rigorous care is needed to exclude errors and even then it is

Sugar	Optical density (640 mμ)	
	No enzyme	With enzyme
Glucose	71	0.5
Fructose	68	73
Ribose	57	56
Maltose	31	1
Sucrose	0	41
Blank	0	1.5

Table 6: The effect of GADH. on the reducing power of various sugars.

advisable to make three or more estimations of each sample. Various means of improving the method have been described, but frequently accuracy is only gained at the expense of convenience (179). The principle upon which it is based is the reduction of an alkaline copper complex forming cuprous oxide, which in turn reduces arsenomolybdic acid, giving rise to molybdenum blue. The main sources of error are variable reduction of alkaline cupric tartrate and its re-oxidation by air. The principal precautions adopted for the present work were the consistent use of thoroughly

deaerated solutions, keeping the reaction under an atmosphere of nitrogen until the arsenomolybdate had been added, and keeping the solutions in iced water except during the heating period.

Eventually the glucose concentrations of Fractions II and III (Fig. 7) were estimated by the cysteine sulphuric acid and the GADH.-Somogyi-Nelson techniques (Expt. 17).

Ribose concentration was determined by a modification of the technique using phloroglucinol proposed by Dische and Borenfreund (180). Pentoses form a chromophore with a maximal absorbance at 552 mμ. Hexoses contribute to the absorption at this wavelength, but their interference can be compensated for by dichromatic readings at 552 mμ and 510 mμ, and also by adding 0.35 mgs. of glucose per sample in the reagent. This, it is argued, is a sufficient excess to minimise the errors produced by any hexose in the solution being examined. However, in Fractions II and III there was about 160 μg. of hexose per ml. compared with only about 10 μg. of ribose. These relative concentrations would have produced a large error in the result sought (Expt. 18). Accordingly a small variation was made in the method. The compensating glucose was omitted from the reagent and instead a calculated quantity of glucose, equal to the hexose in the solution being estimated, was added to each sample of ribose solution used when drawing up the standard curve (Expt. 19).

Maltose concentration in the presence of sucrose was estimated by three different, albeit similar methods. Firstly advantage was taken of the reducing group of maltose to measure its concentration by the Somogyi technique. Secondly, an aliquot of the disaccharide solution (Fraction VI, Fig. 8) was incubated with equine maltase (55), and then the reducing power of the solution correlated with that of a standard glucose solution, again using the Somogyi technique. Both these methods depended upon there being no hydrolysis of the labile sucrose linkage. To check that this was indeed the case, the maltose and sucrose were hydrolysed and then the total glucose concentration estimated as before. Knowing how much of the glucose would have arisen by hydrolysis of the sucrose, this could be deducted from the total so that the remainder must have arisen by hydrolysis of the maltose, the concentration of which could then be deduced. There was only a 2% difference in the results obtained by the last two methods (Expt. 20).

The results of these estimations are summarised in Table 7. It is interesting to note that the concentrations of fructose and glucose were almost identical. This could suggest that these monosaccharides only arose by hydrolysis of sucrose, there being no alternative individual source. The significance of this will be commented upon more fully later on.

SUGAR	µg. extracted/g. of starch
fructose	12.0
glucose	11.9
ribose	3.9
sucrose	71.2
maltose	4.2

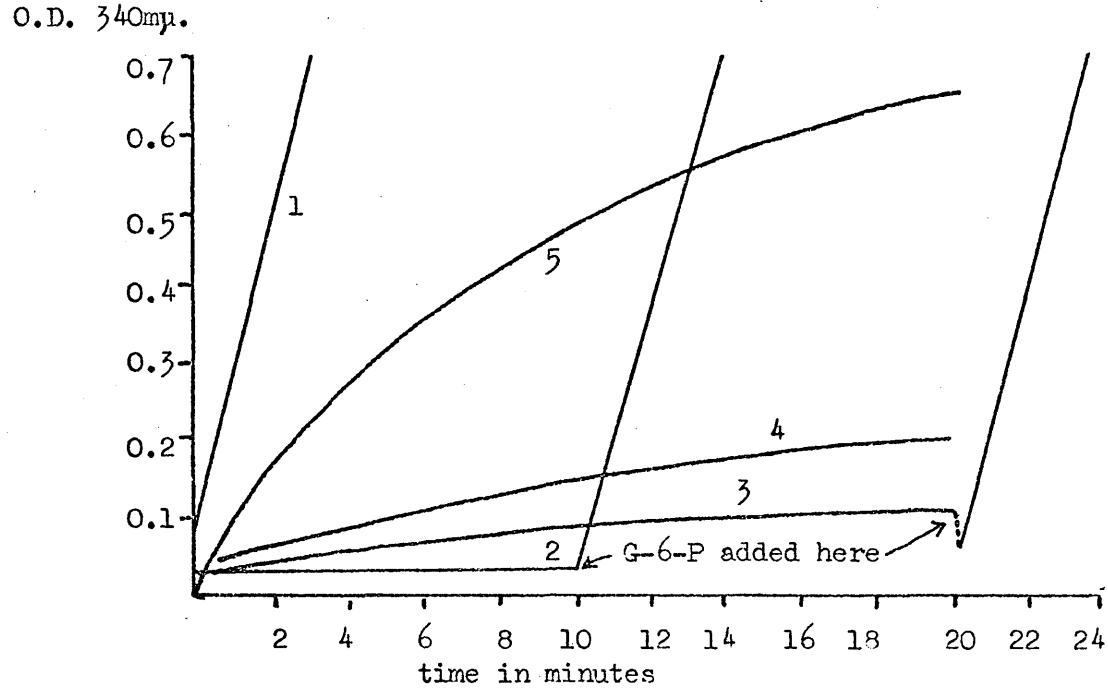
Table 7: Concentrations of the free sugars extracted from starch.

Section III: The analysis of the sugar phosphates which were extracted from potato starch.

1. Preliminary studies.

Before embarking upon an involved programme of ion exchange separations and chromatographic analyses it was decided to seek definite evidence for the presence of sugar phosphates in starch granules. The enzyme, D-glucose-6-phosphate dehydrogenase will catalyse the oxidation of G-6-P concurrently with the reduction of $NADP^+$. The course of this latter reaction can be followed by observing the increasing absorbance of the nucleotide at 340 mμ (181). Since this enzyme is relatively specific for G-6-P it offered a facile method of demonstrating the presence of at least one sugar phosphate in starch granules.

The solution obtained by concentrating the aqueous methanol extract of potato starch would not itself give rise to any reduction of $NADP^+$, under conditions in which standard G-6-P was rapidly oxidised (Fig. 10). However after precipitating the inorganic phosphate with magnesia solution a slow but distinct reduction was observed. The phosphate esters were then separated from the magnesia solution by anhydrous methanol-acetone precipitation. During the course of this separation it was noticed that a portion of the esters were insoluble in anhydrous methanol and it



Curve number 1) Standard G-6-P (4 μmoles)
2) Concentrated 50% aqueous methanol extract of starch.
3) Same solution after precipitating the Pi.
4) Methanol soluble phosphate esters from starch.
5) Methanol insoluble phosphate esters from starch.

Fig 10. The reduction of $NADP^+$ with G-6-P extracted from potato starch granules.

was found that when it had been segregated this insoluble portion could give rise to a rapid reduction of $NADP^+$. This demonstrated that G-6-P was present in isolated starch granules and so by inference were other sugar phosphates.

2. Anion exchange chromatography of sugar phosphates: initial work.

Whilst studying the characteristics of phosphoramidate:

hexose phosphotransferase and D-glucose-1-phosphate:D-glucose-6-phosphotransferase the author had developed techniques for separating sugar phosphates by anion exchange chromatography (182). A sequential elution of sugar phosphates from an anion exchange column of Dowex-1x2 was achieved with a convex gradient elution which gave an increasing chloride ion and a decreasing borate ion concentration. This system of course did not differ radically from the one originally suggested by Khym and Cohn (183) and even less from later improvements of their method such as that of Tarr and Leroux (184). However it gave a good separation of various sugar phosphates particularly G-1-P, G-6-P and F-6-P, from each other and from inorganic phosphate. It was anticipated that these sugar phosphates were very likely to be found in starch granules and so this system appeared to be especially applicable to the present work.

Two minor changes were necessary before it could be utilized. Firstly the resin which was available had a higher degree of cross linking than that previously used, 4% divinyl benzene instead of only 2%. For this reason alone a recalibration of the system was necessary and this seemed a good point at which to introduce a further modification. It is possible to separate sugar phosphates from lithium chloride in the same manner as that habitually used for nucleotides, namely precipitation of their lithium salts by anhydrous acetone from a methanolic solution. This isolation step

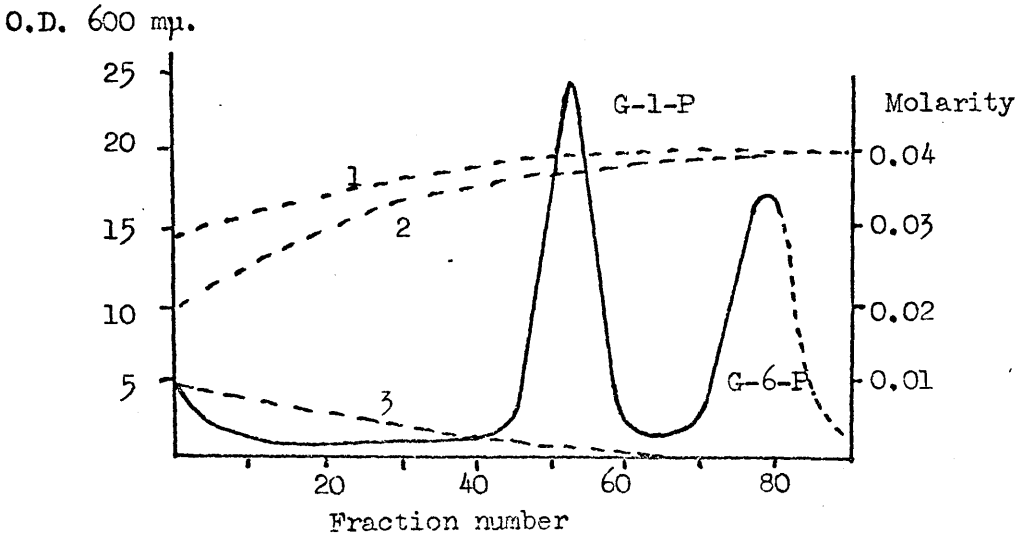
would be simplified if organic phosphates could be eluted from the resin by lithium rather than ammonium salts, and this it was resolved to do in future.

In a model experiment an attempt was therefore made to separate a mixture of G-1-P and G-6-P upon a column of Dowex-1x4, Cl^-, using a convex gradient elution with lithium chloride and lithium borate in the mixing chamber and lithium chloride in the reservoir (Expt. 22) The strength of the eluting solution at any one point was calculated from the formula

$$C=Cr-(Cr-Cm)(1-n/N)^{Ar/Am}$$

where C is the concentration of fraction number n,where there are N total fractions. Cr is the concentration of the solution in the

Fig. 11. The elution of standard sugar phosphates from Dowex-1 Cl^-.



Curve 1, total anion concentration. Curve 2, chloride ion concentration. Curve 3, borate ion concentration.

reservoir having a surface area of Ar, and similarly, with the mixing chamber, Cm and Am. This is merely a simplification of the formula proposed by Pontis and Blumson (185). It had been intended to extend the gradient with a little more of the reservoir solution, but a breakdown of the fraction collector made this pointless (Fig.11). The separation of the G-1-P and the G-6-P was not as wide as in the previous system and they were eluted noticeably later, which was most unsatisfactory. Accordingly it was decided to investigate an alternative method.

A method of separating sugar phosphates has been described which uses Dowex-1 in the borate form and di-triethyl ammonium tetraborate (TEAB) as the eluting salt (186). This promised to give good separations and easy recovery of the sugar phosphates. To assess its potential, a column was run which duplicated the conditions described in reference 186 (Expt. 23). G-1-P and G-6-P were well separated as sharp peaks with practically no tailing (Fig.12a). The fractions which constituted each peak were bulked and evaporated to dryness. Three co-evaporations with methanol sufficed to remove all of the eluting salt.

A second triethyl ammonium borate column was run which, in addition to G-1-P and G-6-P, also separated F-6-P (Fig. 12b). Once again sharp discrete peaks well separated from each other were

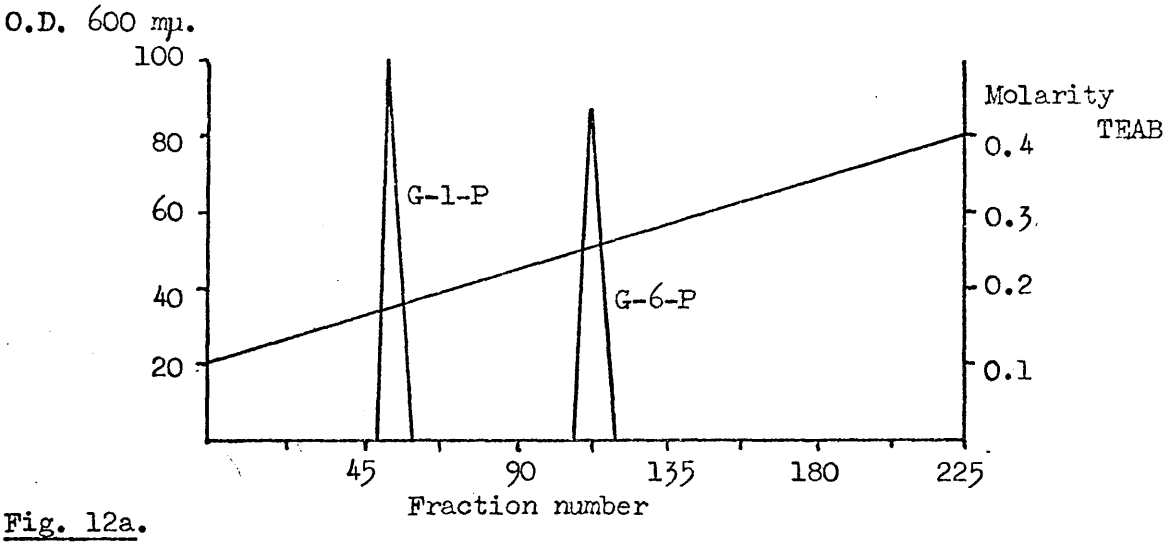


Fig. 12a.

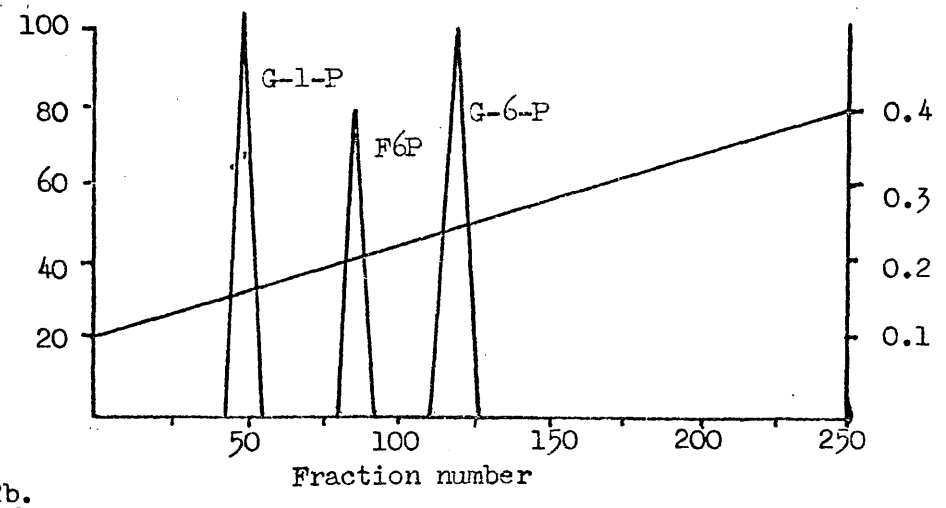


Fig. 12b.

	Molarity of eluting solution		
	Ref. 186	Expt. 23	Expt. 24
G-1-P	0.18-0.19	0.175	0.16
F-6-P	0.21-0.22		0.20
G-6-P	0.29-0.30	0.250	0.245

Fig. 12: The elution of sugar phosphates from Dowex-1 borate by TEAB.

obtained. G-1-P and F-6-P were eluted in almost the same positions as those shown in the paper cited but G-6-P broke through earlier (Expt. 24). On the basis of these two experiments it was decided to adopt the triethyl ammonium borate system for the separation of the sugar phosphates from starch.

3. Purification and isolation of phosphate esters.

A complication arising from the decision to use the triethyl ammonium borate system was the need to remove the free sugars which occur in extracts of potato starch. The success of this system as in any other ion exchange separation depends upon limiting the number of ion exchange sites involved at any one time. The ability of sugars to complex with borate is well known and it is recorded that this system will retain fructose almost to the same extent as it does inorganic phosphate. Thus hexoses occupying anion exchange sites upon the column will reduce the total capacity available for phosphate exchange.

The folly of attempting a separation without removing the free sugars from a starch extract was quickly demonstrated (Expt. 25). Assaying the eluate for carbohydrates revealed a single large peak and nothing which could reliably be ascribed to a sugar phosphate (Fig. 13). The only effect of using more starch extract would have been to broaden this peak to an intolerable extent.

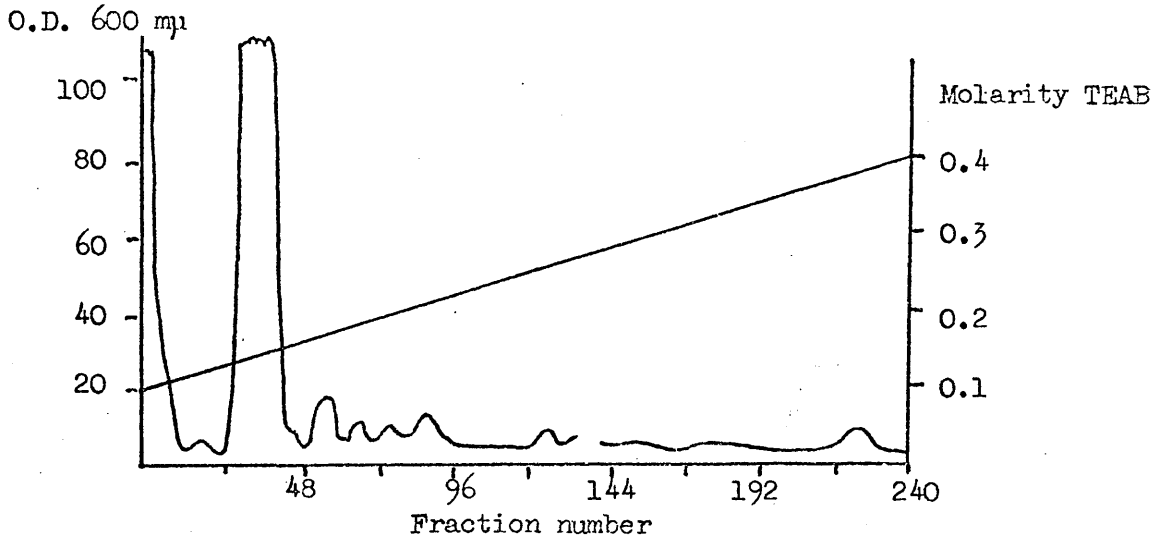


Fig. 13. Attempting to separate the sugar phosphates from starch without prior removal of the free sugars.

The method which was developed for separating the free sugars from the sugar phosphates has already been described in Section II (Expt. 7). It depended upon the absorption of the phosphate esters by an anion exchange resin which had no tendency to absorb the free sugars. The advantages of this method were that the capacity of the column was dictated solely by the amount of phosphate present and that the eluting solutions were so radically different that there should have been no possibility of the two classes of metabolites not separating. It was regretfully noticed that in several experiments a very small proportion of the organic phosphate was eluted with the free sugars; the reason for this is not understood.

Because inorganic phosphate is eluted from the standard

triethyl ammonium borate columns very close to G-1-P it was felt desirable to reduce its relatively high concentration in starch extracts. Various methods for doing this were considered such as precipitating the triethyl ammonium complex of phosphomolybdic acid (187) or forming an insoluble complex with β-stannic accid. It was eventually decided to precipitate magnesium ammonium phosphate with magnesia solution. However, this reagent leaves in solution such a high chloride ion concentration that it could prevent the retention of phosphate esters by ion exchange resin. A possible way of overcoming this anionic problem was to precipitate the inorganic phosphate and then to exchange the magnesium and ammonium ions for lithium ions using a cation exchange resin. The phosphate esters could then be recovered from the lithium chloride solution by anhydrous methanol-acetone precipitation. This method was tried upon a test solution of Pi and G-1-P and also Pi and G-6-P mixed in the proportions 8 : 1, a higher concentration of inorganic phosphate than was extracted from starch granules (Expt. 26). In both cases the recovery of the organic phosphate was 100% and the removal of the inorganic phosphate 98.6%. This was quite satisfactory and so without further modification the method was adopted for the work to be described below.

4. The analysis of the sugar phosphates extracted from potato starch, based upon separations using standardised triethyl ammonium borate columns.

A possibility which was considered at this stage was that these metabolites may be confined exclusively to the surface of the starch granule. If this were the case then they ought to be almost entirely extracted by shaking once with aqueous methanol. If, on the other hand, they permeate deeply into the grain then repeated extractions should each recover fresh material. This is known to be the case with nucleotides and so it is likely that it is also the case with the sugars and the sugar phosphates. However the possibility remains that there may be some metabolites which are confined to the surface of the granule. The experiment which was performed to investigate this possibility has already been mentioned in Section I (Expt. 27).

Potato starch was extracted with 50% aqueous methanol by end-over-end tumbling, the first solution being segregated and analysed separately from the solution produced by combining the three subsequent extractions. The two solutions were concentrated and found to contain respectively 1.86 and 2.29 O.D.U. (260 mμ) per gram of starch extracted. The sugar phosphates were isolated from each solution by absorption onto an anion exchange resin, followed by elution with dilute lithium chloride solution. The inorganic

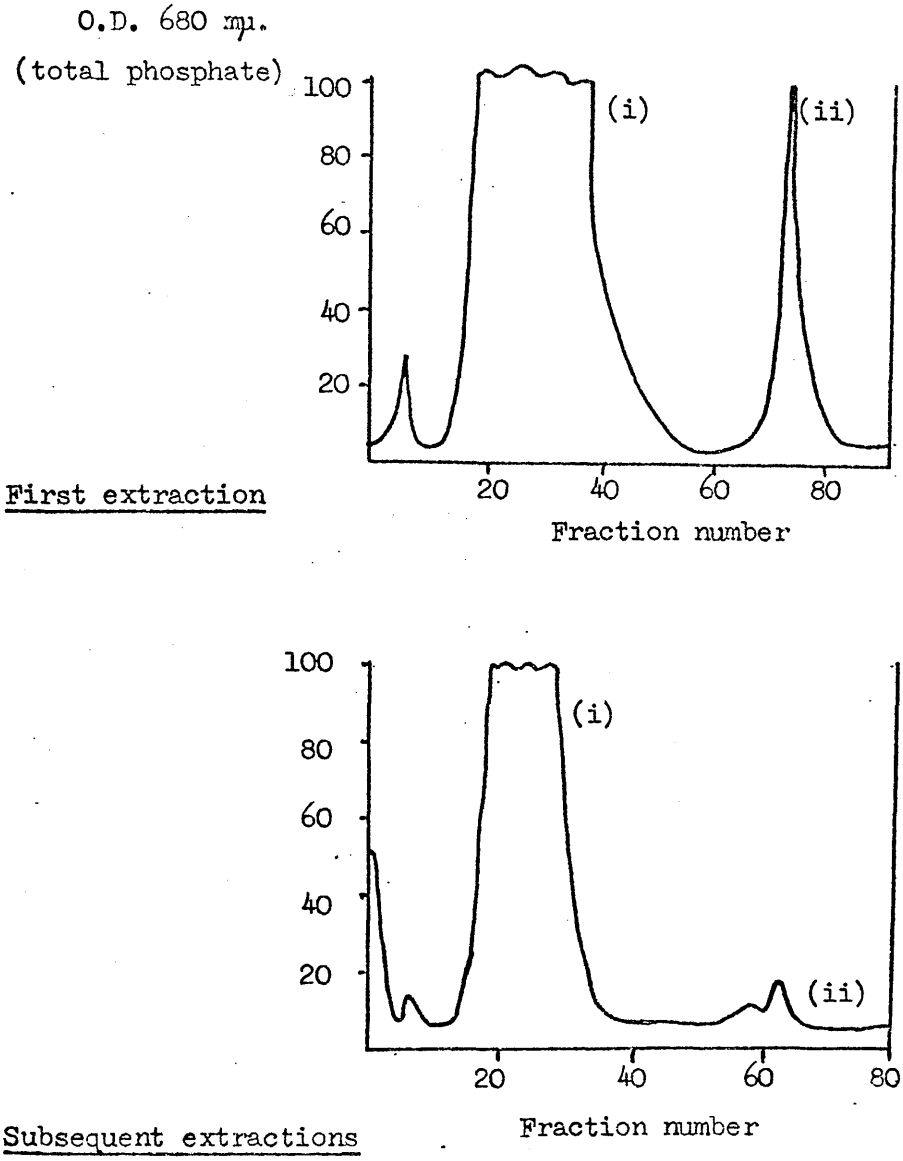
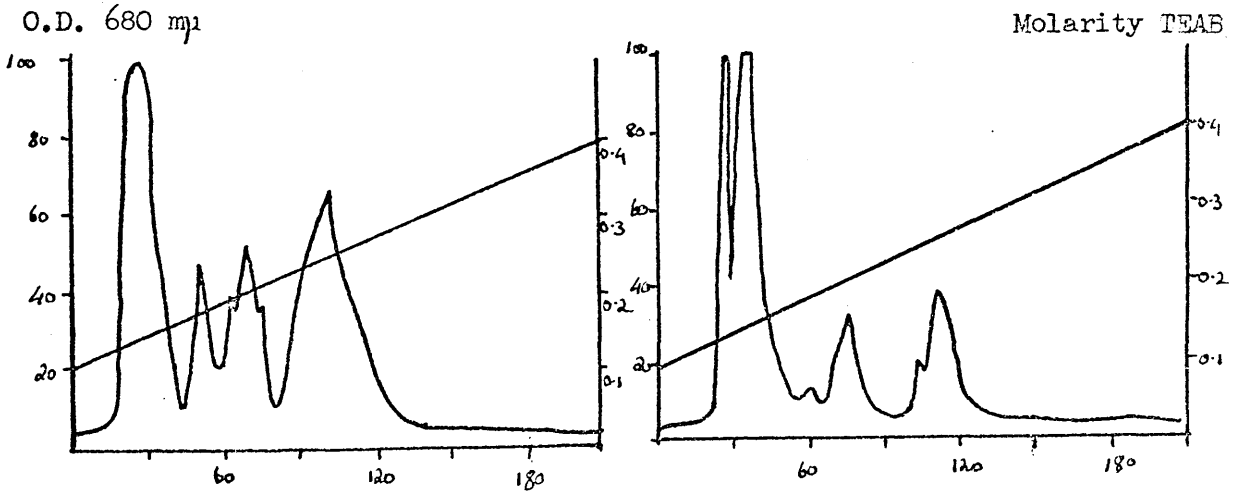


Fig. 14a: Bulk separation of the free sugars from the sugar phosphates showing the elution of the phosphates with 0.1M LiCl (peak i), the free sugars are not shown.



First extraction. Subsequent extractions.

Peak number	Identity by TLC	Molarity of elution		Observed position pilot experiments
		First	Subsequent	
1	?	0.14	0.145	
2	Pi	0.15	0.16	
3	G-1-P	0.175	0.185	0.175, 0.16
4	F-6-P	0.200	0.200	0.20
5	G-6-P	0.231	0.250	0.250, 0.245

Fig. 14b: Elution of sugar phosphates from Dowex-1 borate by TEAB.

phosphate was precipitated and the organic phosphates recovered in the manner described (Expt. 28). The two fractions were found to contain 0.039 μmoles and 0.029 μmoles of organic phosphate per gram of extracted starch, altogether 0.068 μmoles/g. It is interesting to note that there was more sugar phosphate in the first extraction than in the subsequent ones whilst the distribution of the u/v absorbing material was just the opposite. The sugar phosphates were then chromatographed upon standardised triethyl ammonium borate columns. Histograms of each elution pattern can be seen in Figures 14a and 14b, together with a summary of the sugar phosphates identified.

The drastic reduction in the size of peak ii (Fig. 14a) would appear to support the hypothesis upon which this experiment is based, that there can be differences in the distribution of metabolites about the starch granule. However this peak did not form a part of the sugar phosphate fraction and so it was not examined further. The peaks eluted from the triethyl ammonium borate columns (Fig. 14b) were examined by thin layer chromatography (TLC.) and auxiliary techniques(Expt. 29) and some of the sugar phosphates identified. The size of peak 3 was noticeably reduced in the subsequent extractions. This peak could be resolved into three separate spots by TLC., one of which was identified as G-1-P and the other as traces of inorganic phosphate. All three spots however

appeared in both preparations so the diminution in size cannot be ascribed to the disappearance of one of the components.

This experiment provided clear indications of the presence in starch granules of G-1-P, G-6-P and F-6-P amongst other sugar phosphates. It also suggested that they are distributed throughout the grain and not merely confined to the surface. At this stage the limited amount of material originally separated had been exhausted so that a fresh preparation was necessary.

Once again the sugar phosphates were separated from the free sugars (Expt. 8). However, this time elution of the resin with 0.1M lithium chloride was prolonged until the nucleotide monophosphates were eluted (peak E, page 66). This served to demonstrate that the organic phosphate isolated as sugar phosphate was not substantially contaminated with nucleotides. The sugar phosphates were partially separated into two peaks (peaks B and C) but these were subsequently recombined and treated together. All of the carbohydrate in these peaks was retained by a mixed bed resin, which suggested that none of it was uncharged free sugars and so by inference must have been sugar phosphates. The behaviour of uronic acids in these circumstances is not known and of course they too may have been present in peaks B and C.

The starch which was extracted contained originally 20.6 μmoles of phosphate/g. of starch. Of this phosphate 0.8 μmoles

(3.7%) was extracted with 50% aqueous methanol. Of this extractable phosphate 0.63 μmoles were inorganic and 0.17 μmoles organic phosphate. The sugar phosphate fraction comprised 47% of the organic phosphate (Expt. 30).

A rapid indication of the sugar phosphates which were present was sought by an acid hydrolysis of a portion of the fraction followed by paper chromatography of the free sugars (Expt. 31). Comparatively mild hydrolysis resulted in the formation of glucose, glycerol and myo-inositol, together with traces of fructose. Increasing the severity of these conditions merely increased the intensity of the spots without giving rise to any further sugars. So, in addition to the demonstrated presence of glucose and fructose phosphates, there was also glycerol and myo-inositol monophosphate.

The inorganic phosphate was then removed by precipitation with magnesia solution and the sugar phosphates isolated from the magnesia by anhydrous methanol-acetone precipitation. Then 96 μmoles of organic phosphate were separated upon a standardised triethyl ammonium borate column, giving the elution pattern seen in Figure 15 (Expt. 32). After removing the triethyl ammonium borate from the fractions constituting each peak by coevaporation with methanol the sugar phosphates were examined by paper and thin layer chromatography. (Expt. 33). With these it was possible to confirm what was suspected

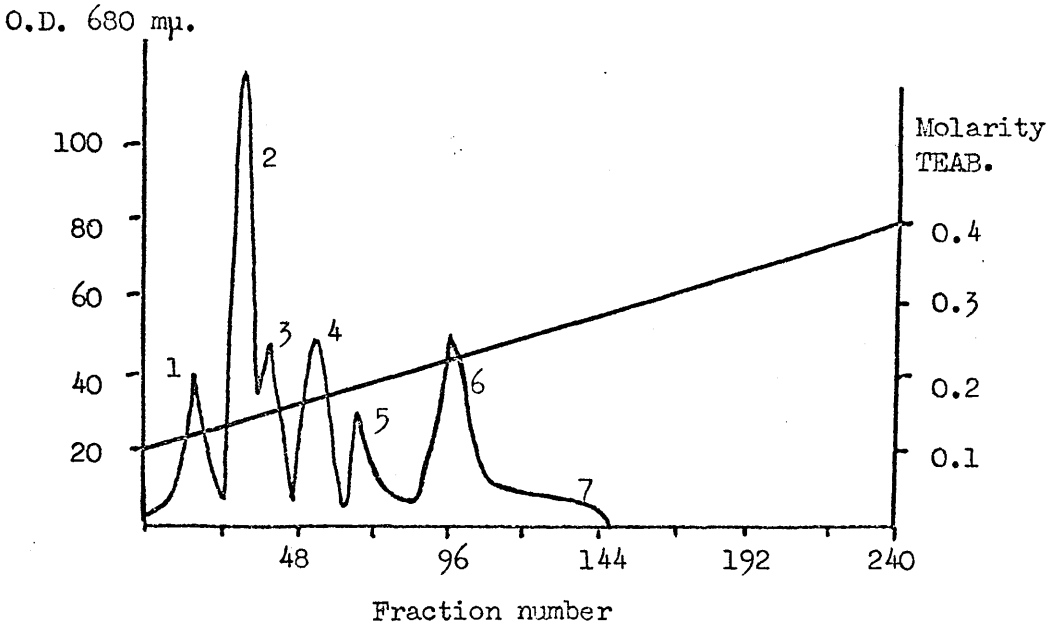


Fig. 15. The elution of sugar phosphates from Dowex-1 borate by TEAB.

upon the basis of previous experience and the elution pattern. Peaks 2 and 3 contained amongst other things Pi and G-1-P; peak 5 contained F-6-P (this was confirmed by positive reactions with two ketose specific reagents); peaks 6 and 7 both consisted of G-6-P.

However there were three peaks completely unidentified and all seven peaks usually showed more than one spot with both thin layer and paper chromatography, not all of which could be identified. In an attempt to identify these other sugar phosphates resort was made to acid hydrolysis coupled with paper chromatography of the sugars. The hydrolysates of peaks 2, 4, 5, 6, and 7 all contained glucose and similarly myo-inositol appeared in peaks 1, 2, 4 and 5. There were

also traces of xylose, ribose, galactose, glycerol, fructose and two unidentified carbohydrates. To some extent this complexity may have been due to the severity of the hydrolysis conditions, 1M hydrochloric acid at 100° for three hours, causing reversion of the sugars. However it suggested that there had been little or no real separation of the sugar phosphates and that the various peaks in the elution pattern were largely fortuitous.

It had been intended to measure the concentrations of each sugar phosphate by estimating the amount of organic phosphate present in each peak. However it was clear that the techniques which were being used were incapable of adequately resolving complex mixtures of sugar phosphates such as occur in starch granules. Furthermore the small amount of material which could be handled at one time imposed serious limitations upon the subsequent examinations. Because of these difficulties it was reluctantly decided that perhaps the triethyl ammonium borate system was not suitable for the present analysis and that an alternative would have to be found. Despite these drawbacks the method had yielded some information. The presence in starch granules of G-1-P, G-6-P and F-6-P had been demonstrated several times, together with strong indications of the additional presence of myo-inositol monophosphate and glycerophosphate.

Digression: A note concerning the difficulties of partition chromatography of sugar phosphates.

For any system of partition chromatography to be effective it must fulfil several criteria.

a) It should be convenient and relatively fast, able to resolve complex mixtures reproducibly.

b) There should be at least one reliably reproducible method of developing the chromatograms.

c) There should be available a wide range of standard compounds which are absolutely pure.

It can fairly be said that none of these conditions are satisfactorily fulfilled for sugar phosphates. Paper chromatography is severely limited by the fact that the standard reagent for developing chromatograms, acid molybdate spray, causes the paper to disintegrate (188). Other reagents are either less sensitive or do not reveal all of the phosphate esters. Thin layer chromatography of course is not limited in this way; on the other hand aqueous solvents such as those of Leloir and Paladini (189) or of Bandurski and Axelrod (190) cause the thin layer to separate from the glass chromatoplates. Because of this it is necessary to use different solvents for the two methods, relatively non-aqueous ones for TLC and aqueous solvents for paper chromatography. Those described by

Waring and Ziporin for TLC (G.M.2, solvents m and n) were found generally to give useful separations and discrete spots (191). As these solvents are relatively non-polar the mobility of phosphate esters was found to be low and it was advantageous to run each chromatogram more than once.

However it was the non-fulfilment of the third criterion which eventually led the author to abandon chromatography of sugar phosphates. The standard phosphates commercially available are generally limited to the more commonly occurring ones. Those which are available tend to be heavily contaminated with other phosphate esters. It proved possible to resolve commercial F-6-P into no fewer than six discrete spots, any one of which could have been authentic. Similarly α-D-G-1-P prepared in the laboratory would occasionally, but by no means always, give three spots. Because of this it was anticipated that little, if any, confidence would be felt in identifications achieved by partition chromatography of phosphate esters.

There is available a wide range of free carbohydrates in a pure condition and many systems for chromatographing them which are facile, reliable and reproducible. Therefore, hydrolysis of the sugar phosphates and identification of the released sugars seemed to be preferable to studying them intact. In addition to acid hydrolysis already mentioned enzymatic hydrolysis with acid and alkaline

phosphatases was used extensively. Both enzymes were purchased commercially, the acid phosphatase being a wheat germ preparation and the alkaline phosphatase obtained from intestinal mucosa. The latter enzyme contained about 10% inorganic phosphate which was removed by dialysis against 0.015M magnesium acetate at pH 6.8 (192). Solutions of these enzymes remained active for about two weeks when stored at 2°, their activity usually being checked by observing the hydrolysis of phenyl disodium orthophosphate. No evidence was found to suggest that there was any isomerisation of the various sugars during hydrolysis.

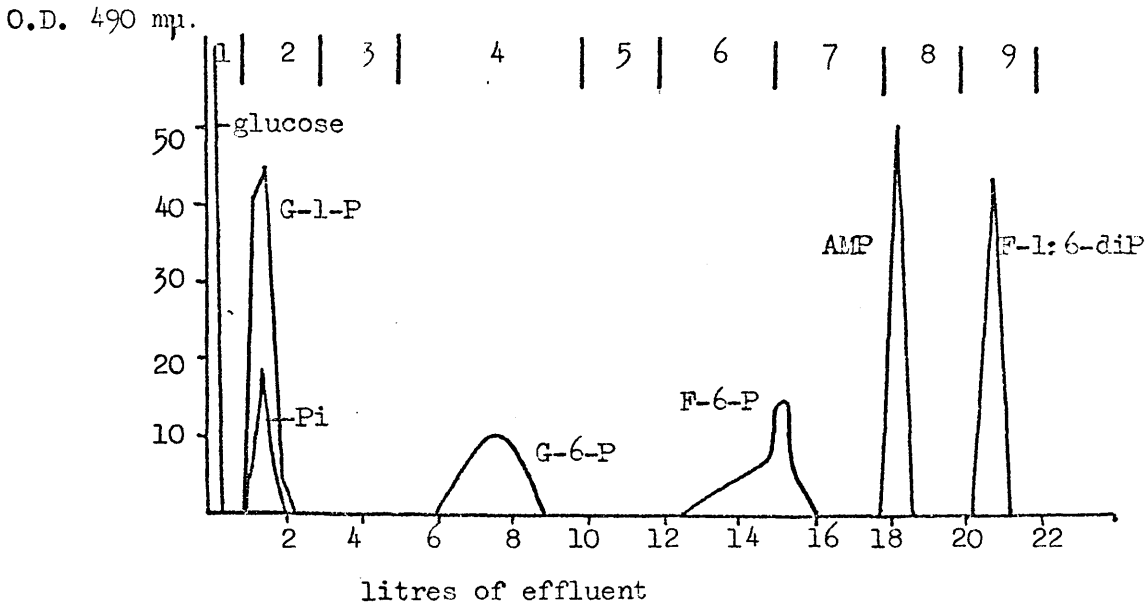
5. The anion exchange separation of sugar phosphates using a stepwise chloride elution system.

In one of the earliest separations of mixtures of sugar phosphates Khym and Cohn used a chloride system with just sufficient borate present for complexing them (183). The sugar phosphates were eluted sequentially by decreasing the borate concentration and lowering the pH stepwise whilst maintaining the anion concentration at more or less the same level. The disadvantages of this procedure are that it is cumbersome and that some of the peaks tend to become very broad. On the other hand it is possible to prolong the use of any single solvent indefinitely so that there is a far greater separation between each peak and the overlapping of separable peaks should be

virtually eliminated. This is obviously a desirable asset for quantitative work.

An advantage particularly applicable to the present work is that chloride forms of anion exchange resins do not react with free sugars (Expt. 7). These therefore should not interfere with the separation of sugar phosphates so that their preliminary removal was no longer necessary.

Khym and Cohn used eluting solutions of ammonium salts but for ease of recovery of the sugar phosphates lithium salts were preferable. Thus a pilot experiment was undertaken attempting to separate a series of standard sugar phosphates upon a column of Dowex-1x4, Cl^-, using lithium chloride and lithium borate solutions. A direct replication of their conditions proved impossible, for one thing lithium hydroxide is a stronger base than is ammonium hydroxide. Also it was found that when solutions were prepared which, as closely as possible, duplicated those used by Khym and Cohn they were not capable of eluting the appropriate sugar phosphates as the sharp, discrete peaks so characteristic of Khym and Cohn's separations (Fig. 16). The elution of G-6-P as a broad shallow peak suggested that slightly higher chloride concentrations were necessary, e.g. 0.03M lithium chloride instead of 0.025M ammonium chloride. A fuller description of the small modifications which were introduced



Details of eluting solutions.

1. 10^{-4}M LiOH, pH 10.0.
2. 0.025M LiCl, 0.04M H_3BO_3, pH 9.0
3. 0.025M LiCl, 0.004M H_3BO_3, pH 9.0
4. 0.025M LiCl, 0.004M H_3BO_3, pH 8.5
5. 0.025M LiCl, 0.00004M H_3BO_3, pH 8.1
6. 0.03M LiCl, 0.00004M H_3BO_3, pH 7.9
7. 0.03M LiCl, pH 6.5 (unbuffered)
8. 0.005M HCl
9. 0.02M LiCl, 0.02M HCl.

Fig. 16. The stepwise elution of standard sugar phosphates from Dowex-1 chloride.

during the course of the experiment to give the satisfactory separation which was achieved is recounted elsewhere (Expt. 35).

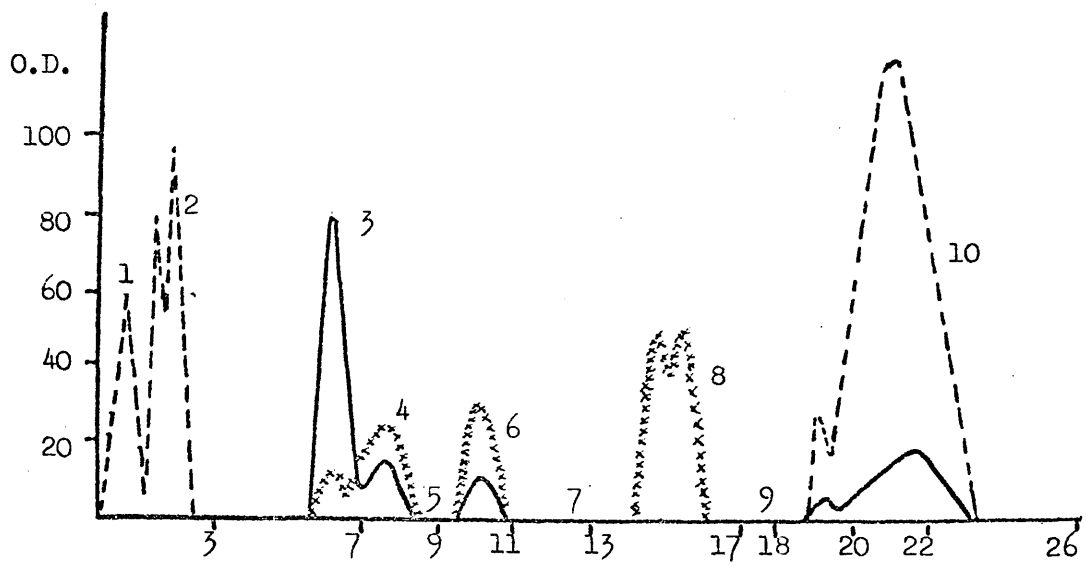
The phosphate esters of each peak were identified by a series of simple colorimetric tests and their percentage recoveries roughly determined by comparing the amount of phosphate in each peak with the weight of the ester which had originally been applied to the column. This was not in fact a very accurate method for estimating the recoveries because it was known that several of the compounds were contaminated with other phosphates, particularly inorganic phosphate. This was reflected in the recoveries which were obtained which, with the exception of inorganic phosphate (148%) were all slightly less than 100%, F-6-P being the worst with only 89% recovered. (Partition chromatography had suggested that the F-6-P which was used was also the most impure preparation.)

This therefore represented a good method for separating various sugar phosphates. Although G-6-P and F-6-P required large volumes of solvent to elute them the separation of these and of the other esters had been absolute. It was realised that the elution pattern could easily be improved by a judicious adjustment of the eluting solutions. Accordingly it was decided to adopt this method for future analyses of the sugar phosphates that were extracted from potato starch granules.

6. The analysis of the sugar phosphates extracted from potato starch involving separations using a chloride based ion exchange system.

An aqueous solution of the 50% aqueous methanol extract of 10 Kg. of potato starch was prepared and the inorganic phosphate precipitated with magnesia solution. The organic phosphates were recovered by anhydrous methanol-acetone precipitation; recovery of the organic phosphate was 100%, of the inorganic phosphate, 7% and of the u/v absorbing material (260 mμ) 36% (Expt. 36).

The preparation was percolated through a column of Dowex-1x4, Cl^- which retained all the phosphate. The free sugars and nucleosides were readily washed from this column with water (10^{-4} M LiOH). Continued washing caused the removal of two more u/v absorbing peaks, the second of which also showed some carbohydrate. The sugar phosphates were eluted from the resin by chloride and borate solutions similar to those already described. These are listed in Figure 17 (Expt. 37). The fractions collected from the column were bulked together so that each peak was segregated. The apparently void fractions between the peaks were also collected on the grounds that they may have contained phosphate at levels below the limits of detection but which would become apparent when the solutions were concentrated. The phosphate concentration in each of these large fractions was estimated and it was noted with satisfaction that almost all of the inorganic phosphate was in peak 3. The



——————— O.D. 680 mμ. Total phosphate.

- - - - - - - - O.D. 260 mμ. x10^2.'Nucleotides'.

+++++++++++ O.D. 490 mμ, carbohydrate

Volume of effluent passed over column	Composition of solution
0 - 3 litres	10^{-4}M LiOH, pH 10.0
3 - 7 litres	Linear gradient, 0 to 0.025 M LiCl and 0.04M H_3BO_3, pH 9.0
7 - 9 litres	0.03M LiCl, 0.04M H_3BO_3, pH 9.0
9 - 11 litres	0.03M LiCl, 0.004M H_3BO_3, pH 8.5
11 - 13 litres	0.03M LiCl, 0.0004M H_3BO_3, pH 8.5
13 - 17 litres	0.03M LiCl, 0.00004M H_3BO_3, pH 7.9
17 - 18 litres	0.03M LiCl, pH 6.5
18 - 20 litres	Linear gradient, 0.03 to 0.05M LiCl
20 - 22 litres	0.05M LiCl
22 - 26 litres	0.005M HCl, pH 2.7

Fig. 17. Ion exchange separation of the sugar phosphates extracted from potato starch.

preliminary experiments had shown that Pi should be eluted in this position so this offered an early indication that the separation being attempted was successful. The results from these estimations are summarised in Table 8, where it can be seen that peak 3 also contained most of the organic phosphate. The sugar phosphates of this and of the subsequent fractions were hydrolysed with both acid and alkaline phosphatase and the carbohydrates identified by paper

Peak Number	Porg µmole	Pi µmole	Carbohydrates in hydrolysate	
			Major	Minor
1, 2	0	0	-	-
3	200	150	myo-inositol	glycerol, sucrose glucose, fructose xylose
4	28	2	glycerol	sucrose, glucose fructose, myo-inositol xylose
5	8	2	myo-inositol glycerol	sucrose, glucose fructose, xylose
6	23	3	myo-inositol	glucose
7	13	10	glucose	myo-inositol, fructose sucrose, glycerol xylose
8	3	5	-	inositol, glucose fructose, sucrose xylose
9	4	8	-	inositol, glucose fructose, glycerol sucrose, xylose
10	9	5	AMP (solvent n)	-

Table 8: Summarising the analyses of the various peaks shown in Fig. 17.

chromatography in four separate solvents. Myo-inositol and glycerol were the predominant carbohydrates, but glucose, fructose and sucrose were also present together with traces of xylose. When similar quantities of each peak were chromatographed without prior hydrolysis there were very faint traces of sucrose, glucose and fructose shown to be present, although not in any way comparable with the levels present after enzymic hydrolysis. There was no difference apparent in the hydrolysates of the different enzymes. However it was somewhat disturbing that peaks 3 to 9 all contained myo-inositol and sucrose for this indicated that this separation technique was no more successful than the previous one. There seemed to be little difference between the successive fractions except that as the concentration of organic phosphate became less so the chromatographic spots became proportionately fainter. It is tempting to suppose that peak 6 was G-6-P and that G-1-P had been eluted entirely with peaks 3 or 4. However there was no justification for assuming that G-1-P behaved any differently from sucrose phosphate.

This lack of definition of the sugar phosphates complicated the estimation of their concentrations. It had been hoped to do this by a combination of phosphate determinations and the techniques used for the free sugars. However, these methods would not have distinguished between G-1-P and G-6-P or between F-6-P

and sucrose phosphate. .Notwithstanding these objections it was decided to follow this method and at least gain an indication of the sum of the glucose phosphate and ketose phosphate concentrations. For this there was little point in estimating the concentrations of each fraction individually so an aliquot of each was mixed together to give a solution theoretically containing all of the sugar phosphates from 2 kg. of extracted starch. This was hydrolysed with acid phosphatase until no more organic phosphate remained and then the concentrations of glucose, fructose and xylose determined. This was the first time that the GADH-peroxidase-ortho-tolidine reagent was used for estimating glucose; fructose was of course estimated by the resorcinol reagent and xylose by the same phloroglucinol reagent that had been used for ribose in Section II (Expt. 38). The results of these estimations are summarised in Table 9.

Unlike the sugars, glycerol and myo-inositol do not possess carbonyl groups which could facilitate colorimetric determination and no direct methods which were sufficiently sensitive and accurate could be found. For inositol the choice appeared to be between a graded bacteriological response (193) and iodometric titration (194), both applicable only upon a millegram scale. Glycerol could be estimated by oxidising it with periodic acid and measuring the formaldehyde produced colorimetrically (195). Myo-

Carbohydrate phosphate	Concentration m.μmoles/g. of starch
glucose phosphates	0.61
pentose phosphates	2.0
fructose + sucrose phosphate	2.8
myo-inositol monophosphate	2.3
glycerophosphate	2.8

Table 9: The concentrations of the various sugar phosphates extracted from starch.

inositol, it was felt could possibly be estimated by following the consumption of periodate spectrophotometrically since it has a chromophore with maximal absorption at 225.5 mμ. This proved to be the case for there was a linear relationship between the optical density of a periodate solution and the quantity of inositol which had been oxidised in that solution (Expt. 40).

However, because periodic acid reacts with most carbohydrates, it was essential that the glycerol and myo-inositol should be separated from each other and from the sugars arising by hydrolysis of the sugar phosphates. This was done by preparative paper chromatography in two stages, isolating first the glycerol and then the myo-inositol (Expt. 41). After subjecting a known amount

of myo-inositol to this treatment, 96% of it was recovered from the filter paper.

The method used for estimating glycerol is a slight modification of the one originally proposed by Ryley (195). This had the effect of doubling its sensitivity. During these preliminary studies it was noted that carbohydrates such as glucose or ribose interfered with the estimation of glycerol which showed that its isolation was necessary (Expt. 42). The estimated concentrations of glycerol and myo-inositol are presented in Table 9.

The results summarised in Table 9 are a little unsatisfactory. Visual comparison of paper chromatograms, admittedly a most unreliable method, had suggested that glycerol and myo-inositol together were the most abundant of the carbohydrate phosphates. Similarly sucrose, glucose and fructose formed the majority of the other carbohydrates, whilst pentose was only present in negligible amounts. From the values shown in Table 8 it can be calculated that peaks 3 to 9 contained organic phosphate equivalent to 28 mμmoles per gram of extracted starch. However only 10.5 mμmoles of this is accounted for in Table 9, merely 37%. Although there may be a small amount of doubt about the precise levels of glucose and pentose phosphates the fact remains that around 63% of the organic phosphate was unaccounted for. This would seem to suggest that there are other, hitherto unsuspected, components present in these fractions.

However beyond the obvious fact that they are organic monophosphates and that they are unlikely to be other carbohydrate phosphates it is difficult to conjecture what their nature might be. As will be seen however a similar result was obtained from a later analysis of the sugar phosphates from starch, when only 34% of the organic phosphate in these same fractions was accounted for.

7. Discourse.

Why were the carbohydrate phosphates not entirely eluted as sharply defined peaks as was desirable? Why do esters like sucrose phosphate appear in almost every fraction? Several hypotheses will be advanced to explain this behaviour but, with the exception of the last one, they all lack experimental proof and must remain tentative.

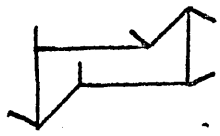
Firstly, the eluting anion concentration may have been too low. To replace phosphate by chloride upon an anion exchange resin requires a certain minimal concentration of chloride. Below this level there can be little or no ion exchange, whilst the more the concentration of chloride exceeds this minimum the more efficient the exchange process becomes. However chloride concentrations around the threshold level replace phosphate inefficiently so that many litres of solution may be required for a complete elution, viz. peak E, figure 6. For lack of the appropriate standard compounds it was not

possible to calibrate this system with myo-inositol monophosphate, glycerophosphate or sucrose phosphate so it may have been that the anion concentration which was used, 0.03M LiCl, was close to their threshold concentrations for ion exchange. For carbohydrates which do not complex with borate such as those mentioned, this effect would be aggravated by the decreasing concentration of the borate anion which has some exchange effect.

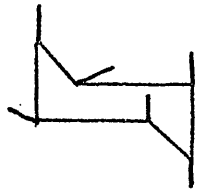
A complicating factor is that isomeric sugar phosphates differ in their ability to form complexes with boric acid. In glucose only the hydroxyls of carbon one and carbon two are involved in borate complexing, thus G-6-P forms a complex with boric acid whilst G-1-P does not do so (196, 197). To remove the G-6-P-borate complex from a resin requires a higher anion concentration or a lower borate concentration than does uncomplexed G-1-P. Thus glucose phosphates are eluted by a wide range of solutions.

A third possibility is that changes may occur in the conformation of carbohydrate phosphates which normally do not complex with borate, so that they form conformers which do so. An equilibrium mixture of myo-inositol and borate contains no more than 0.02% of complex (198). Epi-inositol forms a strong tridentate borate complex by an inversion of its conformation to give an unstable chair form having four axial hydroxyl groups (Fig.18, I, II, III)

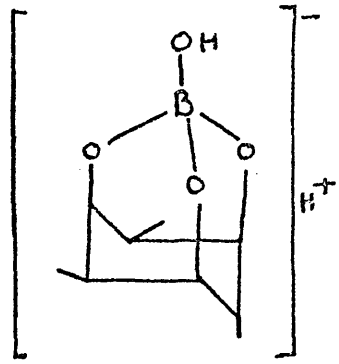
Fig. 18: Conformation of various cyclitols.



I. stable form

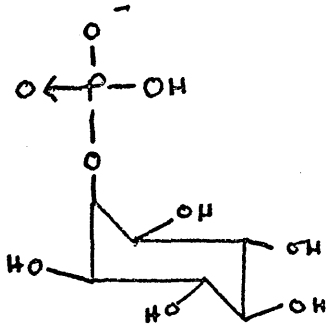


II. unstable form

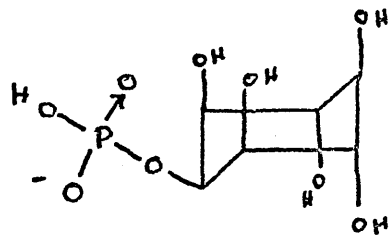


III. tridentate borate complex

Epi-inositol

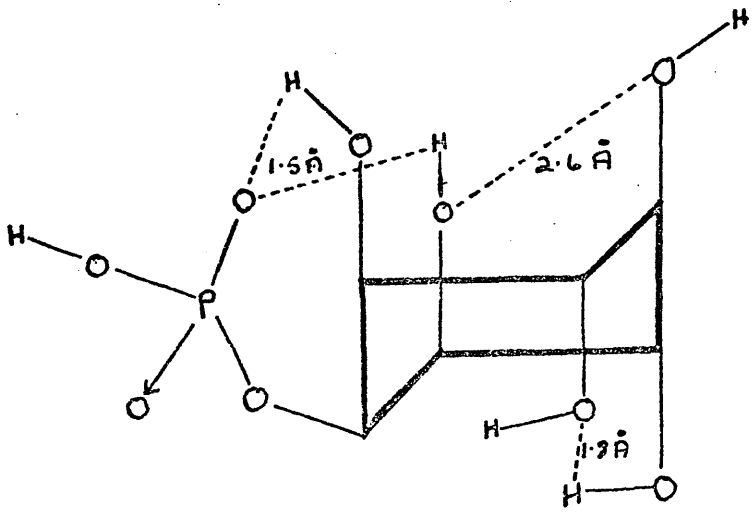


IV



V

Myo-inositol-2-phosphate



VI. inverted form of myo-inositol-2-phosphate showing approximate inter-atomic distances.

Myo-inositol does not exhibit this inversion, but the case of myo-inositol monophosphate does not appear to have been considered. Where this compound has been isolated and studied it is often the only axial hydroxyl group, that of carbon two, which is phosphorylated (199). It seems possible that the bulky phosphate group could promote an inversion of the conformation so that it moves down to an equatorial position (IV and V). If this does occur, there is no reason why the resulting conformer should not form a borate complex. If carbon two was the hydroxyl phosphorylated in the present case, this hypothesis would explain why myo-inositol monophosphate was retained by the anion exchange column to the extent observed. There is an added inducement promoting the inversion of the conformation. This is the increased possibility of hydrogen bonding between an ionised oxygen of the phosphate group and the axial hydroxyls of carbon one and carbon three (VI). The oxygen atoms of the unstable conformer (V) are about 2.6 Å apart whilst in the stable conformer (IV) about 2.8 Å, very little further. The increased possibility of hydrogen bonding with its concurrent stabilisation of the conformer should overcome the slight degree of steric crowding in the inverted form. This effect is observed with 1,3-diaxial cyclohexanediol.

A final explanation for the delayed elution of some carbohydrate phosphates is that borate replaces some of the

exchangeable chloride upon the resin. The fractionations aimed at depend upon gradually decreasing the borate ion concentration of the eluting solution, but this effect will be largely nullified if there are borate ions held on the resin to give locally high concentrations. An experiment to verify this hypothesis was readily performed (Exot.44). Through a column of Dowex-1x4 in the chloride form, identical to that used in the previous experiment, was percolated two litres of the initial eluting solution, 0.025M LiCl and 0.01M $Na_2B_4O_7$. The column was washed well with water and then eluted with 1.0M LiCl. This solution contained 4.4 m.moles of boric acid which had replaced chloride on the ion exchange resin.

It was now apparent that to achieve good separations of sugar phosphates by the Khym and Cohn method the use of borate and the volume of each eluting solution had to be severely curtailed. This, however, is incompatible with a quantitative recovery of the sugar phosphates, for it is seldom that peaks are as sharp as those shown by Khym and Cohn. However, by foregoing some of the possible separations, this difficulty could be largely overcome. A less sophisticated, but more quantitative separation would be merely to resolve those carbohydrate phosphates which complex with borate from those which do not do so. It was felt that the supplementary techniques which had been assembled for analysing sugars and sugar

phosphates should be adequate for the slightly more complicated mixtures which would arise. Furthermore this would fortuitously separate some of the isomeric esters the presence of which had been demonstrated and which could be considered particularly significant to starch metabolism. Thus G-1-P and G-6-P would be separated from each other, as would sucrose phosphate from F-6-P.

Before proceeding to discuss the experiment which was based on these conclusions, something should be said about the analysis of carbohydrates by Gas Liquid Chromatography, which was used extensively in the remaining experiments.

8. Gas Liquid Chromatography of Carbohydrates.*

The technique of gas liquid chromatography (G.L.C.) has received wide acceptance because of its facility, reproducibility and sensitivity. However, the application of G.L.C. to the separation of carbohydrates has tended to lag behind that of other classes of compounds. The major difficulty was the preparation of volatile derivatives of polyhydroxy compounds. It was not until Sweeley et al. pioneered the use of trimethyl silyl derivatives (TMS-ethers) that the G.L.C. of carbohydrates became feasible (200). Their formation simply involves solution of the carbohydrate in anhydrous

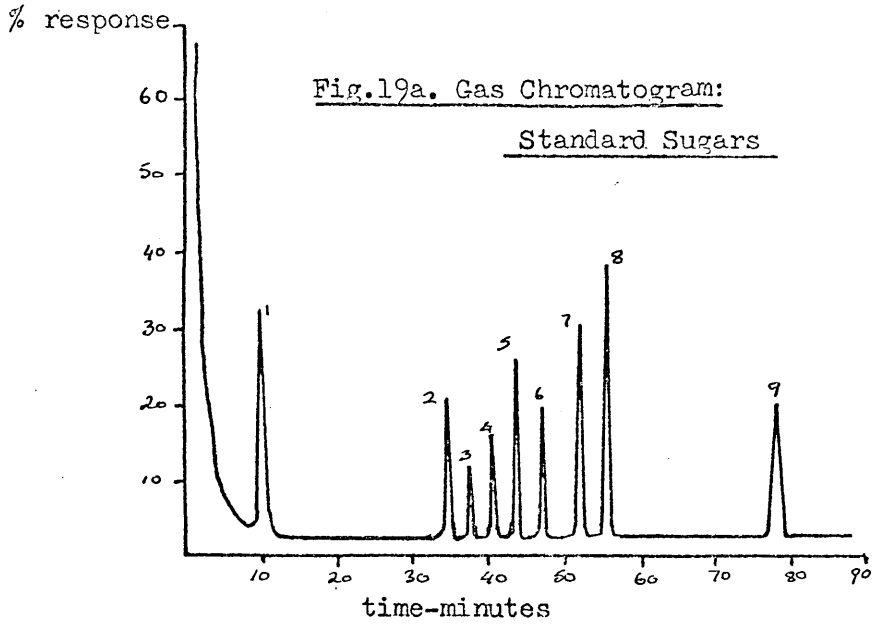
* The author would like to express his gratitude to Dr. J. A. Zabkiewicz for much patient help and guidance in the early stages of this work.

pyridine followed by addition of trimethyl chlorosilane and hexamethyl disilazane. Silanization takes place at room temperature and is virtually completed within a few minutes. This reaction mixture may be used directly or the reagents removed with a stream of dry nitrogen and the TMS-ethers dissolved in hexane. These derivatives are comparatively non-polar so that the stationary phase which is generally chosen is also a non-polar one, the most usual being a methyl silicone gum which is suspended as a 3% solution upon an inert matrix (201).

In Fig. 19(a) may be seen a typical gas chromatogram. This was a temperature programmed separation and it has resolved a wide range of carbohydrates from triol to a disaccharide. By varying the temperature and the rate of change of the temperature, any region of the chromatogram could have been emphasized, e.g. the time between peaks 2 and 8 could be increased to 60 minutes, whilst the time between peaks 1 and 2 was decreased to 5 minutes. This is analogous to changing the temperature in paper chromatography and does not affect the overall separation. Various temperature programming separations are described in Table 10.

Fig. 19(b) shows the carbohydrates produced by enzymatically hydrolysing the sugar phosphates extracted from starch granules. This experiment is typical of several, all of which gave

Fig.19a. Gas Chromatogram:
Standard Sugars



Peak numbers

1) glycerol
2) ribose
3) α-xylose
4) β-xylose
5) fructose
6) α-glucose
7) β-glucose
8) myo-inositol
9) sucrose
10) unidentified
11) unidentified

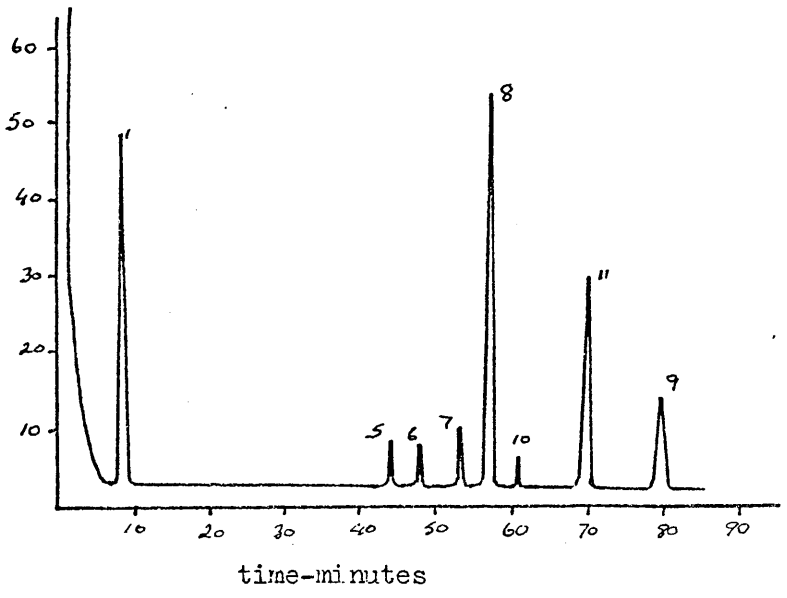


Fig.19b Gas Chromatogram: Sugars produced by hydrolysing the sugar phosphates extracted from starch.

Sample (2μl) injected at 85°, temperature held for 10 mins., then temperature programmed to 230° at 2° per min. Column, 3% SE30, 1.5 metres x 4 mm., gas flow 75 ml. per min. at 150°.

Retention time (mins)				Retention time relative to myo-inositol		
1	2	3	Conditions	1	2	3
-	1.6	9.4	glycerol	-	0.05	0.17
6.7	11.0	34.6	ribose	0.30	0.31	0.62
8.1	13.2	37.2	α-xylose	0.37	0.38	0.67
9.8	15.7	40.2	β-xylose	0.44	0.45	0.72
12.0	19.7	43.3	fructose	0.54	0.56	0.78
14.9	23.8	47.0	α-glucose	0.65	0.68	0.85
18.9	30.9	51.8	β-glucose	0.85	0.86	0.93
17.7	27.4	49.6	sorbitol	0.76	0.78	0.89
22.2	35.0	55.5	myo-inositol	1.00	1.00	1.00
44.6	64.4	78.1	sucrose	2.08	1.85	1.40
39.8	-	72.6	adenosine	1.72	-	1.31
34.2	-	62.7	uridine	1.50	-	1.22
			Peak number (Fig.19b)			
-	-	9.4	1	-	-	0.16
12.0	20.5	45.8	5	0.53	0.58	0.79
14.6	24.0	49.2	6	0.65	0.68	0.85
18.9	30.7	54.5	7	0.84	0.87	0.94
22.4	35.3	58.1	8	1.00	1.00	1.00
50.8	63.3	81.1	9	2.20	1.85	1.40
26.2	40.3	62.2	10	1.17	1.14	1.07
35.6	53.1	71.8	11	1.59	1.51	1.23

Table 10: The gas chromatographic behaviour of various carbohydrates.

1. Temp. Prog. at 2°/min. from 130° to 250°.
2. Hold 5 mins. @ 125° T.P. @ 1.5°/min. to 250°.
3. Hold 10 mins. @ 85° T.P. @ 2°/min. to 250°.

peaks arising from glycerophosphate, fructose phosphate, glucose phosphate, myoinositol phosphate, and sucrose phosphate (Expt. 45). No trace of pentose was found, which was surprising since the analyses described in Part 6 had clearly indicated the presence of traces of xylose phosphate.

It is possible to use gas chromatography quantitatively because the area of each peak is roughly proportional to the total amount present. This means that the response of one sugar can be related to that of another, 1 µg. of A giving rise to a peak having 95% of the area of 1 µg. of B. By adding a known amount of A to B and observing the relative areas of their gas chromatographic peaks, the concentration of B can be determined. The alternative to this internal standard method is to plot a standard curve of peak area against concentration, but this method is particularly vulnerable to small variations between separate chromatograms.

The response factors of several sugars were determined by running a series of chromatograms with various concentrations and measuring the area of each peak by triangulation (Expt. 46).

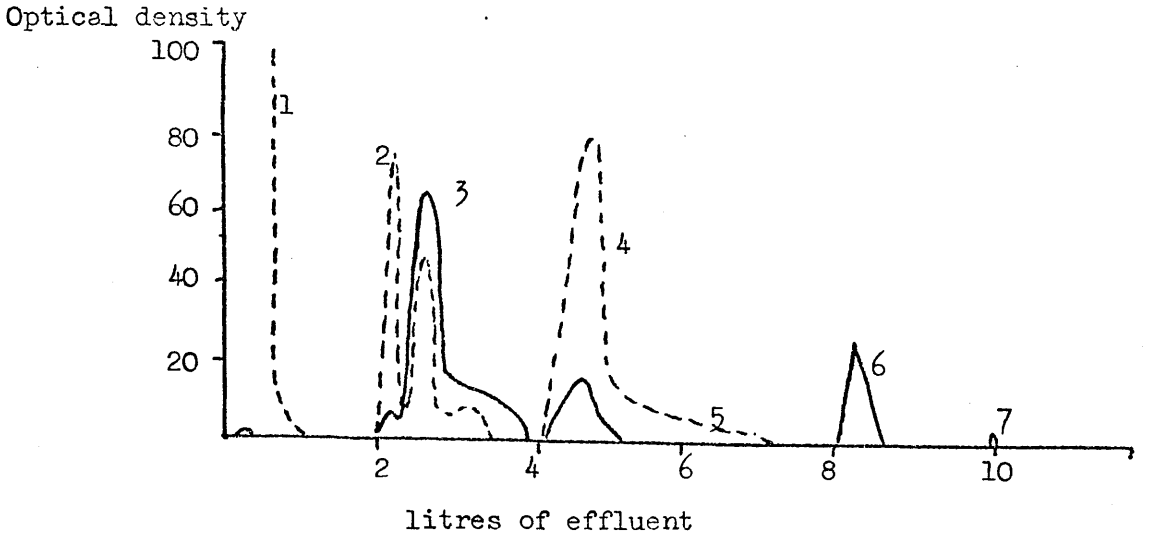
9. Separation and analysis of the sugar phosphates from starch, wherein the use of borate ions is severely curtailed.

Once again the phosphate esters were isolated from an aqueous methanolic extract of 10 Kg. of potato starch. The recovery

of the organic phosphate this time was 103%, of the inorganic phosphate 0.3% and of the u/v absorbing material 38.5% (Expt. 47). A solution of these phosphates was percolated slowly through a column of anion exchange resin which was then eluted with the solutions described in Fig. 20. Only 2 litres of borate-containing solution were used and this was replaced by 0.03M LiCl, followed by dilute HCl to replace the nucleotide monophosphates. As usual, the sugar phosphates were recovered from the eluting solution by anhydrous methanol-acetone precipitation and their recovery ascertained to be quantitative by demonstrating the absence of any phosphate in the supernatant acetone solution.

The phosphate concentration of each peak was estimated and it was noted that the greater part of the sugar phosphate fraction did not form complexes with borate (Peak 3).

Aliquots of each fraction equivalent to 1 Kg. of starch were hydrolysed with acid and alkaline phosphatase and the carbohydrates identified by both G.L.C. and paper chromatography. Peak 3 contained predominantly myo-inositol, glycerol and sucrose, together with glucose and traces of fructose, which almost certainly arose from the cleavage of sucrose, since both F-1-P and F-6-P form strong borate complexes. This meant that some of the glucose would also have arisen from the sucrose, but most of it would have been from



——— Optical density, 680 mμ, phosphate

- - - - - - - - Optical density, 490 mμ, carbohydrate

Composition of eluting solutions.

0 to 2 litres of effluent	10^{-4}M LiOH, pH 10.0
2 to 4 " " "	0.025M LiCl, 0.04M H_3BO_3, pH 9.0
4 to 8 " " "	0.03M LiCl, pH 9.0 falling to pH 6.5.
8 to 12 " " "	0.005M HCl, pH 6.5 falling to pH 3

Fig. 20, part 1. Ion exchange separation of sugar phosphates wherein the use of borate was curtailed.

Peak No.	Position of Elution	Phosphate present mμmoles/g. of starch			Carbohydrates observed after hydrolysis*	
		Pi	Porg	P ål	by paper chromatography	by G.L.C.
1	0-2	-	0.05			
2	2-2.3	0.41	0.07	0.02	m-I, (G, F)	m-I, G, (F)
3	2.3-4	3.3	21.3	1.4	m-I, glyc., S, G, (F)	m-I, glyc, S, G, F.
4	4-5	0.02	5.28	1.17	m-I, (G, S, glyc., F)	m-I, F, G (S, glyc)
5	5-7.5	0.15	0.85	0.20	m-I, G, (S, glyc.)	m-I, G.
6	8-9	0.30	2.50	0.50	m-I, S, G, F.	m-I, G.
7	9-11	0.85	1.40	0.00	(m-I, G, glyc., S)	(m-I)

Figure 20 (Part 2)

* brackets indicate a very faint spot or a dubious peak.

m-I : myo-inositol glyc. : glycerol

S : sucrose G : glucose

F : fructose

Table 11

Concentrations expressed as mmoles/g. of starch.

Peak Number	Carbohydrate	Concentration by G.L.C.	Concentration by colorimetry
2	glucose		0.02
	myo-inositol		0.05
3	glycerol	2.91	2.17
	glucose	0.52	0.55
	fructose	0.11	0.90
	sucrose	0.83	
	myo-inositol	1.97	(17.7)
4	glucose	2.12	2.38
	fructose	0.99	1.4
	sucrose	trace	
	myo-inositol	0.38	-
	glycerol	0.75	-
5	glucose	0.25	0.26
	fructose	0.09	0.19
	sucrose	0.10	
	myo-inositol	0.11	-
6	glucose	0.20	0.26
	fructose	0.08	0.16
	sucrose	0.08	
	myo-inositol	<0.05	-
7	glucose	nil	nil
	fructose	nil	0.06
	sucrose	nil	
	myo-inositol	<0.001	-

A comparison of carbohydrate concentrations estimated by G.L.C. and by colorimetry.

the G-1-P, the concentration of which was probably equal to that of the acid labile phosphate in this fraction. The bulk of the hexoses were eluted in the next fraction (Peak 4), which contained glucose and fructose with traces of myo-inositol, glycerol, and sucrose. The remaining fractions contained diminishing quantities of the same carbohydrates (Expt. 48).

The concentrations of the carbohydrates were with one exception estimated by both quantitative G.L.C. and colorimetry. Because of the lengthy isolation necessary for the colorimetric estimation of myo-inositol, it was estimated only by G.L.C. On account of this, the colorimetric estimation of glycerol in peak 4 had to be omitted, for otherwise the large hexose content of this peak would have introduced errors. It was hoped to estimate myo-inositol by subtracting the total concentration of the other carbohydrates from the phosphate concentration. However, this gave a value nine times higher than the one obtained by G.L.C. In view of the close correlation between the two methods for the other carbohydrates, the direct G.L.C. value was accepted.

Measurement of the glucose concentration of peak 4 by G.L.C. introduced the possibility that there were traces of galactose present in the hydrolysates. Generally, the area of the α-glucose peak is about 65% that of β-glucose, whereas in this case its area was

consistently higher than that, around 80%. Also the peak width was greater than usual. The two anomers of galactose have retention times relative to that of α-glucose of 96% and 105%, so the contaminating presence of a small amount of galactose would appear as part of the α-glucose peak. However, as all attempts to resolve these two sugars in Peak 4 were unsuccessful, the presence of galactose could not be confirmed.

The overall result of these estimations is summarised below.

Carbohydrate phosphate	Concentration m.μmoles/g. of starch
glycerophosphate	3.25
myo-inositol monophosphate	2.51
glucose-1-phosphate	0.54
glucose-6-phosphate	2.77
fructose-6-phosphate	0.52
sucrose phosphate	2.13

Table 12: The concentrations of the various sugar phosphates extracted from starch.

Of the 31.4 m.μmoles/g. of starch of organic phosphate in peaks 2 to 7, 10.5 m.μmoles had been estimated to belong to the sugar phosphates. Only a small proportion of the remaining organic phosphate would have been nucleotide; the 3.5 m.μmoles of peaks 6 and 7 which was unaccounted for. This result is very similar to the previous analysis. Then, 63% of the organic phosphate was unaccounted for; on this occasion, 62%. It would be interesting to know what other organic phosphates there were in these fractions. However, neither paper nor gas chromatography yield any further information, the only indication of their presence being the discrepancy between carbohydrate and phosphate concentrations.

Several of the values in Table 12 compare closely with the results of the previous analyses, Table 9, page 108. Glycerophosphate, inositol phosphate, sucrose and fructose phosphate concentrations were much the same. The glucose phosphate concentration was estimated to be much higher, whilst pentose phosphates were absent. There was some dubiety about these two estimations in the previous experiment, so the later results can be accepted as being more reliable.

10. Assigning the position of the phosphate esters upon their carbohydrate moieties.

Glucose-1-phosphate and glucose-6-phosphate had so far

been distinguished solely upon the basis of their anion exchange properties. More definite evidence for the separate presence of these two entities was now sought.

Apart from a description by Wolf and Kaplan the reduction of hexose phosphates to the corresponding hexitol phosphates by means of sodium borohydride has received little attention (202). It was surmised that esters such as G-6-P or ribose-5-phosphate should be readily reduced by borohydride whilst G-1-P would be unaffected. The validity of this hypothesis was readily demonstrated (Expt. 50). After reduction of G-6-P and R-5-P the phosphates were hydrolysed with acid phosphatase and the carbohydrates identified by paper and gas liquid chromatography as being exclusively sorbitol and ribitol. No trace of the original sugars remained. When this process was repeated with G-1-P the glucose was recovered intact with no production of sorbitol.

Borohydride reduction of the material from Peak 3 isolated in the previous experiment (Fig. 20) did not affect the glucose which was present, thus demonstrating that as had been supposed this ester was G-1-P. Conversely all the glucose in Peak 4 was reduced to sorbitol by borohydride, thereby confirming that it was G-6-P which was present in this peak. The fructose also was reduced but since F-1-P and F-6-P would be equally well reduced by borohydride this

does not provide any further information. However a consideration of the hexose phosphate metabolism of starch suggests that the presence of F-1-P to the exclusion of F-6-P is extremely unlikely so the fructose phosphate was assumed to be F-6-P.

In his description of the enzymatic synthesis of sucrose phosphate Mendicino demonstrated conclusively that it was upon carbon six of the fructose moiety that this disaccharide was phosphorylated. If this also held in the present case then mild acid hydrolysis of the sucrose phosphate from Peak 3 (Fig. 20) should release glucose from both sucrose phosphate and G-1-P, but little or no fructose, which would still be esterified. This proved to be the case. From the amount of glucose produced it was estimated that 76% of the sucrose phosphate was hydrolysed by 1.0M hydrochloric acid at 100° in 10 minutes. On the other hand, there was no more fructose released than was found in enzymic hydrolysates of this fraction. Thus it was demonstrated that the sucrose phosphate from potato starch granules was identical with that synthesized by the enzymes from wheat germ.

11. Phytic acid.

In view of the presence of myo-inositol monophosphate in starch granules, the possibility was considered that there might also be phytic acid present. According to Posternak (168) the best method for isolating phytic acid from other phosphatic metabolites is to

precipitate its ferric salt from an acid medium as described by Young (203). This method had a particular advantage for the present work in that it involved boiling the phytic acid in 0.17 M hydrochloric acid. Thus the precipitating agent could also be used to dissolve the starch. Further, since it was an acid medium, there was a rapid cleavage of the starch molecules so that the normal viscid starch pastes did not ensue (Expt. 52). Eventually a solution was made which contained 10% starch which, after standing for three days, had formed a slight precipitate. This could have been phytic acid or could equally well have been retrogration products from the starch solution.

This precipitate contained a total of 2 μmoles of organic phosphate which, according to Young, could only have arisen from phytic acid. However prolonged acid hydrolysis of this precipitate followed by gas chromatography revealed the presence of glucose only, inositol being entirely absent. From this it was concluded that phytic acid was not present in potato starch granules.

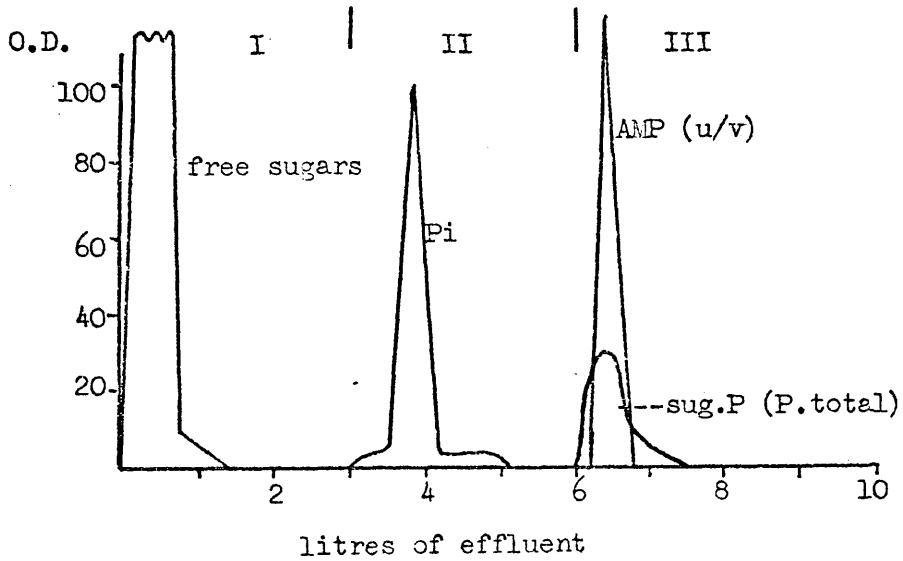
12. Analysis of the free sugars from commercial potato starch by gas liquid chromatography.

In the following Section an account is given of analyses carried out on starch freshly extracted from potatoes. The sugar analyses in this case were to have been done entirely by G.L.C.

Before embarking upon this study it was thought prudent to ensure that the free sugars from starch could indeed be analysed by G.L.C. Accordingly a preliminary experiment was carried out to test the effectiveness of this technique with the sugars obtained from commercial starch grains.

One Kg. of potato starch was extracted with aqueous methanol and the free sugars and sugar phosphates separated by a modification of the Khym and Cohn procedure (Expt. 53). The elution pattern is illustrated in Figure 21.

Fig. 21: Ion exchange separation of the free sugars and the sugar phosphates from potato starch.



I. 10^{-4}M lithium hydroxide

II. 0.03M LiCl, 0.04M H_3BO_3, pH 9.0

III. 0.005M hydrochloric acid.

The use of Solution 3, used in the elution sequence, requires explanation. Having eluted the free sugars and the non-borate-complexing sugar phosphates from the column with solutions 1 and 2, there remained the sugar phosphates which complexed with borate and the nucleotide monophosphates (ignoring the diphosphates). In the previous experiment, these sugar phosphates had not been completely eluted by the solution designated for that purpose, viz. 0.03M LiCl. Thus a small proportion of them were eluted by the following solution and appeared as a contaminant with the nucleotide monophosphate fraction. For the sake of obtaining quantitative results it was decided to accept this situation. The two fractions were combined by omitting the lithium chloride solution and eluting both the borate-complexing sugar phosphates and the nucleotides with dilute hydrochloric acid solution. The supplementary analytical techniques which had been assembled were believed to be sufficiently sophisticated to deal with the slightly more complex mixtures arising. This stratagem also provided incidental protection against a more unlikely eventuality, namely the presence of a phosphate ester which would normally be classified as a sugar phosphate, but whose chromatographic behaviour was that of the nucleotide monophosphates. However, in this experiment the phosphates were not examined beyond the preliminary separation stage, attention being confined to the free

sugars, which were successfully analysed by G.L.C. to give the results shown in Table 13. These are quantitatively not dissimilar to the values described in Section II, Table 7, page 80, except that they are all somewhat lower. The hitherto unsuspected presence of myo-inositol was noted with interest. No attempt was made to confirm these results by paper chromatography or colorimetry.

	Concentration per gram of starch
inorganic phosphate	0.625 μmoles
organic phosphate	0.175 μmoles
fructose	6.1 μg.
glucose	10.4 μg.
ribose	1.2 μg.
sucrose	54.0 μg.
maltose	0.35 μg.
myo-inositol	0.30 μg.

Table 13: Estimated concentrations of various metabolites extracted from potato starch.

Conclusions to be drawn from Sections I, II, and III.

The presence of various free sugars and sugar phosphates was demonstrated in commercial potato starch of uncertain history. Notably these included sucrose, fructose, sucrose phosphate, G-1-P, G-6-P and F-6-P, the presence of all of these being understandable upon the basis of current knowledge of starch metabolism. In addition to these, there was also found glucose, ribose, maltose, myo-inositol, myo-inositol monophosphate and glycerophosphate. The presence of most of these is less readily explained.

The concentrations of all these metabolites were estimated and are summarised in Tables 7, 9, 12, and 13. From these it can be seen that the predominant sugar is sucrose, whilst what glucose and fructose there is present possibly arose by hydrolysis of the sucrose. The most abundant sugar phosphate is glycerophosphate, followed by G-6-P, myo-inositol monophosphate and sucrose phosphate. There were only small amounts of G-1-P extracted from the starch, 0.54 mμmoles/g. compared with 425 mμmoles of inorganic phosphate - a ratio of one part in eight hundred. The equilibrium point of the phosphorylase reaction is reached at a ratio Pi : G-1-P of 2.2 (154). At higher ratios than this, such as apparently prevail in potato starch granules, phosphorylase can only catalyse the degradation of starch.

Section IV: An analysis of various carbohydrates which could be extracted from freshly isolated potato starch granules by aqueous methanol, without disrupting the granule.

All of the work which has so far been described was performed upon starch obtained from a commercial source. Its history was unknown and may have included a variety of treatments, which could conceivably have increased or caused the disappearance of various entities. Because of this it was desirable that freshly extracted starch should be investigated in the same manner. A direct parallel was impossible because so little was known about the commercial starch. Presumably it had been extracted from mature potatoes, but at what stage of maturity, how long they had been stored and under what conditions, and the variety of potatoes were all unknown. These are all reported to have an effect upon the tuber and so they are significant to the present studies upon starch in the tubers (50, 204-207).

Eventually two separate investigations were done at different stages in the growth of the potato. In each case the starch granules were extracted and studied and an attempt made to analyse the remaining tuber homogenate in the same manner, but for a variety of reasons this was less successful.

Kerr's Pink potatoes, grown in an adequately fertilized light loam by a local farmer, were used in these experiments. They had been planted at the end of May and were harvested by the author after about ten and eighteen weeks' growth. In the first instance the tubers were immature and the majority of the smaller ones, below about 1.5 cms. diameter, were rejected, so that the average diameter was about 3-4 cms. The plants appeared to be on the point of flowering. The second batch consisted of potatoes of about 6-7 cms. diameter, and at the time of harvesting the aerial parts of the plant were starting to die away. Starch was extracted from the young potatoes immediately after harvesting, whilst the older ones were stored for three weeks, by which time small shoots had grown from a few of the tubers.

1. The extraction of starch from potatoes.

Considerable thought was given to devising a satisfactory method of isolating starch. For three reasons it was essential that the starch granules should be separated from the cell fluid as rapidly as possible after disruption of the cell. Firstly, the metabolites which were to be studied could be leached from the granule by aqueous media, and so it had to be assumed that extraction commenced immediately the cells were disrupted and the granules

released from their normal environment. Secondly, there was the possibility of the reverse process occurring, namely absorption by the starch granule of metabolites from the cell fluid. Because of these factors, it was desirable to effect an immediate transfer of the granule from the cell fluid to the extracting medium. These hazards were also partly countered by homogenizing the tubers into an environment of about 20% glycerol. This, it was hoped, would inhibit the desorption of metabolites from the granule, whilst glycerol would be absorbed to the exclusion of the normal cell metabolites.

Thirdly, there was the possibility that disruption of the granule's environment could eliminate the usual checks and inhibitions upon enzymic action, leading to a period of uncontrolled and abnormal metabolism. It could be assumed that enzymic activity would cease once the granules were dispersed in the extracting medium, 50% aqueous methanol, and so steps were taken to ensure that this was done as quickly as possible.

Thus a method was developed which was capable of effecting a very rapid transfer of the granules from the potato to the aqueous methanol. For the actual homogenization a simple machine was developed; this consisted of a rapidly spinning abrasive wheel (16,000 r.p.m.), against which segments of the tuber were gently

pressed. A jet of 35% glycerol solution was directed onto the wheel so that it spread out over the abrasive surface and was intimately mixed with the tuber homogenate during the maceration. The cell debris was separated from the starch suspension and the starch recovered from the cell fluid by a brief centrifugation. It was to expedite these two steps that the glycerol solution was introduced, for it gave the macerate a physical consistency which made very rapid separations possible. In fact, it was found that aqueous grinding techniques were quite useless for the present purpose and that the glycerol was essential. The precipitated starch was washed by shaking it with 20% glycerol solution, which was again removed by centrifugation. Finally, the starch was washed out of the centrifuge bottles with 50% aqueous methanol, which was used for the first extracting solvent. With practice, the time taken to reach this stage after the initial homogenization could be as short as five minutes (Expt. 54, summarised in Table 14). When extraction of the starch was complete, it was dried and weighed. Microscopic examination of the preparations showed only starch granules with no appreciable content of cell debris (Fig. 1).

However, if the number of operations in the isolation procedure are curtailed and the starch is washed only once before extracting the metabolites from it, there is a real danger that some

	Young starch	Mature starch
Approx. age after planting to harvesting	10 weeks	18 weeks
Period stored between harvesting and extraction of the starch	18 hours	3 weeks
Mean tuber size	3-4 cms.	6-7 cms.
Weight of potatoes homogenized	21.35 Kg.	24.92 Kg.
Weight of starch recovered	1,800 g.	2,650 g.
% starch of potato wet weight	8.4%	10.7%
Extraction of Starch	O.D.U. at 260 mμ	
1. 50% methanol	5,900	8.700
2. 50% methanol	2,400	3,300
3. 50% methanol	1,200	780
4. 10% methanol	275	540
5. 10% methanol	250	300
Total extracted	10,025	13,590
O.D.U. extracted per g. of starch	5.57	5.13
organic phosphate extracted mμmoles/g. of starch	148	143
Inorganic phosphate extracted mμmoles/g. of starch	212	315
Non-extractable phosphate mμmoles/g. of starch	14,000	6,600
% phosphate extractable	2.6%	7.0%
Number of granules/g. of starch	2.45×10^8	not determined

Table 14: The extraction of starch from potato tubers.

of the cell fluid may remain to contaminate the preparation. This presents a difficulty, since washing the starch sufficiently to remove all the cell fluid could also result in the loss of a significant proportion of the absorbed metabolites. Rather than suffer this loss, it was decided to tolerate the presence of some residual cell fluid and to determine the extent to which it contaminated starch preparations. This was done by adding a measurable amount of a dye during homogenization of the potatoes. Care was taken to choose a dye which was not absorbed by starch but remained in solution. It was noticed that starch can act as quite an effective decolourising agent, However, after rejecting a wide variety of compounds, Blue Dextran (Pharmacia) was eventually used. After isolation the starch was extracted with 50% aqueous methanol as usual and this extract contained only 0.38% of the dye originally present. On this basis it was assumed that 0.38% of all the metabolites in the cell fluid were transferred to the starch extract. When the concentration of each metabolite in the cell fluid was known, this proportion could be subtracted from the amount extracted by aqueous methanol from starch to give the concentrations of the metabolites in the starch granules (Expt. 55).

2. Ion exchange separation of the free sugars and sugar phosphates.

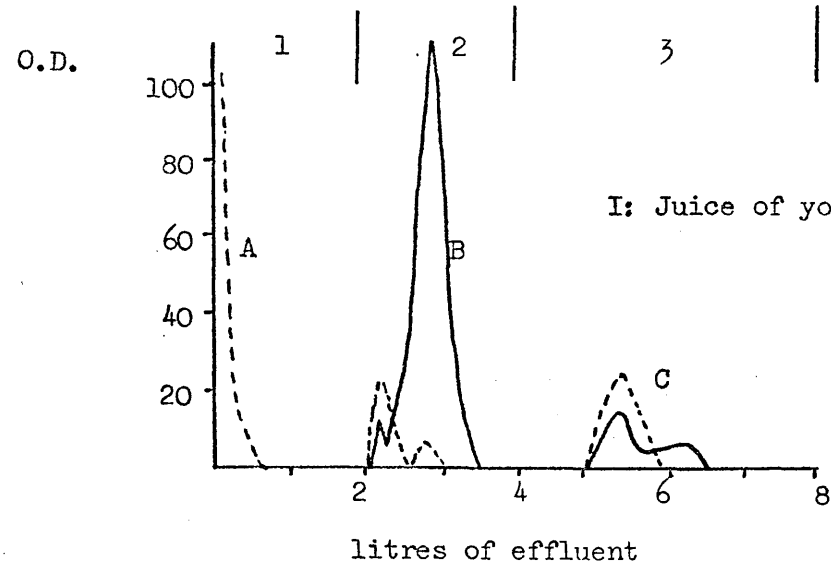
It is necessary to remember at this stage that there were four separate preparations being examined. There were the two extracts from what will be known as the young and the mature starch and also the supernatant potato juice remaining when the starch was centrifuged from the two glycerol-potato homogenate mixtures.

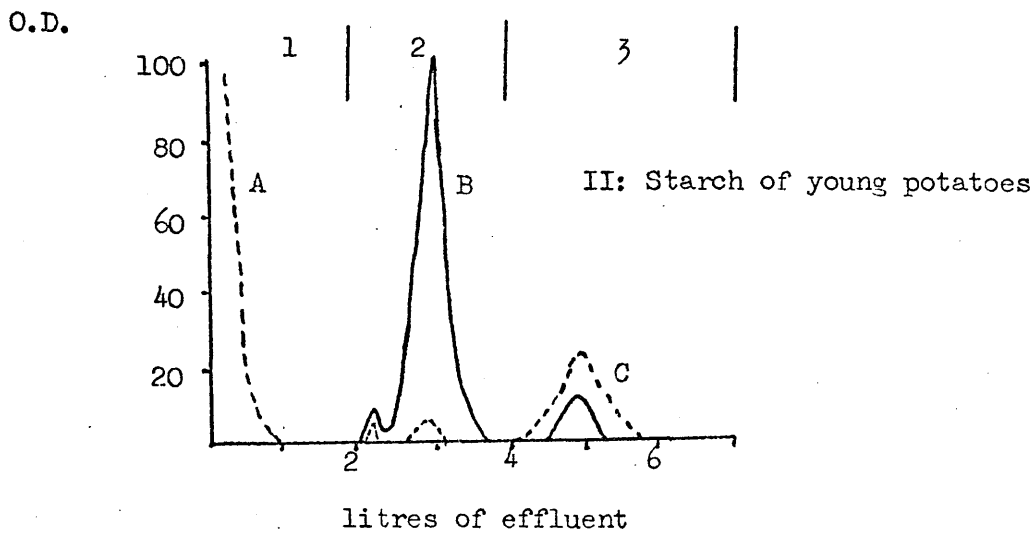
It was anticipated that glycerophosphate would be found amongst the sugar phosphate fraction and so it was necessary to ensure that all the glycerol which was used when isolating the starch was removed from this fraction. To do this it was decided to reintroduce the bulk separation steps used to separate the free sugars from the sugar phosphates. So in each case the preparation was caused to percolate through a column of anion exchange resin which should have retained all the phosphatic material whilst not hindering the passage of the free sugars or glycerol. The columns were washed with several litres of water which removed the remainder of the free sugars. The mono- and diphosphates were eluted from the columns using dilute lithium chloride solution. The esters were then recovered from solution by anhydrous methanol-acetone precipitation and in each case the precipitate was washed twice with acetone to remove any residual traces of glycerol, which is soluble in acetone. (Expt. 56)

Difficulties were encountered during this fractionation

About half of the juice from the young potatoes which had been retained for examination had to be discarded when it became very dark and a precipitate formed. Part of the remainder was lost when the fraction collector which was being used broke down. Because of these vicissitudes no attempt was made to gain quantitative results from this preparation. Difficulty was also experienced in maintaining the rate of percolation of the two extracts from young and mature starch through their respective columns. Several times it was necessary to resuspend the upper layers of the resin with a glass rod in order to break up the crust of impacted resin which appeared to form.

The next stage was to separate the borate-complexing sugar phosphates from the non-complexing ones by anion exchange chromatography using the system finally described in Section III. There were traces of residual acetone still present with the organic phosphates and this was tolerated because it provided a convenient monitor (O.D. 260 mμ) for the elution of the free sugars, of which there should, of course, be none present. The separations proceeded in the usual manner to give the results which are illustrated in Fig. 22. As usual also, the eluate was neutralised, the borate removed by several co-evaporations with methanol and the phosphates recovered by anhydrous methanol-acetone precipitations.





——— O.D. 660 mμ. (phosphate)

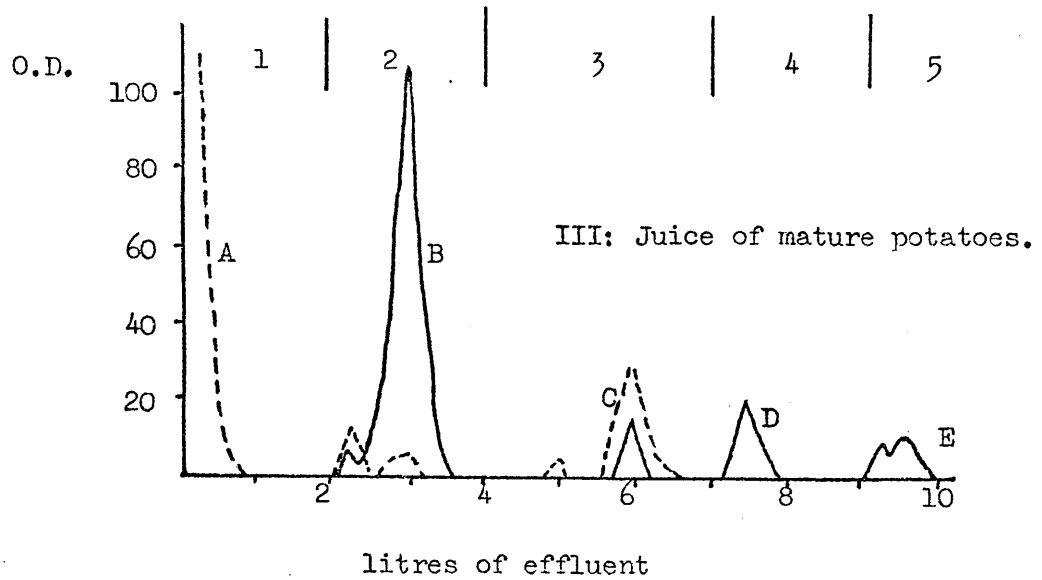
----- O.D. 490 mμ. (carbohydrate)

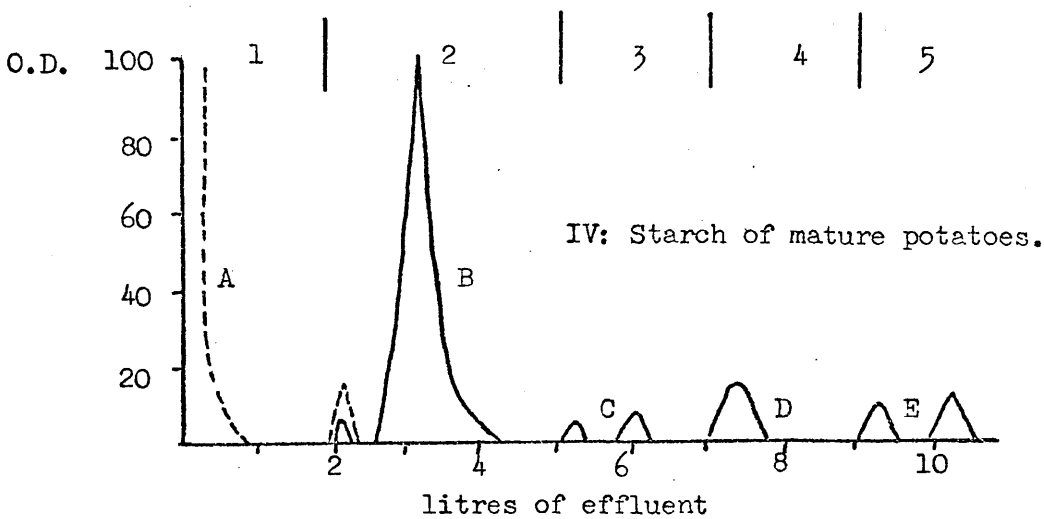
(1) 3×10^{-5} M LiOH, pH 9.5 - eluting the free sugars.

(2) 0.03M LiCl, 0.04M H_3BO_3, pH 9.0 - eluting the non-complexing sugar phosphates.

(3) 0.005M HCl - eluting the complexing sugar phosphates and ATP.

Fig. 22a. The separation of the phosphate esters from young potatoes.





——— O.D. 660 mμ (phosphate)

------ O.D. 490 mμ (carbohydrate)

(1), (2) & (3) as in Fig. 22a.

(4) 0.01M HCl, eluting ADP and ADPG.

(5) 0.02M LiCl, 0.02M HCl, eluting F-1; 6-diP etc.

Fig. 22b. The separation of the phosphate esters from mature potatoes.

Thus as a result of these preliminary separations each potato preparation was subdivided into several fractions, namely:

(A) The free sugars, which fraction contained all the non-anionic metabolites and most of the glycerol.

(B) The carbohydrate phosphates which did not form a complex with borate, and also the inorganic phosphate.

(C) The carbohydrate phosphates forming a complex with borate together with the nucleotide monophosphates.

(D) The nucleotide diphosphates containing a phospho-anhydro bond.

(E) The carbohydrate diphosphates which do not contain a phospho-anhydro bond. (Fractions D and E refer only to the mature potatoes).

The recovery of the inorganic phosphate was determined after each stage of the fractionation (Expt. 58). Overall 96.5% and 97.5% of the inorganic phosphate present in the original extracts of the young and mature starch was recovered but only 82% of the inorganic phosphate in the juice from the mature potatoes. The losses which occured also included the aliquots removed when assaying the columns so a 100% recovery could not have been expected. However since this means that the same proportion of every fraction of the elution was removed it could be assumed that the loss of the other

metabolites was the same as that of inorganic phosphate. The quantitative results which were subsequently obtained were corrected to eliminate the effect of these losses.

3. Analysis of the free sugars in peak A of each of the three preparations.

Because of the glycerol present in these solutions they could not be analysed directly by the methods described in Section II or in Part 12 of Section III. The large amounts of glycerol present in these fractions, deriving from the original extraction procedure meant that paper chromatography was not immediately possible. The glycerol interfered so badly that the comparatively small quantities of hexose which were present appeared as an irresolute blur of spots covering the latitudes of the chromatogram where glucose and sucrose might be expected.

Several attempts were made to remove the glycerol. Vacuum distillation was unsuccessful at temperatures which could be tolerated, merely causing a very slow removal of residual water. Glycerol distills azeotropically with meta-dimethoxy benzene to give a distillate containing 7% glycerol (209). This mixture distills at 212°, 78° lower than the boiling point of pure glycerol; a similar improvement was achieved with vacuum distillation for the mixture distilled quite rapidly at 0.005mm. and 30°. However the distillate

appeared, chromatographically, to contain only very slight traces of glycerol, far less than 7%. With the failure of this expedient no further attempts were made to remove the glycerol by distillation.

Instead resort was made to charcoal column chromatography. The grade of charcoal used in Section II, Ultrasorb SC 120/240, shows a considerable retention of monosaccharides from aqueous solution. It was surmised that this charcoal would show a negligible retention of glycerol and this proved correct (Expt. 59). Aliquots of solutions A were percolated through charcoal columns which were washed with small volumes of water and then with 20% ethanol. Paper chromatography showed that most of the glycerol was in the aqueous eluate and most of the sugars in the ethanolic eluate. Unfortunately some of the sugars were lost in the glycerol fraction, which meant that this method could not be used to precede quantitative estimations.

Paper chromatography of the free sugars in the ethanolic eluates demonstrated that in each case the predominant sugars were glucose and sucrose, in addition to which there were traces of fructose and various slow running carbohydrates which could not be identified (Expt. 60). There were several interesting differences between the young and the mature starch but somewhat disappointingly no apparent difference between the free sugars from the juice and the starch of mature potatoes. Thus the young potato starch contained

traces of ribose and maltose but these sugars were apparently absent from mature potatoes. On the other hand the mature potato juice and the mature starch contained a carbohydrate whose mobility was slightly greater than that of glycerol. This carbohydrate was not identified although several likely sugars were eliminated, namely glyceraldehyde, dihydroxy acetone and glycolaldehyde.

Confirmation of these identities was sought using gas liquid chromatography of the trimethyl silyl derivatives of the free sugars. Once again it was necessary to reduce the glycerol concentration of the solutions since the high levels which were present would have been as inimical to the silanization reaction as would water and for the same reason - because of the excess of hydroxyl groups. Gas chromatograms of the solutions purified by charcoal column chromatography showed the presence of glucose, sucrose, fructose, maltose and definite traces of myo-inositol. The presence of ribose was not confirmed.

An alternative technique was used to reduce the glycerol concentration so that it did not interfere with the G.L.C. separations. This was to take advantage of the fact that the TMS-ether of glycerol is considerably more volatile than the TMS-ethers of the common sugars, viz. the wide separation between glycerol and arabinose (Table 10, page 118). By adding a large excess of the

silanizing reagents to small volumes of the free sugar solutions the derivatives could be prepared. The glycerol was then removed by a crude fractional distillation, by heating the TMS-ethers at 50° in a current of dry air for about 20 minutes. This removed most of the glycerol but of course it offered no guarantee that some of the sugars, particularly those with low molecular weights were not also removed. It was clear that accurate quantitative estimation of the sugars present by gas chromatography could not follow this purification.

There were noticeable differences between samples prepared in this manner and those prepared after purification upon charcoal columns. The chromatograms were considerably more complicated, the free sugars from the starch of mature potatoes giving rise to about fifteen definite peaks and many more smaller or minor peaks (Fig. 23). Clearly discernible amongst these were glucose, sucrose, fructose, maltose and myo-inositol together with remnants of the glycerol. The presence of ribose could not be confirmed because the ribose peak, if any, was obscured by a large neighbouring peak. By the use of a wide range of standard compounds it was demonstrated that most of the unidentified peaks were not commonly occurring sugars. Amongst others, erythrose, xylose, mannose, galactose and sedoheptulose were all absent or present only in very

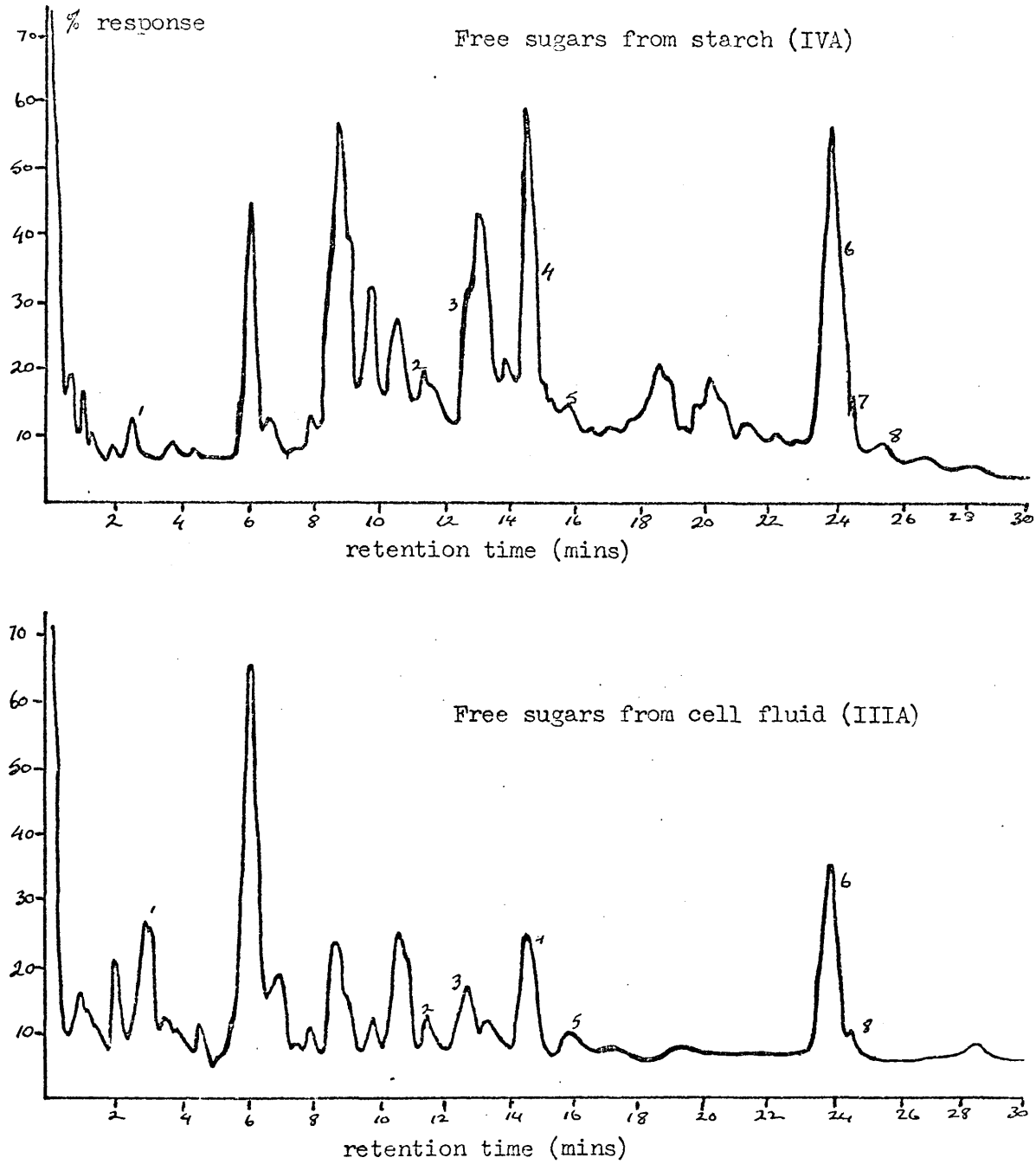


Fig. 23. Gas chromatograms. 2.5% SE 30, 1.5 metres long. temperature programmed from 100° to 250° @ 6°/min.

Peak 1, glycerol, 2, fructose. 3, α-glucose. 4, β-glucose. 5, myo-inositol. 6, sucrose. 7, α-maltose. 8, β-maltose.

small traces which could not be discovered.

With the exception of those numbered in Fig. 23 none of the gas chromatographic peaks were identified. This was perhaps unfortunate for there were differences between those from starch and those from the cell fluid. Particularly noticeable was a complex of peaks with retention times between 18 and 22 minutes (Fig. 23) from the mature potato starch which were not present in the cell fluid. It would have been interesting to know the identity of these metabolites although they were not necessarily carbohydrates. One of the disadvantages of gas chromatography is the inclusive nature of the detection devices. The flame ionisation detector cannot be confined to carbohydrates and it will reveal the presence of any extraneous compounds in the effluent gas including, for example, the TMS-ethers of nucleosides or hydroxylated steroids.

4. Estimating the concentration of the free sugars.

Neither of the methods which were used to prepare volatile derivatives of carbohydrates for G.L.C. could be relied upon to give quantitative yields. Because of this G.L.C. could not be used for estimating the free sugars. This was unfortunate because it would have been much easier and more specific than the colorimetric methods which were eventually used.

In Section II the monosaccharides were separated from the

disaccharides by charcoal column chromatography prior to the estimation of their concentrations. However in the present case the glycerol would interfere to some extent with this separation. Because of this and because a simpler method presented itself this step was omitted.

It was surmised that fructose and sucrose could be estimated in the presence of each other by selectively destroying the fructose with borohydride; similarly that glucose, maltose and sucrose could all be estimated together in a mixture by a combination of enzymatic hydrolyses and borohydride reductions. Firstly, it was ascertained that after it had been reduced with borohydride, fructose gives no reaction with the resorcinol reagent. Sucrose on the other hand survives this treatment unaffected since the reducing group is protected by a fructoside bond (Expt. 61). This method did admit one source of error, this being the possible presence of oligosaccharides other than sucrose, such as raffinose, which contained ketohexose and would have been determined as part of the sucrose fraction. However paper chromatography had demonstrated that sucrose was by far the most abundant disaccharide and that any other oligosaccharide which was present would constitute only a very small proportion of the sucrose giving rise to a negligible error. By taking advantage of this the fructose and sucrose concentrations

of each of the three free sugar solutions were estimated without difficulty (Expt. 62).

Concentrations as µg. per g. of starch or potato.

	Young potato starch	Mature potato starch	Mature potato juice
fructose	28	69	935
sucrose	450	230	1900

Glucose was determined using the GADH-peroxidase reagent. This reagent is buffered at pH 4.15 and at this pH the maltase and invertase activities which contaminated the GADH preparation (Expt.16) were imperceptible. The estimation was further complicated by finding that the equine maltase preparation which was available would not catalyse the hydrolysis of maltitol to glucose and sorbitol (Expt. 61). However by the action of maltase, invertase and borohydride in different sequences it was possible to arrive at values for the concentrations of glucose, maltose and sucrose (Expt.62).

Concentrations as µg. per g. of starch or potato.

	Young potato starch	Mature potato starch	Mature potato juice
glucose	290	110	1870
maltose	19	30	160
sucrose	475	230	1880

It was noted with satisfaction that the values obtained for sucrose

were very similar to those obtained by estimating the ketose moiety of the carbohydrate.

5. The carbohydrate monophosphates.

In Table 15 is summarised the phosphate content of the fractions A to E into which the various potato preparations were subdivided (Expt. 58). Fractions A, being the non-anionic compounds or the free sugars contained no phosphate, as indeed should have been the case. Fractions B, the non-borate-complexing phosphates, should have contained all the inorganic phosphate and this was virtually true except for the fractions from the juice of young potatoes, thus offering an indication that the attempted separations had been successful. The carbohydrate phosphates from the juice of young potatoes was not examined further because of the contamination of Fraction C by Fraction B. It was interesting to note that a substantial proportion of the organic phosphate which was eluted from the columns was also contained in Fraction B.

It had been intended to analyse the carbohydrate phosphates by qualitative and quantitative G.L.C. This technique had already been used successfully for this purpose and no difficulties were anticipated. Aliquots of fractions B and C of each preparation were hydrolysed enzymatically until no more organic phosphate remained and then the carbohydrates were isolated and treated in the usual

Potato Preparation	Fraction	Total phosphate in fraction mμmoles/g.			
		Pt	Pi	Porg	P á labile
Juice from young potatoes * (I)	A	This fraction was lost.			
	B	0.69	0.46	0.23	0.035
	C	0.325	0.115	0.210	0.005
Starch from young potatoes (II)	A	0	-	-	-
	B	249	204	45	0
	C	19	4	15	0
Juice from mature potatoes (III)	A	0	-	-	-
	B	540	423	117	13
	C	60	0	60	0
	D	43	2	41	-
	E	29	0	29	-
Starch from mature potatoes (IV)	A	0	-	-	-
	B	371	305	67	6.4
	C	97	3	94	4
	D	25	2	23	-
	E	16	0	16	-

Table 15: Phosphate contents of the various fractions into which the potato preparations were subdivided.

* The concentrations expressed for this fraction are μmoles/ml. of solution, since the weight of potatoes represented in this fraction could not be determined.

manner to prepare the volatile TMS-ethers (Expt. 63). Apparently there was no silanization for there was no trace of any identifiable carbohydrates except glycerol. The procedure was repeated taking elaborate precautions with each step, but to no avail. Variations were made by repeating or omitting each step in turn and by changing the proportions of the reagents used and the method of hydrolysis, but always without success. By increasing the sensitivity of the gas chromatograph ten or twentyfold, peaks which were recognisable as carbohydrates became discernible in these preparations but they were always much smaller than was indicated by the amount of organic phosphate which was present in each fraction, or by paper chromatography of comparable hydrolysates. Some silanization was occurring but clearly it was far from quantitative and the extent to which it took place or indeed the very occurrence of silanization was unpredictable. The reasons for this unfortunate inhibition are unclear. However a similar inhibition of silanization has been observed with other metabolites in some circumstances (210). Thus the information yielded by G.L.C. was solely of a qualitative nature and even the qualitative results were unreliable. However the identifications which were achieved were adequately confirmed by paper chromatography using two different solvents.

Qualitatively there were no marked differences between

the various preparations of potato juice and starch, with one exception. Fraction B from mature potato juice and starch produced predominantly glycerol and myo-inositol together with traces of glucose and sucrose after enzymatic hydrolysis of the phosphate esters. However no glucose appeared in this fraction from the starch of young potatoes although the other three carbohydrates were present. Hydrolysis of this, and of the other fractions, was repeated several times with consistent results. This interesting observation, suggesting that there was no G-1-P in the starch of young potatoes, was corroborated by the absence of any acid labile organic phosphate in this particular fraction (Table 15).

Fraction C from all three preparations produced myo-inositol, glycerol, glucose and fructose after hydrolysis of the esters.

Blank chromatograms of each fraction were prepared by deionizing suitable aliquots without prior hydrolysis. In this manner the carbohydrate phosphates would have been removed whilst any contaminating free sugars which were present would have remained in solution so that their presence would be revealed. The chromatograms showed barely discernible traces of glycerol but no other carbohydrates were detected.

Perhaps it is worth commenting upon these traces of

glycerol. Large quantities of glycerol were used in the isolation of the starch. From the studies of commercial starch it was anticipated that glycerophosphate would be found, so precautions were taken to ensure that there was no possibility of the glycerol remaining to contaminate the fractions containing glycerophosphate. These were, in sequence:

(1) The mixture was passed over an anion exchange resin which could retain the glycerophosphate whilst allowing the glycerol free passage. Each column was then washed with 3 litres of water.

(2) The phosphates were recovered from the resin and isolated by anhydrous methanol-acetone precipitation. The precipitate was washed twice with anhydrous acetone.

(3) The anion exchange separation was repeated, washing the column once again with water.

(4) The anhydrous methanol-acetone precipitations and washings were also repeated.

Although these somewhat disparate steps cannot perhaps be classified together there were no fewer than eight separate stages in this separation, each one of which should theoretically have eliminated the glycerol. In view of this it is perhaps surprising that even traces of glycerol remained.

Upon the basis of the experiments calibrating these methods it was concluded that G-1-P, G-6-P, F-6-P and sucrose phosphate were present in trace amounts in potato starch whilst the major phosphate esters were myo-inositol monophosphate and glycerophosphate. Since these results were the same as those obtained from commercial potato starch further tests to confirm the identity of each carbohydrate phosphate were not performed.

6. Estimations of the concentrations of the carbohydrate phosphates.

Once again quantitative G.L.C. could not be considered. This was particularly unfortunate in the present case because there is no other convenient method of estimating myo-inositol in the presence of other carbohydrates. Also, the method which was used for estimating glycerol was subject to certain small errors in the presence of other carbohydrates. However chromatography had indicated that glycerol was the predominant carbohydrate in the hydrolysates whilst the sugars which could interfere with its estimation by producing formaldehyde with periodate were present only in trace amounts. Thus any interference which did occur would have constituted only a fractional percentage of the overall result.

Glycerol was determined after enzymatic removal of the phosphate group by the periodate-chromotropic acid method, glucose with the GADH-peroxidase-ortho-tolidine reagent, and fructose with

the resorcinol reagent. The results are summarised in Table 16. The concentration of glucose-1-phosphate was assumed to be that of the acid labile phosphate in Fractions B. Sucrose phosphate was taken to be equivalent to the ketose in these fractions and fructose-6-phosphate equivalent to the ketose present in Fractions C. Similarly glucose-6-phosphate concentration was assumed to be the same as the glucose concentrations of Fractions C.

		G-1-P	G-6-P	F-6-P	sucrose phosphate	glycerol phosphate	inositol phosphate
Young potato starch	B	0.00	-	-	1.54	present	present
	C	-	5.4	1.54	-	"	"
Mature potato starch	B	6.2	-	-	5.4	47	"
	C	-	15	5.5	-	25	"
Mature potato juice	B	13	-	-	6.2	74	"
	C	-	16.4	1	-	20	"

Table 16. Estimated concentrations of various sugar phosphates as mumoles per g. of starch or potato.

7. The carbohydrate diphosphates.

The class of compounds referred to by this title is exemplified by glucose-1:6-diphosphate (G-1:6-diP). It has been demonstrated that these esters are eluted from Dowex-1x4 anion exchange resin by solution 5 (Fig. 22) and not by any weaker eluting solution (Expt. 35); this is in full accord with Khym and Cohn's results. Therefore it could be assumed that the small double peak of Fraction E of the mature potato preparations represented this class of metabolites. This assumption was further supported by the lack of any u/v absorption by these fractions.

Paper chromatography of the carbohydrates released by enzymatic hydrolysis of these esters demonstrated the presence of myo-inositol, glucose and fructose. Myo-inositol was apparently by far the most abundant carbohydrate, the other two being present only in trace amounts. The myo-inositol was in fact so much more abundant than the two sugars that it could almost be said that its concentration was approximately equivalent to that of the total organic phosphate in these fractions. On this basis the cell fluid would have contained not more than 14.5 mμmoles of myo-inositol diphosphate per g. of mature potatoes and similarly there was not more than 8.0 mμmoles of this ester extracted per g. of starch. Possibly the double peak arose from the inositol being phosphorylated upon different hydroxyl groups.

8. Summary of results and discussion.

The results of the examinations of both the commercial and the fresh potato starch are presented together in Table 17. The levels of all the metabolites except for G-1-P and sucrose phosphate were increased in the fresh starch preparations, glycerophosphate and glucose showing the largest increases. There was less G-1-P and sucrose phosphate in young potato starch than in commercial starch but substantially more in the mature potato starch. A significantly greater proportion of the organic phosphate present in the sugar phosphate fractions was accounted for this time, 73% in mature potato starch, not including myo-inositol monophosphate. In the two studies upon commercial starch, which included inositol monophosphate, only 37% and 38% of the organic phosphate was accounted for, the rest remaining unidentified.

The high levels of glycerophosphate were somewhat disconcerting in view of the large amounts of glycerol which were used when isolating the starch. Elaborate steps were taken to remove this glycerol and the remaining traces of it were estimated so that they could be subtracted from the measured concentrations, before arriving at the values shown for the concentrations of glycerophosphate. These results would almost seem to suggest that some phosphorylation of the glycerol may have been occurring during the

	Commercial starch			Fresh potatoes		
	Sect.2 part 4	Sect.3 part 9	Sect.3 part 12	Young Potato Starch	Mature Potato Starch	Mature Potato Juice
Non-extractable phosphate	20,600	15,300	15,600	14,000	6,600	-
Extractable organic phosphate	170	120	120	148	143	412
Extractable inorganic phosphate	630	425	625	212	315	515
G-1-P		0.54		0.0	6.2	13
G-6-P		2.77		5.4	15	16.4
F-6-P		0.52		1.54	5.5	1
sucrose (F6) phosphate		2.13		1.54	5.4	6.2
glycerophosphate		3.25		pres.	72	94
myo-inositol monophosphate		2.51		pres.	pres.	pres.
myo-inositol diphosphate					<18	<14.5
glucose-1:6-diphosphate					pres.	pres.
fructose-1:6-diphosphate					pres.	pres.
sucrose	208		158	1320	674	5,500
glucose	66		53	1610	612	10,400
fructose	67		34	155	383	5,200
ribose	26		8			
maltose	12.3		1	56	83	1,170
myo-inositol			2.6	pres.	pres.	pres.

Table 17: Estimated concentrations of various metabolites found in potato starch granules.

All values are expressed as mμmoles per g. of starch or potatoes.

isolation of the starch, precisely the kind of abnormal metabolism which was feared at the outset. This possibility was briefly investigated by incubating some freshly isolated starch with 20% glycerol solution (Expt. 67). Aliquots were taken at intervals from which the glycerophosphate was separated and the amount which was present estimated colorimetrically. There was virtually no change in the glycerophosphate concentration with time and on this basis it was concluded that phosphorylation of the glycerol was not occurring.

However, the values shown in Table 17 do not express the true situation in potatoes. Correction has not yet been made for the contamination of the starch preparations by the traces of residual cell fluid. It is because of the need to make this correction that the loss of the young potato juice was so particularly unfortunate since valid comparisons between the two stages of the potato's growth could not be made. Another irregularity is that the concentrations of the metabolites in the potato juice are expressed in relation to the weight of potatoes macerated, although the cell fluid could not have comprised more than about 85% of the total weight of the tuber. For comparison the concentrations of the metabolites extracted from starch should be expressed on the same basis, in relation to the weight of potatoes macerated. These corrections have been made in Table 18 which of course refers only to the mature potatoes.

Concentration per g. of potatoes in			Concentration per gram of	
starch	juice		starch	juice
11.8	412	organic phosphate	111	350
27.5	515	inorganic phosphate	257	437
0.53	13.0	G-1-P	5.0	11
1.34	16.4	G-6-P	12.5	13.9
0.52	1.0	F-6-P	4.9	0.9
0.49	6.2	sucrose (F6) phosphate	4.6	5.3
6.39	94	glycerophosphate	60	80
<0.7	<14.5	myo-inositol diphosphate	<6.5	<12.3
39	5,500	sucrose	365	4,700
11.5	10,400	glucose	108	8,800
16	5,200	fructose	150	4,400
6.5	470	maltose	61	400

Table 18: Concentrations expressed as mμmoles.

In order to facilitate comparison between the levels of metabolites in starch and in the medium in which the granules were suspended, Table 18 also shows the concentrations expressed per g. of starch and per g. of cell fluid. From this an interesting relationship becomes apparent. The concentrations of the phosphate esters are not dissimilar in starch and cell fluid but the concentrations of the free sugars are, very approximately, ten times higher in cell fluid than in starch. Whether or not this is significant is doubtful but it could be yet another indication, if such were needed, that the sugar phosphates are more closely linked with starch metabolism than are the free sugars.

The presence in tapioca starch granules of considerable quantities of sucrose and reducing sugars, including fructose has been reported (211). Similarly it has been reported that the principal sugars of potatoes are sucrose, fructose and glucose together with various oligosaccharides (212). In a recent comprehensive study of the low molecular weight carbohydrates from potatoes, Urbas reported the presence of galactose, glucose, fructose, myo-inositol and various oligosaccharides including sucrose (213). He says that these oligosaccharides could be divided into four groups. The first contained sucrose and members of the raffinose family; the second myo-inositol, galactopyranosyl myo-inositol and glucopyranosyl

myo-inositol. The third group consisted of di- and trigalactosyl glycerol whilst the fourth group contained three disaccharides and maltotriose. Since it is unlikely that these carbohydrates should be entirely confined to the tuber cell fluid the presence of some of them in starch granules might be expected. Maltose is a surprising exception to this list since its presence in potato starch granules was conclusively demonstrated.

The presence of galactosyl compounds in potato starch granules has been reported (214). Considering this, and again considering the work of Urbas, it is somewhat surprising that they were not found in the sugar or sugar phosphate fractions extracted from either fresh or commercial starch granules.

The sugar phosphates and nucleotides in potato tubers have been examined qualitatively before. Schwimmer et al. did this by fractional precipitation of the barium salts of the esters and they identified G-6-P, F-6-P and glycerophosphate amongst others(225). They also found traces of F-1:6-diP and five other phosphate esters which they were unable to identify. In an interesting comparable study using ion exchange chromatography Mori et al. found G-1-P, G-6-P and F-6-P. They also found phosphoglyceric acid which was not sought in the present work. However they did not find any myo-inositol monophosphate or glycerophosphate although their results

could allow for the presence of these esters and also possibly for inositol diphosphate (215).

Similarly Murata et al. found that when they supplied radioactive sucrose to developing rice grains a large part of the activity was incorporated into a peak which they did not identify (89). Its chromatographic behaviour was that of a carbohydrate phosphate which did not form a complex with borate and also it did not react with the phenol-sulphuric acid reagent. Taken together these factors suggest that this peak could well have been glycerol or inositol monophosphate or both esters together.

Many of the compounds shown in the cyclic representation of starch metabolism, Fig. 2 (page 51) have been identified in extracts from starch granules. It is interesting to note that sucrose phosphate, which features in this pathway only during the degradation of starch was present only in comparatively low levels in young potato starch. It is, though, difficult to understand why there should have been any sucrose phosphate at all in this preparation since its involvment with sucrose synthesis is the only metabolic rôle postulated for it. A developing potato tuber actively depositing starch should be receiving a large and almost continuous supply of sucrose transported from the photosynthetic regions of the plant. In these circumstances sucrose synthesis within the

tuber would not be expected to occur.

G-1-P was found to be completely absent in the starch granules of young potatoes. The synthesis of starch by phosphorylase requires the maintenance of high levels of G-1-P, particularly in the presence of inorganic phosphate. Synthesis will only occur by this pathway when the ratio G-1-P : Pi is less than 1 : 2.2. The ratios of G-1-P : Pi in the starch and in the cell fluid of mature potatoes were found to be 1 : 51 and 1 : 40 respectively. These were less than was found in commercial starch (1 : 800) but they are still large enough to preclude starch synthesis by phosphorylase.

The presence of myo-inositol, myo-inositol monophosphate and diphosphate is interesting. Both penta- and hexaphosphoinositol are relatively abundant in tubers (216) so it is not inconceivable that the tri- and tetraphosphates should also be present especially since the breakdown of phytic acid is reported to be by the stepwise removal of phosphate groups from inositol (217). It has been suggested that by controlling the availability of inorganic phosphate phytic acid exerts a regulating effect upon starch metabolism (216, 218). There is however, very little evidence to support this hypothesis. Gatt and Racker showed that simulated carbohydrate metabolism is slowed down by limiting concentrations of inorganic

phosphate, but since the enzymes they used were of animal origin it would be dangerous to conclude too much from their results (219). In potatoes and in rice the level of phytic acid increases with maturity whilst the level of phosphate bound to starch decreases (218, 220). It is obvious that the successful degradation of starch by phosphorylase requires a continuing supply of large amounts of inorganic phosphate and phytic acid may be a convenient source of this. The synthesis of phytic acid need not necessarily be closely connected with starch metabolism. It is, though, necessary to conjecture that the factors which stimulate phosphorylase to activity when dormancy is broken will also have a stimulatory effect upon phytase activity.

EXPERIMENTAL SECTION

INDEX

General Methods.

Experiments.

GENERAL METHODS AND PROCEDURES

G.M. 1. Paper chromatography.

For descending elution of chromatograms samples were applied at intervals along a line 7 cms. from the top of a sheet of filter paper (Whatman Chromatography Paper, No. 1) and not less than $3\frac{1}{2}$ cms. from the edge of the paper. They were dried in a current of cold air since warm air can cause degradation of some sugars. The bottom of the paper was serrated to encourage an even flow of solvent from the paper. The following solvents were used for carbohydrates.

a) Ethyl acetate - pyridine - water (12 - 5 - 4, v/v/v).

b) n-Butanol- acetic acid - water (12 - 3 - 5, v/v/v).

c) ter-Butanol - methyl ethyl ketone - formic acid - water (8 - 6 - 3 - 3, v/v/v/v) (224).

d) n-Propanol - ethyl acetate - water (7 - 1 - 2, v/v/v).

e) Phenol - water (4 - 1, w/v).

f) Methyl ethyl ketone - ammonia (0.88) (99 - 1, v/v) (171).

g) Methyl ethyl ketone - acetic acid - water, saturated with boric acid (9 - 1 - 1, v/v/v) (147).

The solvents used for phosphate esters were:

h) Ethyl alcohol(95%) - 1M ammonium acetate ($7\frac{1}{2}$ - 3, v/v) adjusted to pH 3.8 (189)

i) Same solution as h), adjusted to pH 7.5.

The mobility of the carbohydrates was measured by drawing a line around the outside of a spot and measuring the distance to the centre of this area from the origin. Mobilities were usually related to glucose since the solvent front was allowed to run off the paper.

G.M. 2. Thin layer chromatography.

Chromatoplates spread with cellulose powder (Whatman Chromedia) 0.3 mm. thick were used. The cellulose was washed by stirring it overnight with 1% citric acid solution and then with methanol. A suspension of cellulose powder (33 g.) in water (69 mls.) was used for preparing the plates which were dried in air. The samples were applied with a fine pointed capillary tube along a line 1 cm. from the bottom edge of the cellulose layer. The plates were then placed in a sealed tank with the bottom edge of the cellulose immersed in the solvent. For carbohydrates, solvents a), b), c), and e) listed in G.M. 1. were suitable. For sugar phosphates, the following solvents were used:

j) ter-Butanol - methanol - 0.1M HCl (3 - 1 - 1, v/v/v) (146).

k) Methanol - ammonia(0.88) - water (7 - 1 - 2, v/v/v) (190).

l) Methanol - acetic acid - water (16 - 3 - 1, v/v/v) (190).

m) ter-Amyl alcohol - water - p-toluene sulphonic acid, using the water poor phase (30 - 15 - 1, v/v/w) (191).

n) Isobutyric acid - water - ammonia (66 - 33 - 1, v/v/v) (191).

G.M. 3. Detection of compounds upon filter paper.

1) Carbohydrates.

a) alkaline silver nitrate

Unless stated otherwise in the text this reagent was always used for developing chromatograms.

Reagents i) Silver nitrate (5.0 mls. of a saturated solution) was diluted with acetone (1 litre) and water added until the precipitate just redissolved

ii) Sodium hydroxide (10.0 mls. of a saturated solution) was diluted with ethanol (300 mls.). Solution prepared fresh daily.

iii) 6M ammonia solution in 33% aqueous ethanol.

Procedure The paper was dipped rapidly through reagent (i), allowed to dry in air and then dipped through (ii) and left until the spots had developed the required intensity or until the background showed signs of becoming too pronounced. The paper was dipped through (iii) and the excess reagents removed by immersion in constantly running water for two hours.

b) ketohexoses; α-naphthol - phosphoric acid (172).

Reagent α-Naphthol, a 1% solution in a mixture consisting of 10 parts ethanol and 1 part phosphoric acid.

Procedure Paper sprayed evenly and dried in air for 30 mins. and then heated at 90° for 5 mins.

c) vicinal hydroxyl groups; periodate-benzidine. CARCINOGENIC

Reagents i) 0.04M $NaIO_4$ (75mls.) with ter-butanol (25 mls) ii) A 0.03M solution of benzidine in ter-butanol (0.27 g. in 50 ml) with 0.6M NH_4NO_3 (2.4 g. in 50 ml. of water).

Procedure Spray lightly with (i), dry at room temperature (30 mins.), then spray heavily on both sides of the paper with (ii).

d) reducing sugars and non-reducing sugars with an acid labile linkage; benzidine-TCA. CARCINOGENIC

Reagent Benzidine (0.5 g.) dissolved in acetic acid (10 ml) mixed with trichloracetic acid (TCA, 10 ml. of 40% w/v) and ethanol (80 ml.)

Procedure The reagent was sprayed lightly on the paper which was then heated at 100° until the spots reached maximal intensity.

N.B. G.M. 3-1, c&d, Benzidine solutions were sprayed only in a well ventilated fume cupboard whilst wearing a surgical face mask.

e) reducing sugars; aniline phthalate.

Reagent Aniline (0.93 g.), phthalic acid (1.66 g.) dissolved in n-butanol (100 ml. water saturated).

Procedure Sprayed lightly and heated at 105° for 5 mins.

2) Phosphate esters.

a) These could sometimes be located with alkaline silver nitrate (G.M. 3-1 a)

b) acid-molybdate spray (188).

Reagent Perchloric acid (72%, 4.3 ml.), ammonium molybdate (5%, 20 ml.), HCl (2M, 5.0 ml.), the mixture being diluted to 100 ml. with acetone.

Procedure Paper sprayed lightly and dried at 85°, then placed under a u/v light for several minutes.

3) Nucleotides.

These were located by viewing the paper under a u/v light. The visibility of the spots was greatly improved by spraying the paper lightly with a fluorescent compound such as Rhodamine 6G (0.1% soln.).

G.M. 4. Detection of compounds upon thin layer plates.

a) organic compounds, ceric ammonium nitrate

Reagent Ceric ammonium nitrate (1 g.) dissolved in H_2SO_4 (2M, 100 ml.)

Procedure Plate sprayed lightly and then heated at about 150° for several minutes.

b) phosphate esters, acid molybdate

Reagent Ammonium molybdate (2.7 g.), perchloric acid

(15 ml.), HCl (5M, 5ml.), the mixture being diluted to 100 ml. with water.

Procedure: Apart from the reagent being more concentrated, this method is identical to that described for paper chromatography.

With this exception and also that of alkaline silver nitrate, the reagents described above for filter paper were suitable for thin layer plates without modification.

G.M.5. Gas liquid chromatography.

1) Preparation of the liquid phase, 2.5% SE30.

Silicone Gum Rubber, SE30 (0.62 g., Analabs Inc.) was dissolved in methylene chloride (approx. 100 ml.) and added to the support material, celite (25 g.), washed with acid and alkali, then silanized to render it inert, (Anakrom 100/110 ABS, Analabs Inc.). These were thoroughly mixed and the solvent removed with a gentle vacuum using the very minimum of agitation necessary to maintain an even distribution of celite and SE30 solution. The last traces of solvent were removed by heating on a steam bath for 12 hours.

2) Preparation of the columns.

Two columns (1.5 m. long, internal dia. 2.5 mm.) were prepared together, care being taken to ensure that they were

identical. The glass was deactivated by filling the columns with a 5% solution of dimethyl dichlorosilane in toluene and allowing them to stand for 30 mins. One end was blocked with a small plug of glass wool (deactivated) and the column filled with the prepared stationary phase, using a slight vacuum, and the end closed with a second plug of glass wool. Volatile compounds were purged from the columns by heating them for 18 hours at 250° with a stream of nitrogen passing through. These columns could then be used up to but no higher than 250°.

3) The preparation of volatile derivatives of carbohydrates (200).

Reagent: Anhydrous pyridine (1.0 ml.), hexamethyl-disilazane (0.2 ml.) and trimethylchlorosilane (0.1 ml.). Stable for up to a week at room temperature, if kept dry.

Procedure: An aqueous solution of the sugar (1 mg.) was made anhydrous by several co-evaporations with anhydrous pyridine. The reagent was added (1.0 ml.) and shaken for five minutes. This preparation could be used directly. To avoid an excessive injection peak, the silanizing reagent was often removed by evaporation in a current of dry air. The residue was triturated with hexane and the precipitate removed by centrifuging. Finally, the excess hexane was removed and the TMS-ethers dissolved in a small volume of hexane.

4) The choice of conditions for chromatography.

A twin column Pye Series 104 Chromatograph having a flame ionisation detector was used, coupled to either a Hitachi Perkin Elmer 165 Recorder or a Goerz Servoscribe Recorder. British Oxygen Company gases were used. The flow rate of the carrier gas (N_2) was adjusted to 75 ml./min. at 150°; this would, of course, have varied considerably during temperature programmed runs. The carbohydrate solution (generally between 1 and 5 µlitres) was injected using a Hamilton Syringe. The conditions of individual analyses tended to vary. Isothermal runs at 80° and 140° gave good results for glycerol and monosaccharides respectively. Temperature programming between 120° and 180° at 1°/min. also gave a good separation of the monosaccharides, and between 200° and 250° at 1°/min. for the di- and tri-saccharides. Glycerol and the mono- and disaccharides could be analysed together by temperature programming from 100° to 250° at 6°/min.

G.M. 6: The preparation of column chromatographic materials.

1) Charcoal (Ultrasorb SC 120/240) and Celite 535.

These were separately stirred overnight with a large volume of 0.3N HCl, which was afterwards removed by decantation with water (about five times), this also serving to remove

the fines from the charcoal. They were then collected in a Buchner funnel, washed twice with water, and dried overnight at 80°. The celite was passed through a 100 mesh sieve.

2) Anion exchange resins, Dowex 1x4 (200-400 mesh).

(a) Chloride form: The resin was pretreated by suspension in a large volume of water and decanting those fines which failed to settle within 10 mins. The resin was then suspended successively in 1N HCl (15 mins.), water, and 1N NaOH (15 mins.), this cycle being repeated three times before terminating it with two successive washes with 1N HCl.

(b) Borate form: This was prepared directly from the chloride form of the resin by suspending and decanting the resin several times with saturated sodium borate solution.

3) Mixed bed resin.

Biodeminrolit (Permutit) was suspended in water and stirred vigorously for 24 hours and then washed by decantation with water a dozen times. (The initial decantations yielded a white cloudy suspension which had not settled after standing undisturbed for three weeks).

G.M.7: The packing of chromatographic columns.

1) Charcoal columns.

Equal weights of charcoal and celite (10-15 g.) were

mixed to form a thick slurry. This was poured into a glass column to rest upon a bed of celite supported by glass wool. A thick slurry was necessary in order to inhibit the differential sedimentation of the charcoal and celite. Finally the column was washed with about 10 bed volumes of water.

2) Anion exchange columns.

Trapped air was removed from the Dowex-1 borate by placing the resin in a slight vacuum for 10 mins.; this was not necessary with the chloride form. The resin was suspended in either 1M HCl or 0.5M $Na_2B_4O_7$ and poured into the column to rest upon a bed of acid washed sand.

Care was exercised to place and maintain the columns in a vertical position.

G.M. 8: Methods for assaying column chromatography fractions.

These are similar to the general colorimetric methods to be described in G.M. 9, but more emphasis was placed upon broad applicability and convenience for assaying a large number of fractions at once, rather than upon accuracy.

1) For carbohydrates.

(a) Anthrone-sulphuric acid.

Reagent: Anthrone (2g.) dissolved in 80% H_2SO_4 (1 litre). Prepared fresh every 5 days.

Procedure: Sample (0.5 ml.) mixed with reagent (2.5 ml.) and heated at 100° for 10 mins. O.D. measured at 600 mμ.

(b) Phenol-sulphuric acid (169).

Reagents: i) 5% aqueous phenol. ii) H_2SO_4 (A.R. grade essential).

Procedure: Sample (1.0 ml.) mixed with phenol (1.0 ml.) and H_2SO_4 (5.0 ml.) added as rapidly as possible whilst shaking. After standing for 10 mins. the solutions were cooled and the O.D. measured at 490 mμ.

2) Phosphate esters.

Reagents: i) Perchloric acid 60% (S.G. 1.54). ii) ammonium molybdate (0.6% solution). iii) reducing agent, 1-amino-2-naphthol sulphonic acid (0.5 g.), $NaHSO_3$ (30g.)(Or if $NaHSO_3.H_2O$ 34.5 g.), Na_2SO_3 (6 g.), dissolved in water to 250 ml. and filtered if necessary. The solution was stable for 2 weeks when stored in a dark bottle.

Procedure: Samples were taken in batches of 22 or less (0-2 ml.) and to each were added 2 or more small boiling chips. They were evaporated to dryness using a micro-Kjeldahl digestion unit (Gallenkamp). Allowed to cool briefly (30 secs.) and (i) added (1.2 ml.) using an automatic pipette; digested for several mins. or until any brown colour had disappeared. Removed from the

digestion unit and (ii) added (8.5 ml.) again automatically. Then (iii) (0.5 ml.) added and the samples shaken. After standing for at least 15 mins. the O.D. was measured at 660 mμ.

3) Nucleotides.

These were assayed upon the eluate directly by measuring the O.D. at 260 mμ. using a Unicam SP 500.

G.M. 9. Methods for estimating compounds in solution.

Colour intensity measurments were usually made with a Unicam SP 500 or, rarely, with an EEL Absorbtiometer. Latterly, Section IV, a Bausch and Lomb Spectronic 20 was used for all measurments.

1) Phosphate esters.

Reagents: As in G.M. 8-2 except for (ii) being 5% ammonium molybdate.

Procedure.

a) Inorganic phosphate. To a solution of the sample were added reagents (i) (1.0 ml.), (ii) (1.0 ml.), (iii) (0.5 ml.) and the volume diluted to 10.0 ml. with water. The contents were mixed and the solution allowed to stand for 15 mins. after which the colour was measured at 660 mμ. The phosphate content was estimated by comparison with a standard curve prepared in the same manner and whose continuing accuracy was frequently checked.

b) 'Acid labile phosphate' was measured as the inorganic phosphate after heating the sample with 1.0M HCl for 7 mins. in boiling water.

c) Total phosphate. was determined as inorganic phosphate after combustion of the organic matter by heating the sample with (i) (1.2 ml.) until a clear solution resulted. If necessary more perchloric acid was added to complete the process, otherwise the inorganic phosphate was determined without adding more perchloric acid.

2) Carbohydrates.

For reasons discussed in Section II a fresh standard curve was determined for each separate estimation. For this solutions containing 100.0 µg/ml. were customarily used.

a) reducing power by the Somogyi(1952)-Nelson colorimetric method (177,178).

Reagents: (i) Somogyi (1952), Rochelle salt (12 g.) and anhydrous Na_2CO_3 (24 g.) were dissolved in water (250 ml). A solution of $CuSO_4.5H_2O$ (4 g.) in water was added followed by $NaHCO_3$ (16 g.). A solution of Na_2SO_4 (180 g.) in water (500 ml.) was boiled to expel air. The solutions were combined and diluted to 1 litre; after standing for one week in a warm place the clear solution was used.

(ii) Nelson, ammonium molybdate (25 g.) dissolved in

water and H_2SO_4 (21 ml.) added. To this was added a solution of Na_2HAsO_4 (3 g.) in water (25 ml.) and the mixture kept at 37° for 48 hours. Solution stored in the dark.

Procedure: 10 ml. volumetric flasks were fixed rigidly into a large test tube rack to minimise vibration. Standing these in iced water the air was flushed out of them with a stream of nitrogen and up to 5 ml. of sugar solution added. The flasks were refilled with N_2 and (i) (1.0 ml.) added leaving the stoppers loosely in place; they were then placed in vigorously boiling water for 30.0 min. After about a minute of heating the flasks were refilled with N_2 and the stoppers tightened to minimise the ingress of air. The flasks were cooled in iced water for 5 min. and (ii) (1.0 ml) added and the contents shaken by gently swirling the rack. 15 mins. from this time the solutions were ready to have their colour intensity measured. Meanwhile the flasks were removed from the rack and the solutions diluted to 10.0 ml. with water. It was found that consistently better results were obtained if the differences in O.D. between 640 mμ and 480 mμ were correlated to sugar concentration rather than O.D. at a single wavelength.

b) cysteine-sulphuric acid (175).

Reagents; (i) H_2SO_4 (86%) (ii) L-cysteine hydrochloride

monohydrate (3% solution).

Procedure: To the sample (1.0 ml.) was added under cooling (i) (5.0 ml.). After 2 min. the sample was gently shaken in the iced water then taken out and stirred vigorously, placed 1 min. in tap water then heated (3 min.) in vigorously boiling water. Samples were cooled in tap water and (ii) added (0.1 ml.). O.D. measured at various wavelengths after not less than 15 min.

c) GADH-Somogyi method.

Reagents: (i) Enzyme solution, prepared in a crude form from a commercial preparation (Deoxygenase, Takamine Laboratory Inc.) by mixing the powder with 10 volumes of water (w/v) for 5 min. and removing the undissolved material by centrifuging. Portions of this were deactivated by boiling the preparation for 5 min. and removing the coagulated protein.

(ii) Buffer solution, 1.0M sodium acetate adjusted to pH 5.5 with dilute acetic acid.

Procedure: Oxidations were carried out in a wide mouthed boiling tube wherein sugar solution, (5ml) (i) (0.5ml) and (ii) (2.0 ml.) were shaken vigorously overnight. The residual reducing power was estimated by G.M. 9-2a.

d) GADH-peroxidase-ortho-tolidine (174).

Reagent: o-Tolidine HCl (0.7 g. CARCINOGENIC) dissolved in

2M acetic acid adjusted to pH 4.15 with NaOH. Also Deoxygenase (0.2 g.) and peroxidase (0.015 g., B.D.H., preparation from horse radish) dissolved in a small volume of water. The solutions were mixed and diluted to 300 ml. with buffer. Stable for 6 weeks at 3°.

Procedure Sample (2.0 ml.) and reagent (1.5 ml.) incubated at room temp. for not less than 1 hour. O.D. read at 365 mμ.

e)Ketohexose, resorcinol reagent (170).

Reagents (i) resorcinol, recrystallized from benzene, was dissolved in ethanol (0.15%, w/v). (ii) HCl containing $FeCl_3$ (7.5 mg. per litre)

Procedure: The sample (1.0 ml.) was mixed with (i) and (ii), (1.5 ml. each) and heated at 75° for 30 min. in a tube loosely stoppered with a glass marble. After cooling the colour was measured at 480 mμ.

f)Pentose, phloroglucinol reagent (180).

Reagent: Acetic acid (110 ml.), HCl (2 ml.), phloroglucinol in ethanol solution (5% w/v, 4.5 ml.), glucose (1% soln. w/v, 0.3 ml) For reasons and on occasions discussed in the text glucose was sometimes omitted from this reagent.

Procedure: Sample (0.4 ml. or 0.5 ml. when glucose was omitted from the reagent) mixed with reagent (5.0 ml. freshly prepared)

and heated in boiling water for 15 min. After cooling the O.D. was measured at 552 mμ.

g) Inositol

Reagents: (i) 1M H_2SO_4, (ii) 0.03M $NaIO_4$.

Procedure: Sample (<1 ml.) made up to 1.0 ml. and (i) and (ii) added (0.1 ml. each). Shaken and kept in the dark for 18 hours. Volume diluted to 10.0 ml. with water and O.D. measured at 230 mμ.

h) Glycerol (195)

Reagents: (i) 1M H_2SO_4, (ii) 0.1M $NaIO_4$, (iii) 13.13% w/v $NaAsO_2$, (iv) Na salt of chromotropic acid (1.08 g., 4,5-dihydroxy-2,7-naphthalene disulphonic acid) dissolved in water (100 ml.) and mixed with 65% (v/v) H_2SO_4 (450 ml.). Stable for 1 week kept in a dark bottle.

Procedure: Sample (1.5 ml.) mixed with (i) and (ii) (0.5 ml. each). After standing for 5 min. (iii) added (0.5ml.). Shaken and allowed to stand for 10 mins. then water (2.0 ml.) added. An aliquot, (1.0 ml.) was heated with (iv) (10.0 ml.) in boiling water for 30 min. After cooling, the O.D. was measured at 580mμ.

G.M. 10. The precipitation of inorganic phosphate.

Magnesia mixture was prepared having the concentrations 1.0M $MgCl_2$ and 1.0 M NH_4Cl, being adjusted to pH 8.7 with NH_4OH (approx 1.8M)

The mixture was added to 20 volumes of solution. After standing at 0° for 1 hour the precipitate was filtered off leaving a solution free of inorganic phosphate. The precipitate was usually suspended in water and redissolved by lowering to pH 4 with dil. HCl and then reprecipitated by readjusting to pH 8.7 with ammonia and then the solution filtered. The filtrate was added to the previous one.

G.M. 11. The isolation of phosphate esters; anhydrous methanol-acetone precipitation.

The ester-containing solution was if necessary converted to the lithium salts by passage over a column of cation exchange resin in the lithium form and neutralising the solution with HCl. Water was removed upon a rotary evaporator at a low bath temperature; if there were borate ions to be removed frequent additions of methanol were made during the concentration. The pH of the solution was checked when it had concentrated to a small volume and readjusted if necessary. The residue was rendered anhydrous by azeotropic distillation with dry methanol, or, if there were more than traces of LiCl present, by alternate co-evaporations with dry pyridine and benzene, finishing with methanol. The residue was dissolved in a small volume of anhydrous methanol and the phosphate esters precipitated by the addition of an excess volume of dry acetone. Not all carbohydrate phosphates are soluble in methanol but these could safely be ignored since the

purpose of this treatment is to remove LiCl which is itself readily soluble in methanol. After allowing the precipitate to stand overnight it was collected by centrifugation and washed with a few cc's of acetone. The supernatant acetone was always examined for the presence of phosphate and if any was found to be present the isolation procedure was repeated.

G.M. 12. The preparation of anhydrous solvents.

In this work 'Analar Grade' chemicals were used wherever possible. When in G.M. 9 and 10 an acid is mentioned it is always the Analar concentrated chemical which is referred to. Except for those itemised below Analar solvents were sufficiently pure to be used without further drying.

a) Methanol. This was prepared by a Grignard reaction for which a small amount of dry methanol was essential to initiate the reaction (250 ml.). To a reaction flask (5 litres) containing clean dry magnesium turnings(15 g.) and a trace of resublimed iodine, dry methanol was added dropwise with the liberation of heat and hydrogen. After a brisk reaction had been established the methanol to be dried (1.9 litres) was added dropwise with stirring. When addition was completed the contents were refluxed for 2 hours and then the dry methanol recovered by distillation.

b) Acetone. This was dried by shaking it continuously for about 18 hours with anhydrous Na_2SO_4 followed by distillation. The dried acetone was stored over molecular sieve.

c) Ethanol., (4 litres) was dried by adding Na (28 g.) followed by diethyl phthalate (110 g.) under anhydrous conditions. After refluxing for 2 hours the ethanol was redistilled, the first 25 ml. being rejected.

d) Pyridine. For general use.

This solvent was purified by refluxing (2.5 litres) with BaO (250 g.) under anhydrous conditions until the refluxing temperature rose to 113-115°; if this was not achieved after 6 hours a further addition of BaO was made (250 g.) and the refluxing continued until the temperature was between these limits. The fraction distilling at 113-115° was collected and stored over NaOH pellets.

For G.L.C.

The pyridine was further purified by refluxing 500 ml. over p-toluene sulphonyl chloride (100 g.), distilled and refluxed over solid KOH (100 g.) and again redistilled; the dry pyridine was stored over KOH pellets (221).

G.M. 13. The isolation of carbohydrates for chromatography.

1) Hydrolysis of phosphate esters.

(a) Acid hydrolysis was done in a sealed vial in an oven at

100°, or for short periods, in boiling water, using various strengths of acid.

b) Enzymatic hydrolysis

Acid phosphatase (preparation from wheat germ, B.D.H.) substrate (1.0 ml.), 0.2M Mg acetate pH 5.0 (0.1 ml.) enzyme solution (10 mg/ml., 0.05 ml.), Total volume 1.15 ml. or multiples thereof.

Alkaline phosphatase (preparation from intestinal mucosa, B.D.H.), substrate (1.0 ml.), 0.2M glycine pH 9.0 (0.1 ml.) 0.1M $MgCl_2$ (0.1 ml.), enzyme solution (10 mg/ml., 0.05 ml.) Total volume 1.25 ml. or multiples thereof. Before using it this enzyme was purified by dialysis for 12 hours against 0.015M magnesium acetate (10 litres) at pH 6.8 and at 4°.

Both digests were incubated at 37° for 2 days or until such time as the inorganic phosphate approached the total phosphate (95% or more).

2) Purification of the carbohydrates.

a) Removal of protein (208).

Reagents (i) $ZnSO_4.7H_2O$, 2.0% w/v. (ii) $Ba(OH)_2.8H_2O$ 1.8% w/v.

Procedure. To the sample (1.0 ml.) was added (i) and (ii)

(0.5 ml. each) and the mixture centrifuged until clear.

b) Deionisation.

The supernatant solution was passed through a column of Biodeminrolit mixed bed resin (1x10 cm.) which was washed with about 10 bed volumes of water and the total eluate evaporated to dryness.

G.M. 14. Maintenance of sterile conditions.

To inhibit the growth of bacteria, chloroform (1-2 ml.) was added to solutions likely to become so contaminated.

EXPERIMENTS OF SECTION I

Expt. 1: The extraction of nucleotides from starch by dissolution of the granule in boiling water.

Commercial potato starch (140 g., Hopkins and Williams) was suspended in a small volume of cold water, poured into boiling water (7 litres) and boiled for 1 hour. The starch was coagulated and removed by repeated additions of an equal volume of methanol followed by concentration to a smaller volume until the solution was once again viscid with starch. This was continued until the solution was concentrated to 14 ml. whereupon it was poured into methanol (58 ml.) and the light precipitate removed by centrifuging to give a clear yellow solution. The u/v spectrum of this solution was strongly reminiscent of nucleotide, the $O.D._{260\ m\mu}$ and the phosphate content are recorded in Table 3 (page 57).

Expt. 2: The extraction of starch by a batchwise treatment with 50% aqueous methanol.

Commercial potato starch (5.2 kg.) was mixed with 50% methanol and shaken mechanically for 5 hours at room temperature. The starch was allowed to settle overnight and the supernatant decanted; if cloudy it was filtered. Two further treatments with aqueous methanol were carried out as described above; the volumes of solution and the $O.D._{260\ m\mu}$ readings were recorded and the extracts combined.

The results of this and other starch grain extractions are recorded in Table 3.

Expt. 3. The extraction of starch with 50% aqueous methanol by a continuous flow column technique.

Commercial starch (14 kg.) was mixed with aqueous methanol and poured into a large glass column (30 cms. internal diameter) to rest upon a broad plug of glass wool. Elution was started immediately and monitored by following the $O.D._{260\ m\mu}$ of the eluate as it rose from an initially low value (0.6) to a maximum (1.8) and then declined gradually to 0.05 whereupon elution was stopped. The combined eluate was concentrated by vacuum distillation below 30°.

Expt.4. The extraction of 48.5 kg. of potato starch.

The starch was extracted by a column process as described in Expt. 3. 6 separate columns were used taking 14, 10, 10, 7.5, 4 and 3 kg. of starch. The overall results are summarised in Table 3; in the course of these extractions several observations were made:-

a) Initial delay in the elution of metabolites.

Elution from the starch was initially slow and rose to a maximum before tailing off gradually. This though was not as might be expected because the first few litres had not passed entirely through the column, coming into contact with all the starch. Since

the density of starch is approximately 1.5 g./ml., 14 kg. of starch would occupy about 9.3 litres. The void, intergranular volume of such a column, or indeed the void volume of any packed starch was not determined but it would have been less than 8.8 litres, which as may be seen in Fig. 3 (page 56) was the volume of effluent which passed before the elution of metabolites was maximal.

b) The cause of the dual peak in the elution of 14 kg. of starch, Fig.3.

After this column had been eluted with 11.4 litres of aqueous methanol, the flow of effluent was stopped for 24 hours. Upon resumption, the level of metabolites in the effluent had increased to a new maximum and a high level of elution was maintained for several litres. This suggests that elution of metabolites from the grains is a continuous process dependent more upon the concentration within the granule than in the extracting fluid.

c) The efficiency of extraction of variously sized columns.

Considering the extraction of 2 columns of 14 and 3 kg. of starch respectively: The initial rate of elution gave O.D.$_{260\ m\mu}$ of 0.59 and 0.71 respectively. These rose to maxima of 1.72 after 8.8 litres and 1.49 after 2.2 litres and then fell gradually to 0.065 after 59.6 litres and the 3 kg. to 0.07 after 24.2 litres. Thus to effect the same diminution in the level of elution of metabolites required respectively 4.25 and 8.1 litres of aqueous methanol per kg. of starch extracted.

Expt. 5. The exhaustive extraction of starch with 50% and 10% aqueous methanol.

Starch (1kg.) was eluted with 50% aqueous methanol (15 litres) until the O.D.260 mμ of the eluate had fallen to 0.035. After further elution (10 litres) it had only decreased to 0.02; continued elution at this level would have removed altogether 200 O.D.U. Percolating 10% methanol (10 litres) through the column of starch eluted 400 O.D.U. together with further organic and inorganic phosphate (G.M. 9-1) but no more sucrose or glucose. These results are summarised in Table 4 (page 59, see also Expt. 59).

EXPERIMENTS OF SECTION II

Expt. 6. Identification of the sugars extractable from starch with 50% aqueous methanol.

(1) Preliminary identification of the sugars.

A 50% aqueous methanol extract of starch was concentrated and a portion of it deionised (G.M. 13-2b). This was concentrated to a yet smaller volume (0.5 ml., approx. 100 g. of starch extracted) and examined by paper chromatography using solvents a-e (G.M. 1), the chromatograms being developed with alkaline silver nitrate and α-naphthol-phosphoric acid (G.M. 3-1a&b). The results, summarised in Table 5 (page 64) were consistent with the presence of sucrose,

glucose, fructose, maltose and ribose.

(2) Confirmatory chromatography.

None of the conventional solvents mentioned above would separate fructose from mannose and arabinose. For this, it was necessary to use solvent f, methyl ethyl ketone - ammonia (99 - 1, v/v). The addition of ammonia to the ketone caused the formation of a fine precipitate which could not be redissolved and after standing for 24 hours the solvent turned bright yellow. This was ignored and chromatography proceeded with freshly prepared solvent. The descending solvent front reached the bottom of the paper after 6 hours (50 cms.) but after irrigation for 72 hours, glucose had only migrated 1.6 cms. whilst glycerol was eluted from the paper. Inositol, maltose and sucrose all remained at the origin.

Expt. 7: The separation of free sugars from sugar phosphates and nucleotides - a model experiment.

A solution (1 litre) of glucose (2 g.) and G-1-P (20 mg.) was adjusted to pH 8.4 with LiOH and applied to a column of Dowex-1x4, Cl^-, (200-400 mesh), 2x14 cms. The column was washed with water (500 ml.) until the eluate contained no more material reacting positively with anthrone. The water was then replaced by 0.1M LiCl (unbuffered, 1 litre), which eluted carbohydrate and phosphate coincidentally (Fig. 5, page 66). The recovery of the phosphate was

estimated to be approximately 106%.

Expt. 8. The separation of the free sugars from the sugar phosphates and nucleotides extractable from starch.

This was virtually a repeat of the previous experiment using the solution (900 ml.) prepared by extracting 5.4 kg. of starch. This solution is described more fully in Table 3 and also under Expt. 27. The column (2x14 cms.) was washed with water (3 litres) adjusted to pH 8.4 with LiOH, followed by 0.1M LiCl (1 litre) Fig. 6 page 66. Peak A contained no phosphate. Aliquots (20 ml.) of peaks B & C were passed through a small column of mixed bed resin; the eluate of this contained no carbohydrate (G.M. 8b) or lithium ions (flame test). The absence of carbohydrate was confirmed by chromatography (solvent a) of the entire concentrated eluate from the mixed bed resin. Peak A contained free sugars, peaks B & C, sugar phosphates, peak D unknown, peak E , AMP and there were traces of UMP between peaks D & E (G.M. 1). Peak D gave a purple chromophore with phenol-sulphuric acid (λmax, 590 mμ), previously unreported. The following compounds were tested with this reagent but did not give the same reaction:- glycerol, sorbitol, 2-deoxyribose, myo-inositol, gluconic acid, glucuronic acid, glycerophosphate, ethanolamine phosphate, glycine, alanine, aspartic acid, cysteine, taurine, myristic acid, stearic acid, and oleic acid.

Expt. 9. The separation of mono- and disaccharides using charcoal column chromatography - a model experiment.

Charcoal (Ultrasorb SC 120/240) and Celite (535) were purified for chromatography (G.M. 6-1) and a column prepared (3x20 cm). To this was applied fructose, sucrose, maltose and raffinose (50 mg. each). The column was then eluted successively with water (2.7 litres), 4% ethanol (1.3 litres), 7.5% ethanol(4 litres) and 15% ethanol (2 litres). Fractions (25 ml.) were assayed by the phenol-sulphuric acid method after an attempt to use optical rotation had failed; the results can be seen in Fig. 7, page 67.

Expt. 10. The separation of the mono- and disaccharides extractable from starch.

With slight modifications this experiment was a repeat of the previous one using the concentrated extract (45 ml.) from 960 g. of starch. Elution was commenced immediately with 2% ethanol (2.5 litres) followed by 7% ethanol (1.6 litres) and 9% ethanol (1.9 litres). The separation was performed in a constant temperature (19°) almost dust free room, collecting fractions (25 ml.) at the rate of 40 per day; the fractions were assayed by the phenol-sulphuric acid procedure (Fig. 8, page 69). The fractions containing carbohydrate were bulked together and a few grams of $BaCO_3$ added. They were concentrated and made up to a standard volume after

removing the $BaCO_3$ by filtration. In this way any risk of hydrolysing the acid labile sucrose linkage was avoided. A small aliquot of each was further concentrated and examined by paper chromatography (solvent a).

Expt. 11. Attempts to identify the unknown carbohydrate in Fraction III of the previous experiment.

Several chromatograms of Fraction III were run and developed with various reagents in an attempt to elucidate the nature of this carbohydrate.

(1) Alkaline silver nitrate (G.M. 3-1a); a positive reaction, $R_{glucose} = 26$.

(2) Periodate-benzidine (G.M. 3-1c); a positive reaction.

(3) Benzidine-TCA (G.M. 3-1d); no visible reaction.

(4) Aniline phthalate (G.M. 3-1e); no visible reaction.

(5) α-Naphthol-phosphoric acid (G.M. 3-1b); no visible reaction.

Expt. 12. Confirming the identities of maltose and sucrose enzymatically.

Fraction VI, isolated in Expt. 8, appeared chromatographically to contain equal proportions of maltose and sucrose, (solvent a. G.M. 3-1a). Aliquots (1.0 ml.) were incubated overnight with (i) invertase (B.D.H., yeast concentrate) (ii) maltase (equine serum, 55)

(iii) maltase and invertase together. The incubate was examined directly by paper chromatography. In digest (i) there was present maltose, glucose and fructose; in digest (ii) sucrose and glucose and in (iii) only glucose and fructose.

Expt. 13. Demonstrating the specificity of the resorcinol reaction for ketohexoses (G.M. 9-2e).

This reaction was demonstrably linear with respect to fructose concentration up to 120 µg/sample. Samples were prepared containing no fructose but between 50 and 400 µg. of glucose and ribose. When treated with the resorcinol reagent these gave solutions virtually indistinguishable from blank specimens containing only water. Similarly, samples were prepared containing 50 µg of fructose and between 0 and 300 µg of glucose or ribose. These were all indistinguishable regardless of the aldose content.

Expt. 14. Estimating the fructose concentration of the fractions whose isolation was described in Expt. 10.

For these estimations the resorcinol reagent (G.M. 9-2e) was used in the conventional manner; (a) & (b) refer to separate estimations with different standard curves.

1) Fructose.

(a) Fraction II: 21.5 µg/ml. equivalent to 11.6 µg/g. of

starch extracted.

Fraction III: 20.5 µg/ml. equivalent to 0.5 µg/g. of starch extracted.

(b) Fraction II: 21.25 µg/ml. equivalent to 11.4 µg/g. of starch extracted.

Fraction III: 20.0 µg/ml. equivalent to 0.5 µg/g. of starch extracted.

Hence the mean fructose concentration of Fractions II & III was equivalent to 11.5 µg and 0.5 µg/g. of starch and the total was 12.0 µg. of fructose per g. of starch extracted.

2) Sucrose.

(a) Using the standard curve drawn up to estimate fructose in (a) above.

Fraction V: 59 µg/ml. equivalent to 31.4 µg of fructose per g. of starch or 59.6 µg. of sucrose per g. of starch.

Fraction VI: 68 µg/ml. equivalent to 7.1 µg/g. of starch or 13.5 µg of sucrose per g. of starch.

(b) Using a standard curve made up with sucrose.

Fraction V: 107 µg/ml. equivalent to 56.9 µg of sucrose per g. of starch extracted.

Fraction VI: 118 µg/ml. equivalent to 12.3 µg/g. of starch extracted.

Hence the mean sucrose content of Fractions V & VI was

equal to 58.2 and 12.9 µg. of sucrose per g. of starch and the total was 71.1 µg. of sucrose per g. of starch.

Expt. 15. Studies upon the cysteine-sulphuric acid reaction (G.M.9-2b).

(1) Attempts to eliminate the interference of pentoses from the estimation of hexoses.

Using the cysteine-sulphuric acid reaction two standard curves for fructose were drawn up; one without ribose and one with ribose present in decreasing amounts as the fructose concentration increased. Thus samples contained 100 µg. of ribose and 0 µg. of fructose, 90 µg. of ribose and 10 µg. of fructose, 80 µg. of ribose and 20 µg. of fructose and so on up to 0 µg. of ribose and 100 µg. of fructose. Using the sample containing only ribose the O.D. at 414 mµ was measured and the isochromatic wavelength sought, this rising with time from 358 mµ to 380 mµ. Readings were taken when the isochromatic wavelength was 360 mµ. The results gave two convergent standard curves showing that the interference of ribose was not eliminated by this method.

(2) Estimating glucose in the presence of a constant amount of ribose.

Two standard curves for glucose were derived, one without ribose and the other with the addition of 10 µg. of ribose per sample. The O.D. was measured at a single wavelength (414 mµ). Parallel

curves were obtained, the one containing ribose being slightly higher than the other.

(3) Attempts to obtain a correlation between the fructose and glucose moieties of sucrose.

Using standard solutions of fructose and glucose it was found the fructose gave 112% of the reaction given by the same amount of glucose. Taking a sucrose solution of known fructose content (58.2 µg/ml., G.M. 9-2e), the carbohydrates were estimated by the cysteine-sulphuric acid reaction (O.D.$_{414\ m\mu}$ = 1.50) and the contribution of the fructose to this absorbance determined from the appropriate standard curve (0.96 @ 414 mµ). Hence the absorbance due to glucose was determined (1.50 - 0.96 = 0.54) to be equivalent to 38 µg. of glucose per ml.

Expt. 16. Studies upon Glucose aerodehydrogenase (GADH).

(1) Progress curve to determine the time necessary for a complete reaction to occur.

A digest was set up containing twice the volumes listed in G.M. 9-2c and the reaction initiated by the addition of enzyme solution; a comparative digest using deactivated enzyme was also set up. Aliquots (1.0 ml.) were withdrawn at intervals (30 secs, then ½ hourly) and the reaction stopped immediately by pipetting the aliquot into a tube immersed in liquid nitrogen. After 8 hrs. the residual

reducing power of all the aliquots was estimated (G.M. 9-2a), adding the copper reagent before the aliquots had thawed so that no further oxidation could occur. The results are illustrated in Fig. 9, page 76.

(2) Specificity of the enzyme.

Digests were set up containing various sugars - glucose, fructose, ribose, maltose, sucrose, water (300 µg. each). After shaking for 10 hrs. the residual reducing power was estimated (Table 6, page 77). Paper chromatography (solvent a) confirmed the results. Note that the solution containing protein but no carbohydrate exhibited no reducing power which meant that the protein did not interfere with the Somogyi-Nelson technique.

Expt. 17. Estimations of glucose concentration.

These were done by the cysteine-sulphuric acid method and by the GADH-Somogyi Nelson technique.

1) Cysteine-sulphuric acid technique.

Fraction II: Mean O.D. 414 mµ = 0.77. Interference due to 21.5 µg. of fructose per ml. = 0.35 O.D.U. hence glucose accounted for 0.77 - 0.35 = 0.42 O.D.U. which equalled 26 µg/ml. equivalent to 13.8 µg/g. of starch extracted.

Fraction III: Mean O.D. 414 mµ = 0.48. Interference due to 20 µg. of fructose per ml. = 0.34 O.D.U., hence glucose accounted for 0.48 - 0.34 = 0.14 O.D.U. = 6 µg/ml. equivalent to 0.2 µg/g. of starch.

2) Somogyi-Nelson technique.

Δoptical density 640 - 480 mμ. (These trivial values are quoted here because this method is so notoriously unreliable).

Fraction II: Before GADH oxidation, 0.500, 0.515, 0.474, 0.525, mean value 0.50.

After GADH oxidation, 0.295, 0.266, 0.330, 0.298, mean value 0.30. Hence change in O.D. due to oxidation of the glucose = 0.20 = 22 μg. per ml. which was equivalent to 11.7 μg/g. of starch extracted. For reasons explained in the text this value was more acceptable than the previous one so that the glucose content of Fraction II was taken as being equivalent to 11.7 μg/g. of starch extracted.

Fraction III: Before GADH oxidation 0.251, 0.290, 0.269, mean value 0.27.

After GADH oxidation 0.251, 0.239, 0.270, mean value 0.25. Any difference in these figures cannot be regarded as significant so it was necessary to accept the result of the cysteine-sulphuric acid method for the glucose content of Fraction III.

Hence the mean glucose content of Fractions II & III was equivalent to 11.7 and 0.2 μg/g. of starch and the total 11.9 μg. of glucose per g. of starch extracted.

Expt. 18. The effect of hexose upon the phloroglucinol reaction with pentoses.

A standard curve for ribose (0 - 50 µg) was drawn up using the reagent containing glucose (350 µg/sample, G.M. 9-2f). A similar curve was then constructed containing an additional 100 µg. of fru ctose per sample. Convergent straight lines were obtained showing that the fructose was not adequately compensated for by the glucose present in the reagent.

Expt. 19. Estimations of ribose concentration.

Both the phloroglucinol reagents were used for estimating ribose (G.M. 9-2f).

1) Uncompensated for hexose, with glucose in the reagent.

Fraction II. 8.75 µg/ml. equivalent to 4.6 µg/g. of starch.

Fraction III. 10.6 µg/ml. equivalent to 0.3 µg/g. of starch.

2) With the fractions adjusted to have the same hexose concentration and adding the same amount of glucose to each sample of the standard curve, there being no glucose in the reagent.

Fraction II. 7.0 µg/ml. equivalent to 3.7 µg/g. of starch.

Fraction III. 9.25 µg/ml. equivalent to 0.2 µg/g. of starch.

These latter values were taken as being the correct ones and so the total ribose content was 3.9 µg. per g. of starch extracted.

Expt. 20. Estimations of maltose concentration.

As this fraction contained only sucrose and maltose, a

variety of techniques could be employed to estimate the maltose.

1) By the reducing power of the maltose using the Somogyi-Nelson technique. (G.M. 9-2a)

Δ O.D. 640 - 480 mμ: 0.23, 0.43, 0.25, 0.32. mean value = 0.32 = 46 μg. maltose per ml. which was equivalent to 4.8 μg per g. of starch extracted. However the values which were averaged to give this figure varied widely, and it could not be taken as an accurate result.

2) By the reducing power of glucose following hydrolysis with equine maltase.

An aliquot of the solution (5.0 ml.) was incubated with a preparation of equine maltase (0.25 ml.) and the total reducing power measured.

O.D. 640-480 mμ: 0.44, 0.44, 0.44, 0.44. Mean value = 0.44 = 41 μg. glucose/ml. or 41 μg. of maltose/ml. (The solution being assayed was diluted 5% in the course of the assay; adjusting for this dilution cancelled the difference of a molecule of water between glucose and maltose). This was equivalent to 4.3 μg./g. of starch extracted.

3) By measuring the total glucose concentration following hydrolysis with maltase and invertase, then subtracting the glucose which arose from sucrose.

ΔO.D. 640-480 mμ: Before GADH oxidation: 0.87, 1.06, 0.96.

Mean value = 0.96.

After GADH oxidation: 0.46, 0.47, 0.59.

Mean value = 0.51.

Hence the change in the O.D. due to the oxidation of glucose was 0.96 - 0.51 = 0.45 O.D.U. = 32.5 μg. glucose, which, after compensating for the various dilutions involved in the estimation was equivalent to 103 μg. glucose/ml. Of this, 62 μg. would have arisen by hydrolysis of the sucrose present, leaving 41 μg./ml. from the hydrolysed maltose, or 39 μg. maltose/ml., which was equivalent to 4.1 μg./g. of starch extracted.

EXPERIMENTS OF SECTION III

Expt. 21: Demonstrating the presence of G-6-P in the 50% aqueous methanol extract of starch.

Various preparations of starch extract were incubated with D-glucose-6-phosphate dehydrogenase, and the course of their oxidation, if any, followed by noting the changes in absorbance of the solution at 340 mμ. The standard digest contained: 0.1M Tris-acetate buffer pH 6.45 (0.3 ml.), 0.01M $NADP^+$ (0.2 ml.), G-6-P-dehydrogenase (1 mg.), water, and starch extract preparation to a total volume of 2.0 ml. (182). The reaction was started by addition of the enzyme and followed using

a Unicam SP 500. The results can be seen in Fig. 10, page 82.

Curve 1: This is the standard digest to which was added G-6-P (4 μmole) in order to demonstrate that the system was viable.

Curve 2: Starch (5.2 kg.) was extracted in a batchwise manner with 50% aqueous methanol and the solution concentrated to 500 ml. by vacuum distillation (Table 3, Page 57). An aliquot of this solution (1.0 ml. equivalent to 10 g. of starch) was added to a standard digest. No reduction of $NADP^+$ was observed and after 10 mins. inactivity, G-6-P (6 μmole) was added and was rapidly oxidised.

Curve 3: To an aliquot of the starch extract (100 ml., equivalent to 1.04 kg. of starch) was added $Mg(CH_3COO)_2.H_2O$ (2.0 g.), and the solution adjusted to pH 8.5 with NH_4OH. The mixture was allowed to stand at 0^o for 3 hours and the precipitate removed by centrifuging to give a clear bright green solution. 1.0 ml. of this solution gave a very slow reduction of $NADP^+$ and after 20 mins. G-6-P (6 μmole) was added, to be rapidly oxidised.

Curves 4 and 5: After precipitating the Pi from some of the starch extract with magnesia, the magnesium and ammonium ions were exchanged for lithium ions by passage over a small column of Dowex-50 Li^+ (10xlcm.), and the phosphate esters isolated by anhydrous methanol-acetone precipitation (G.M.11). The material

insoluble in methanol was segregated from that which dissolved. The latter was precipitated with anhydrous acetone. Both precipitates were dissolved in a small volume (5.0 ml.) of water and tested in standard digests. The acetone-precipitated material gave a slow reduction of $NADP^+$ (Curve 4), whilst the "methanol precipitate" gave rise to a comparatively fast reduction of $NADP^+$ (Curve 5). It would appear that, compared with nucleotides and other sugar phosphates, G-6-P is only slightly soluble in anhydrous methanol.

Expt. 22: The anion exchange separation of sugar phosphates as borate complexes, using a chloride based system - a model experiment.

A mixture of G-1-P and G-6-P (20 µmole each) was converted to the lithium salts by passage over a small (1x10 cms.) column of Dowex-50, Li^+ form, the eluate being adjusted to pH 8.5 with LiOH. This was applied to a column of Dowex-1x4, Cl^- (1.2 x 20 cms.) and washed into the resin with a few c.c.s of water. The sugar phosphates were then eluted from the column using a convex gradient consisting of:

Mixing Chamber:- 600 ml., 0.02M LiCl and 0.04M H_3BO_3, adjusted to pH 8.4 with LiOH.

Reservoir:- 1,200 ml., 0.04M LiCl, pH 8.4.

The column was run at 4° and fractions (21 ml.) were collected at the rate of two per hour. They were assayed by the anthrone procedure to give the histogram seen in Fig. 11, page 84. Unfortunately, the fraction collector stopped before elution of the G-6-P was completed, but sufficient fractions had been collected to ascertain the separation between G-6-P and G-1-P.

Expt. 23: Anion exchange chromatography of sugar phosphates using Dowex-1 borate and ditriethyl ammonium tetraborate (TEAB) - a model experiment (1).

Dowex-1x4 Cl^- was converted to the borate form (G.M.6-2b) and a column prepared (0.5x45 cms.). To this was applied a solution (10 µmole each) of G-1-P and G-6-P adjusted to pH 8.0 (NH_4OH), which was washed into the resin with water (5 ml.). The column was then eluted using a linear gradient:

Mixing chamber:- 180 ml., 0.1M TEAB.

Reservoir:- 180 ml., 0.4M TEAB.

(The eluting species was prepared by dissolving boric acid (98.8 g.) and triethylamine (113 ml., redistilled, B.P. 89-90°) in water and making the volume up to 1 litre - giving a 0.4M solution of TEAB). Fractions (2 ml.) were collected at the rate of five per hour and assayed by the anthrone procedure (Fig. 12(a), page 86).

The fractions of each peak were bulked, evaporated to

dryness, and the TEAB removed by co-evaporation with methanol, three co-evaporations being sufficient. The residue was dissolved in water (1 ml.) and the peaks identified by T.L.C. (G.M.2, solvent m). Peak 1, G-1-P, was eluted at 0.175M TEAB and Peak 2, G-6-P, at 0.250M TEAB.

Expt. 24: The separation of sugar phosphates upon Dowex-1x4 borate using triethyl ammonium borate as the eluting species (2).

The specifications of the column and the eluting solution used were identical to those of Expt. 23. To the column were applied 10 μmole each of G-1-P, G-6-P, and F-6-P. Fractions (1.38 ml.) were collected, assayed, and the peaks identified as before. The relative ionic strengths of elution were:- G-1-P, 0.18-0.19M TEAB; F-6-P, 0.21-0.22M TEAB; G-6-P, 0.29-0.30M TEAB (Fig. 12(b)). It is difficult to understand why G-6-P should have been eluted so much later than in Expt. 23; however, such a delay can only represent an amelioration of the technique.

Expt. 25: To demonstrate the necessity of purifying the sugar phosphates present in aqueous methanol extracts of starch before chromatography by borate-based systems.

10 kg. of potato starch granules were extracted with 50% aqueous methanol and the solution concentrated to 1.0 litre.

Assayed by the anthrone procedure, this solution contained 12.2 µmole of "hexose" per ml., of which about 25% would have been monosaccharide capable of complexing with borate. (This estimation is based upon the results described in Section II).

5 ml. of this solution were chromatographed using the system described in Expt.23, and the fractions assayed by the anthrone procedure, to give the histogram shown in Fig. 13, page 88.

Expt. 26: The precipitation of inorganic phosphate with magnesia mixture and the isolation of the organic phosphates from the mixture - a model experiment.

A solution was prepared containing Pi (5.0 µmole/ml.) and G-1-P (0.6 µmole/ml.). Magnesia mixture was also prepared: 1.0M $MgCl_2$, 1.0M NH_4Cl, adjusted to pH 8.7 with NH_4OH; ($Mg(OH)_2$ does not start precipitating visibly until pH 8.9). This mixture was added to the phosphate solution, being diluted twenty times to give a concentration of 50 µmole/ml. of Mg^{++}. After adding the magnesia mixture (0.75 ml.) to the phosphate solution (15.0 ml.), the precipitate was allowed to form for 30 mins. at 0° and then removed by filtration. The vessels and filter papers were washed twice with 0.06M NH_4OH (total volume, 5.0 ml.), and the entire solution passed over a small cation exchange column in the lithium form

(Dowex-50, 1x10 cms.) held at 0°. The emergent effluent was at pH 11.1, and it was immediately neutralised with dil. HCl. This was evaporated to dryness, and the last traces of water removed by co-evaporation with methanol. The G-1-P was recovered by anhydrous methanol-acetone precipitation. After collecting the precipitate by centrifugation, it was dissolved in water (15.0 ml.). At each stage of the procedure the recovery of the G-1-P and the removal of the Pi were checked using the phenol sulphuric acid (G.M.8 -1b) and the King (G.M.9 -1a) methods respectively. These are summarised below.

Stage of the procedure	Optical density measurements			
	Carbohydrate		Inorganic phosphate	
	Observed	Theoretical	Observed	Theoretical
1. Starting material	49	49	285	285
2. After magnesia precipitation	34	35	3	0
3. After conversion to Li^+.	30.5	32	2	0
4. Final solution	47.5	47	4	0

The theoretical O.D. is based upon that of the starting material, taking into account successive dilutions and removal of samples. Repeating the experiment using a mixture of G-6-P (0.6 μmole) and Pi (5.0 μmole) gave similarly satisfactory results.

Expt. 27: The batchwise extraction of potato starch, segregating the first and subsequent extractions.

Starch (6 kg.) was shaken with 50% aqueous methanol (15 litres) for six hours, allowed to settle overnight, and then decanted. The supernatant was concentrated using a climbing film evaporator, and found to contain 11,152 O.D.U.$_{(260\ m\mu)}$. The starch was then extracted three more times in a like manner, which recovered a further 13,784 O.D.U.$_{(260\ m\mu)}$

Expt. 28: The fractionation of the sugar phosphates from the two segregated extracts of potato starch.

1. Separation of the sugar phosphates and free sugars.

Both extracts were treated in an identical manner, being diluted to 500 ml., adjusted to pH 8.5, and poured through columns of Dowex-1x4 Cl^- (2x14 cms.), washed with water and then the sugar phosphates eluted with 0.1M LiCl. The large initial peak (i), (Fig. 14(a), page 91), was assumed to be the sugar phosphates. Peak (ii) was not examined further.

Each peak was concentrated (100 ml.), the Pi precipitated with 1.0M magnesia (5.0 ml.), and the sugar phosphates recovered from the filtrate in the accustomed manner. Both anhydrous methanol-acetone precipitations were successful, and the precipitates

proved readily soluble in water (25.0 ml.). The two solutions contained respectively 233 µmole and 175 µmole of organic phosphate or 0.039 and 0.029 µmole/g. of extracted starch.

2. Ion exchange separation of the isolated sugar phosphates.

This was done using the same conditions as were described in Expt. 23. To columns (0.5x45 cms.) of Dowex-1x4 (borate form) were applied 46.5 µmole and 35.1 µmole of organic phosphate. The columns were then eluted with a linear gradient of increasingly more concentrated TEAB. Fractions (1.50 and 1.67 ml.) were assayed by measuring their total phosphorus content. Histograms of the elution of sugar phosphates can be seen in Fig. 14 (b), page 91.

Expt. 29: Chromatographic identification of the sugar phosphates isolated in the previous experiment.

The fractions constituting each peak were evaporated to dryness and the eluting solute removed by co-evaporation with methanol (4 times). Each residue was dissolved in a small volume of water and examined by T.L.C., using solvents m and n (G.M.2) separately, unidirectionally, or combined in that same order for two dimensional T.L.C.

Peak 1:- remained unidentified, since its mobility agreed with none of the standards available. Further tests were

undertaken. It was a phosphate which was not acid labile, i.e. hydrolysed by 7 mins. heating at 100° in 1M HCl, nor indeed was it hydrolysed by 4M HCl at 100° in 30 mins. Very strong alkali, 2.5M NaOH at 100° for 30 mins. would eventually hydrolyse the ester to produce Pi. It gave no visible reaction with anthrone, orcinol, ninhydrin or cysteine-sulphuric acid. There was no u/v spectrum and the nuclear magnetic resonance spectrum only revealed the protons of the triethyl ammonium cation.

Peak 2:- This was identified by both solvents as Pi and this was confirmed colorimetrically. However, the pronounced shoulder which can be seen in both histograms (Fig. 14(b)) suggests that there may be more than one component in this fraction.

Peak 3:- Was shown by both solvents to contain G-1-P, Pi, and a third phosphate which was not identified.

Peak 4:- Was identified as F-6-P, and this was further confirmed by a positive reaction with the resorcinol reagent (G.M.9 -2e).

Peak 5:- Chromatographically this peak was indistinguishable from G-6-P.

Expt. 30: Estimations of the amount of phosphate isolable as sugar phosphates and comparison with the original extract.

1. The isolation of these sugar phosphates was described in Expt.8 and they constituted peaks B and C of Fig. 6 (page 66). The two peaks were bulked together and concentrated (250 ml.), following which the phosphate content was measured and compared with the original extract.

	Original starch grain extract (per g. of starch)	Peaks B & C (per g. of starch)
Inorganic phosphate	0.63 µmole	0.56 µmole
Organic phosphate	0.17 µmole	0.071 µmole
Acid labile phosphate (G.M.9 -1b)	-	0.020 µmole
Non-acid labile phosphate	-	0.051 µmole

It was noted that the recovery of the inorganic phosphate was only 89%; most of this loss would have arisen from the aliquots removed from each fraction when they were being assayed. In subsequent work, the concentrations of the organic phosphate were adjusted to compensate for this loss.

2. Concentration of phosphate originally present in the starch.

A solution of the same batch of potato starch granules was prepared by boiling the starch (2 g.) with 0.1M HCl (75 ml.)

and adjusting the volume to 100 ml. The total phosphate content of this solution was readily estimated (G.M.9 -1c), although it was necessary to add altogether 4.0 ml. of $HClO_4$ to digest the organic matter completely. The result was 20.6 μmole of phosphate per g. of starch.

Expt. 31: Acid hydrolysis of the sugar phosphates from starch.

An aliquot (42 ml.) of the sugar phosphate solution described in the previous experiment was made 1.0M with respect to HCl, and heated (15 mins.) at 100°. After neutralising the solution (NaOH), it was deionised using a mixed bed resin (Biodeminrolit column 1x20 cms.). The eluate contained no traces of phosphate, chloride, or sodium ions; it was concentrated to a small volume and examined by paper chromatography using solvents a, b, and c (G.M.1). These all suggested that the major components of the hydrolysate were glucose, glycerol, and myo-inositol, with traces of fructose. Making the conditions of hydrolysis more severe, 2.5M HCl, heated at 100° for 2½ hours produced no more sugars in the hydrolysate, merely increasing the density of all the spots.

Expt. 32: Anion exchange separation of the sugar phosphates.

1. Isolation of the sugar phosphates from Peaks B & C (Expt. 8).

As described in Expt. 30, the fractions of these

peaks had been concentrated to 250 ml., equivalent to 5.4 kg. of starch extracted. The inorganic phosphate was precipitated from 150 ml. of this solution (equivalent to 3.4 kg. of starch extracted) by the addition of 1.0M magnesia solution (7.5 ml.). After exchanging the Mg^{++} and NH_4^+ for Li^+, the carbohydrate phosphates were recovered by anhydrous methanol-acetone precipitation and the precipitate dissolved readily in 10 ml. of water, yielding 192 µmole of phosphate (105% recovery - this would have included some inorganic phosphate)..

2. Anion exchange separation of the isolated sugar phosphates.

96 µmole of phosphate (5.0 ml.) were applied to a standard TEAB column, which was eluted in the usual manner and the fractions, when assayed for total phosphate, gave the histogram seen in Fig. 15 (page 95). Upon the basis of previous columns, the peaks were tentatively identified by the molarity of TEAB which eluted them and their relative positions, (see overleaf), although it must be noted that all these are lower molarities than previous usage of this system had indicated. The fractions of each peak were bulked, concentrated and the

TEAB removed by co-evaporation with methanol, so that the peaks could be examined chromatographically.

Peak Number	Molarity of elution	Suspected identity
1	0.12	-
2	0.135	-
3	0.150	G-1-P
4	0.170	
5	0.185	F-6-P
6	0.215	G-6-P
7	0.230	

Expt. 33: Chromatographic characterization of the phosphate esters separated in the previous experiment.

1. Filter paper chromatography using the Leloir solvents (h and i, G.M.1)

This technique proved to be entirely analogous to paper chromatography of the free sugars. The spots could be localised using alkaline silver nitrate, provided the papers were dried at about 100° for an hour beforehand to remove most of the ammonium acetate. Reducing sugar phosphates appeared rapidly in the usual manner but non-reducing esters such as G-1-P appeared only slowly, (slower even than non-reducing carbohydrates such as sucrose) and

unless the NaOH stage was unduly prolonged they gave only a faint brown spot. The results could always be confirmed using acid molybdate spray upon separate chromatograms.

Irrigation times of 36 to 48 hours were necessary, so the mobilities were related to G-6-P, which usually gave a single compact spot. Under these conditions, free carbohydrates were eluted completely from the chromatograms, except for myo-inositol which, however, was always distinguishable from the sugar phosphates. The results are summarised below:-

Peak number or standard sugar phosphates	Mobility pH 3.8	Mobility pH 7.5
1	177	177
2	59	
	110	108
	178	
G-1-P	110	104
3	128 streaked	193 streaked
4	129	110
		193
5	117	
	135	116
F-6-P	133	116
6	100	99
G-6-P	100	100
7	100	100
myo-inositol	134	260

Mobilities are related to standard G-6-P.

Thus of eleven different spots observed from seven different peaks, only four were identifiable as G-1-P, G-6-P, and F-6-P.

2. Thin layer chromatography.

Several solvents were used to separate sugar phosphates upon thin layers (0.3 mm.) of cellulose powder. Solvents h and i did not yield such good separations as had been observed using filter paper chromatography, whilst solvents j, k, and l (G.M.2) tended to promote streaking or the separation of the thin layer from the plate. Only the relatively non-polar solvents m and n gave satisfactory results. Using these two solvents, separately or together, two dimensionally, and with a combination of acid molybdate spray, keto-hexose spray (G.M.3 -1b) and alkaline silver nitrate, omitting the ammoniacal reagent and not washing the plate, several tentative identifications were made. Multiple irrigation of the plates and the multiplicity of spots given by most standard compounds made relative mobilities meaningless, but generally G-6-P travelled about 33% of the distance travelled by Pi, G-1-P about 37%, and F-6-P about 45% in solvent m. G-1-P and G-6-P could also be distinguished since G-1-P gave a green colour with acid molybdate spray. The results can be summarised:-

Peak 1: gave a single spot slower even than G-6-P.

Peak 2: gave at least three spots, although the

pattern was somewhat obscured by salt effects. One of these, however, appeared to be G-1-P, and another Pi, whilst the third remained unidentified.

Peak 3: showed only two spots, G-1-P and an unidentifiable spot.

Peak 4: with alkaline silver nitrate three spots appeared indicating carbohydrate, but only one of these showed up with phosphate spray.

Peak 5: a single spot running concurrently with F-6-P and also giving a positive reaction with α-naphthol phosphoric acid. (A positive reaction with the resorcinol reagent could also be obtained from the solution).

Peak 6: this showed three spots, one of which was probably G-6-P. They gave a positive reaction with silver nitrate and no visible reaction with α-naphthol.

Standard Compounds:

These gave variable chromatograms for reasons which were not entirely understood. Frequently the esters migrated uniquely to give a single spot, which could then be used for tentative identification purposes. However, equally often these supposedly unique compounds gave a multiplicity of spots from two to six in

number, not all of which could be identified. A possible cause of this was the advent of "ghost" spots when a plate was run twice, i.e. when the solvent front reached the top of the plate the latter was taken out, dried and then replaced in the solvent. Thus if F-6-P contained traces of G-6-P and Pi, as was several times demonstrated, and each of these split into two spots when the plate was run twice, then the six spots observed with F-6-P could all be accounted for. However, this sophistry did not engender confidence in the results, especially when the slowest spot faded, leaving only five visible. Other standards showed similar behaviour, although to a lesser extent.

3. Acid hydrolysis followed by paper chromatography of the carbohydrates.

Aliquots of each peak (0.1 ml.) were mixed with 2N HCl (0.1 ml.) and sealed into a small glass vial. These were heated at 100° for three hours, cooled, neutralised by the addition of 0.1N NaOH (2.0 ml.), deionised using a small column of Biodeminrolit (1x8 cms.), and the eluate concentrated to a small volume (approx. 0.1 ml.) so that it could be examined by paper chromatography (solvent a). In Peak 1 the major component was myo-inositol with traces of two other unidentified carbohydrates (R_G 27 and 80). Peak 2 was somewhat similar, although, in addition, there were traces of

xylose, ribose and glucose. Peak 3 showed nothing at all. Peak 4; once again myo-inositol was the major component with traces of galactose, glucose, xylose, ribose and glycerol. Peak 5 showed predominantly fructose with traces of glucose, myo-inositol and the two carbohydrates R_G 27 & 80. Peak 6 apparently had only traces of glucose and the carbohydrate R_G 80. Finally Peak 7 showed definite spots of glucose and fructose together with traces of the two unknown carbohydrates.

Expt. 34. Phosphate hydrolysing enzymes: purification, assaying and determination of specificity.

Commercial acid and alkaline phosphatases were dissolved in water and the inorganic phosphate content of each solution determined. There was none at all in the acid phosphatase but the alkaline phosphatase contained about 100 µg per mg. of protein or 10%. This enzyme was purified by dialysis with 0.015M magnesium acetate, pH 6.8, at 2° (208). It is recommended that the dialysis should be curtailed after 4 hrs, however the Pi concentration of the dialysate continued to rise for 12 hrs. after which the process was stopped. The activity of each preparation was checked in a standard reaction mixture (G.M. 13-1b) using a solution of phenyl disodium orthophosphate, which was diluted so that the Pi in 1 ml. of the reaction mixture gave a 95% colorimeter scale deflection following complete

hydrolysis; this required only 10 mins. at 37° by both enzymes in the digests described. Stored in a refigerator the preparations remained active for two weeks.

Isomerisation of carbohydrates.

Each enzyme was incubated with about 1 mg. of G-6-P, F-6-P, R-5-P, glycerophosphate, and galactose-6-phosphate following which the hydrolysate was deionised, evaporated to dryness and the residue examined by paper chromatography (solvent a). Ribose, glycerol and galactose appeared only from the appropriate ester whilst glucose and fructose arose from the hydrolysis of both G-6-P and F-6-P. This was not as disturbing as might at first appear since the presence of each of these esters contaminating the other had already been established by T.L.C. (Expt 33, part 2). It was therefore concluded that these enzymes did not bring about the interconversion of carbohydrates during hydrolysis of sugar phosphates.

Expt. 35. The ion exchange separation of sugar phosphates using a chloride system and borate complexes - a model experiment.

This experiment set out to duplicate the separation of sugar phosphates described in reference 183 with two major differences: firstly, the solvents used were to be based upon lithium as the cation, and, secondly, a slightly larger column was to be used so that greater amounts of phosphate could be handled at one time. To a column of

Dowex-1x4, Cl^- (1.3 x 18 cms.) was applied a mixture of phosphates totalling 1 millé-equivalent or 3.8% of the total exchange capacity of the resin; dissolved in a solution adjusted to pH 10.0. This mixture contained G-1-P (76 µmoles by weight), G-6-P (99 µmoles), Pi (66 µmoles) F-6-P (88 µmoles), F-1;6-diP (67 µmoles), AMP (29 µmoles) and also glucose (250 µmoles). The column was washed with 10^{-4}M LiOH (pH 10.0, 1 litre) which quantitatively eluted the glucose together with traces of u/v absorbing material. There was no trace of phosphate in these fractions (Fig. 16, page 101). Following this a solution being 0.025M NH_4Cl and 0.01M $K_2B_4O_7$ was prepared and found to have pH 9.0. This was duplicated by taking 0.025 M LiCl, 0.04M H_3BO_3 and adjusting the solution to pH 9.0 with 0.1M LiOH. To prepare 2 litres of this solution required 400 ml. of 0.1M LiOH which corresponds to the formula $Li_2B_4O_7$. This solution eluted G-1-P and Pi together as a single peak, the Pi being identified by the King method (G.M. 9-1a) and the G-1-P by observing that the organic phosphate was completely acid labile (G.M. 9-1b).

This procedure was repeated with the next ammonium solution which was found to have pH 8.5; duplicating this with 0.025M LiCl and 0.004M H_3BO_3 required only half the stochiometric amount of LiOH (20 ml. of 0.1M) to adjust the solution to pH 8.5, suggesting that the $B_4O_7^=$ concentration was only 0.0005M instead of 0.001M. Adding the stochiometric amount of LiOH, 40 ml. at 0.1M, raised the solution to

pH 9.0. However this solution would not elute G-6-P so it was replaced by another of the same concentrations, adjusted to pH 8.5. After some delay this solution eluted G-6-P as a broad peak spread over 2.5 litres of eluate. It was decided that in future 0.03M LiCl would be used instead of 0.025M, which should yield a more compact peak. The G-6-P was partially identified by observing that the phosphate was not acid labile and that the carbohydrate moiety was not a ketose. In addition to this, all of the other phosphates added to the column were eventually accounted for in other peaks. The fourth solution, 0.025M NH_4Cl, 0.00 001M $K_2B_4O_7$ and 0.0025M NH_4OH pH 8.1, was duplicated exactly using lithium salts and this solution would not elute any sugar phosphate at all. Raising the anion concentration to 0.03M LiCl and lowering the pH to 7.9 gave rise to the gradual elution of F-6-P, reacting positively with the resorcinol reagent (G.M. 9-2e). Replacing this solution with 0.03M LiCl, pH 6.5, which corresponds to the next solution used by Khym and Cohn eluted the rest of the F-6-P as a compact peak.

The sixth solution used by Khym and Cohn, 0.005M HCl, was used to elute AMP as a well defined peak, as observed by u/v absorption of the eluate. Finally the column was eluted with 0.02M HCl and 0.02M LiCl which eluted F-1:6-diP as a sharp peak. This ester was identified by measuring the acid labile phosphate which was found to be 33%, compared with a theoretically calculated value of 31% using

the values quoted in reference 222.

Recoveries: Except for glucose these were determined by bulking together the individual fractions of each peak and estimating the phosphate concentrations of the resulting solutions. This was compared with the weight of the ester which had originally been applied to the column.

Esters	Amount added µmoles	Amount recovered µmoles	% recovery
G-1-P	76	73	95
Pi	66	98	148
G-6-P	99	92	92
F-6-P	88	78	89
AMP	29	26	90
F-1:6-diP	67	60	90

Expt. 36. Purification of the sugar phosphates from starch.

An aliquot of the prepared solution (1,030 ml., equivalent to 10.0 kg. of starch, Expt. 4) was extracted three times with chloroform (50 ml. each) the residual traces of which were removed by reduced pressure, giving a clear brown solution. To this was added freshly prepared magnesia solution, pH 8.7 (50 ml. G.M. 10) and the mixture allowed to stand for 2 hours at 0° before removing the

precipitate by filtration. The filtrate was passed over a cation exchange column in the lithium form (Biorad AG 50 Wx8, 5x30 cms.) which was washed with water and the combined eluate (pH 9.5) ne tralised with HCl. The organic phosphates were recovered in the usual manner from this solution by anhydrous methanol-acetone precipitation. (The free sugars would mostly have remained in solution at this stage.) As usual the supernatant acetone was demonstrably free of phosphate. After washing the precipitate with acetone the residual traces were removed using a vacuum dessicator containing paraffin wax. This yielded a light gray powder completely soluble in water to form a dark brown solution, pH 6.95 (250 ml.) This solution contained organic phosphate equivalent to 0.12 µmoles per g. of starch extracted, which by comparison with Table 3 can be seen to be a 100% recovery. There was also recovered 0.03 µmoles of Pi per g. of starch, a 7% recovery. Recovery of the $u/v_{(260\ m\mu)}$ absorbing material was only 36%

Expt. 37. Ion exchange separation of the purified organic phosphates.

The solution prepared in the previous experiment containing a total of 30 m.eq. of phosphate, was caused to percolate slowly through a column of Dowex-1x4, Cl^-, (1.8x18 cms) at pH 10.0. The phosphate was entirely absorbed by the resin thus occupying about 6% of the available ion exchange sites. The column was eluted with

a series of solutions as described in Fig. 17, page 104. The remaining free sugars and bases were eluted directly from the column as had been anticipated. The first solution would have caused the elution of a mixture of phosphates which do not complex with borate. Besides Pi and G-1-P this would have included myo-inositol monophosphate and glycerophosphate, the presence of which had been suggested by Expt. 31. In an attempt to resolve this mixture, the eluting anion, chloride, was introduced to the column by means of a gradient whilst maintaining the borate concentration at its original level. A slight resolution was achieved but the separation was not really great enough to warrant segregation of the peaks as was done. The LiCl solutions were extended to 0.05M LiCl in an attempt to ensure that all the sugar phosphates were eluted before the nucleotides broke through.

Characterization of the various peaks.

(1) The fractions constituting each peak and the apparently void fractions between the peaks were bulked together, neutralised and concentrated to a convenient volume in order to estimate the phosphate concentrations (summarised in Table 8, page 105). The majority of the Pi (83%) was eluted in Peak 3 (5.5 - 6.5 litres) whilst the remaining 17% emerged gradually throughout the rest of the elution. Similarly the first phosphate-containing fraction held most of the organic phosphate (72%). Peak 8 was a little surprising,

a prominent peak when assayed for carbohydrate but containing very little phosphate at all.

(2) Attempts to hydrolyse the phosphate esters in these fractions and to chromatograph the carbohydrates were unsuccessful, probably because the borate present led to the retention of the carbohydrates by the resin used to deionise the hydrolysates (G.M. 13). Accordingly the borate was removed by co-evaporation with methanol and the phosphate esters recovered by anhydrous methanol-acetone precipitation.

(3) The isolated esters were then chromatographed before hydrolysis using the Lelcir solvents (solvents h & i, G.M. 1). Beyond demonstrating the presence of Pi in peak 3, no conclusive results were obtained, although there were faint indications of the presence of glycerophosphate and G-1-P in Peak 3 and G-6-P in Peak 6. These chromatograms demonstrated once again the necessity for hydrolysing the phosphate esters before attempting a chromatographic identification.

(4) The phosphate esters were hydrolysed using both acid and alkaline phosphatase and the carbohydrates isolated (G.M. 13). Paper chromatography (solvent a) of peak 3 hydrolysate revealed that the major component of this fraction was myo-inositol. The presence of glucose, fructose, sucrose and glycerol was also clearly demonstrated and this was confirmed by chromatography in solvents b, c, & e.

The presence of sucrose was further confirmed by use of a ketohexose specific spray (G.M. 3-1b). Since both F-1-P and F-6-P react strongly with borate it seems probable that the fructose present in this fraction arose by hydrolysis of the sucrose linkage. A similar quantity of the fraction was desalted and chromatographed without prior hydrolysis; this showed faintly discernible traces of sucrose, glucose and fructose, by no means comparable with those arising from the hydrolysate. The remaining peaks were hydrolysed and chromatographed in the same manner to give the results summarised in Table 8, page 105.

Expt. 38. Determining the concentration of the various carbohydrate phosphates.

An aliquot of each fraction was taken, appropriate to the volume of the solution, so that altogether 2 kg. of starch were represented in the solution. This was hydrolysed with acid phosphatase for 72 hours at 37°, at the end of which period the inorganic and total phosphate were equal.

Fructose determination: A 2.0 ml. aliquot of the hydrolysate, equivalent to 200 g. of starch, was determined to contain 101.5 µg. of fructose or 0.5 µg/g. of starch. This represented the sum of the sucrose and fructose phosphates, being 2.8 mµmoles/g. of starch extracted. (G.M. 9-2e)

Xylose determination: Aliquots of the hydrolysate (1.0 ml.) were

evaporated to dryness and dissolved in water (0.4 ml) to which the reagent, containing glucose, was added. Thus the xylose concentration was determined to be 2.0 mμmoles/g. of starch. Since this reaction is given by nucleotides and the hexoses which were present were not compensated for, this almost certainly represents an over estimation of the true value (G.M. 9-2f).

Glucose: (See subsequent experiment) Using the GADH-peroxidase ortho-tolidine reagent (G.M. 9-2d), glucose concentration was determined to be 0.61 mμmoles/g. of starch extracted, this being the sum of the G-6-P and G-1-P concentrations.

Expt. 39. To demonstrate the specificity of the GADH-peroxidase-ortho-tolidine reagent.

This repeated to some extent the results of Expt. 16, part 2. Glucose (50 μg.) was incubated with the reagent (G.M. 9-2d) alone and in the presence of various additional carbohydrates. The following results were obtained. Glucose(50 μg), O.D.$_{365\ m\mu}$ = 0.275

glucose (50 μg.) + fructose (150 μg.) O.D. = 0.273

glucose (50 μg.) + ribose (150 μg.) O.D. = 0.273

glucose (50 μg.) + xylose (250 μg.) O.D. = 0.278

glucose (50 μg.) + sucrose (150 μg.) O.D. = 0.276

glucose (50 μg.) + myo-inositol (150 μg.) O.D. = 0.274

glucose (50 μg.) + glycerol (200 μg.) O.D. = 0.275

Expt. 40. Estimating the concentration of myo-inositol by the consumption of periodic acid.

Myo-inositol (0-100 µg.) in solution (1.0ml.) was incubated overnight in the dark with H_2SO_4 (100 µmoles) and $NaIO_4$ (3 µmoles). The solutions were diluted to 10.0 ml. and the optical density measured at 230 mµ. This was found to be inversely proportional to the inositol concentration between 0 and 80 µg. of myo-inositol (G.M. 9-2g).

Expt. 41. The isolation of glycerol and myo-inositol.

Filter paper (Whatman No. 3) was washed with a descending solvent (a) for about 48 hours, dried and an aliquot of the acid phosphatase hydrolysate (Expt. 38), equivalent to 500 g. of extracted starch, applied as a band across the paper. This was irrigated for 18 hours (solvent c) and the bottom one third of the paper cut off. The irrigation of the chromatogram was then continued for a further 24 hours in the same solvent. A comparative chromatogram bearing standard sugars was similarly treated and then developed to reveal the position of the bands. Following this the appropriate bands were cut from the chromatogram and the carbohydrates eluted with water (250 ml.).

When a known amount of myo-inositol was subjected to this treatment 96% of it was recovered from the filter paper.

Expt. 42. Estimating the concentration of glycerol (G.M. 9-2h).

The method described by Ryley for estimating glycerol concentrations requires the original solution to be diluted from 1.5 ml. to 10.0 ml. (195). It was felt that this unnecessarily reduced the sensitivity of the method, 100 µg. of glycerol giving only 35% deflection on the colorimeter scale. By diluting the original solution to only 5.0 ml. the sensitivity was doubled. At lesser dilutions than this a heavy brown precipitate was formed upon addition of the chromotropic acid reagent to a 1.0 ml. aliquot of the periodate incubate.

The necessity of isolating the glycerol was demonstrated by addition of other carbohydrates and observing their interference. Thus glycerol (50 µg.) gave an $O.D._{580\ m\mu}$ of 38; in the additional presence of glucose (50 µg.) an O.D. of 41.5, and of ribose (125 µg.) an O.D. of 48.

Expt. 43. The estimation of glycerol and myo-inositol in the hydrolysates of starch sugar phosphates.

Inositol was measured by determining the consumption of periodate spectrophotometrically and the concentration was found to be 2.3mµmoles/g. of starch extracted (G.M. 9-2g).

Glycerol was determined by incubating it with periodate and then estimating the formaldehyde formed using chromotropic acid

(G.M. 9-2f). Using the isolated glycerol described in Exot. 41 the glycerol concentration was determined to 2.8 mmoles/g. of starch extracted.

Expt. 44. Determination of the extent to which Dowex-1x4 chloride will retain borate ions from solution.

A column of the resin (2x22.3 cms, 77 m.eq. capacity) was washed with water until the eluate was free of chloride ions. Then 2.0 litres of 0.025M LiCl and 0.01M $Na_2B_4O_7$ were percolated slowly through the column. This solution, initially at pH 8.90, was eluted at pH 8.70 suggesting that some of the borate had been removed. Titration of this solution established the borate concentration as being 0.0078M $B_4O_7^=$ although it was noted that some dilution of the solution by residual water held in the column would have occurred (223). The resin was then eluted with 1.0M LiCl (250 ml.) at pH 6.0, the emergent pH being 10.8, followed by a second volume of LiCl, emerging at pH 8.6. Titration demonstrated that the first solution contained 4.4 m.moles of H_3BO_3 which was 5.5% of that originally applied to the resin. The amount of borate in the second eluate was too small to be accurately titrated. Thus when 77 m.eq. of Dowex-1 chloride was exposed to 40 m.eq. of $B_4O_7^=$ accompanied by 50 m.eq. of Cl^- then 2.2 m.eq. of $B_4O_7^=$ were retained

by the resin.

Titration: Aliquots of each solution (25.0 ml.) were taken and a few drops of phenolphthalein added, then 0.1M HCl until the colour was discharged. Each solution was heated upon a steam bath for 1 hour to remove any carbonate present and allowed to cool; 0.02M NaOH was added until the solution was faintly pink. Then a few grams of mannitol were added which immediately discharged the colour, following which the borate concentration was established by titration with 0.02M NaOH.

Expt. 45: G.L.C. of the carbohydrates arising by hydrolysis of the sugar phosphates extractable from starch granules.

A volume of the starch extract solution described in Expt. 4, equivalent to 1 kg. of starch was adjusted to pH 10.0 (LiOH) and percolated slowly through a small column of Dowex-1, Cl^- (1x10 cms.) which was then washed with water (1 litre) at pH 9.0. The column was eluted with 0.01M HCl (200 ml.) to recover the monophosphates. This eluate was neutralised, concentrated (20 ml.) and the sugar phosphates hydrolysed with alkaline phosphatase until the inorganic and the total phosphate reached the same value (48 hours). The carbohydrates were purified (G.M. 13) and the TMS-ethers prepared using 0.5 ml. of silanizing reagent (G.M. 3-3). This was analysed by G.L.C.; a typical chromatogram may be seen in Fig. 19b, page 117.

Expt. 46: Determining the gas chromatographic response factors for various carbohydrates.

A mixture of TMS-ethers was prepared by silanizing (1.0 ml. reagent) 1 mg. each of glycerol, ribose, xylose, fructose, glucose, myo-inositol and sucrose. A portion of this was diluted ten times with anhydrous pyridine so that the final solution had a concentration of 0.1 µg/µlitre. A series of chromatograms were run injecting 1, 2, 3, 4 and 5 µlitres, a total of 15 chromatograms. The area of each peak was measured by triangulation with a 12" ruler, ignoring the trailing edges of each peak. (For later work a fixed arm planimeter was acquired so that the entire peak could be considered). Standard curves were plotted of response against concentration and the slopes of these taken as the specific response from which the relative response of each sugar to ribose was determined.

Sugar	Peak area (cm^2/µg.)	% response of ribose
glycerol	18	109
fructose	17	103
ribose	16.5	100
-glucose	8.5	51.5
-glucose	11	66.7
total glucose	19.5	118.2
myo-inositol	26	157.8
sucrose	20.5	124.2

Expt. 47. Ion exchange separation of sugar phosphates wherein the use of borate was severely curtailed.

The manipulations and conditions of Expt. 37 were duplicated, once more using a volume of solution equivalent to 10 kg. of extracted starch. The recovery of the organic phosphate was 103%, of the inorganic phosphate approximately 0.3% and of the u/v (260 mμ) absorbing material 38.5%. The organic phosphate solution was adjusted to pH 10.0 and percolated through a column of Dowex-1x4, Cl^- (1.8x12 cms) at 4° which retained 99.5% of the organic phosphate. The column was then eluted with the solutions listed in Fig. 20, part 1, page 121.

Expt. 48. Chromatographic identification of the isolated sugar phosphates.

The fractions of each peak (Expt. 47) were bulked together, neutralised and the sugar phosphates recovered in the accustomed manner. Peaks 6 & 7 were included in order to determine to what extent the sugar phosphates overlapped the nucleotides. The phosphate content of each fraction was determined and aliquots hydrolysed with both acid and alkaline phosphatase so that the carbohydrates could be isolated and identified chromatographically (G.M. 13) by both paper and G.L.C. The two enzymes gave essentially the same results and similarly paper and G.L.C. corroborated each

other in a satisfactory manner. The results are summarised in Fig.20

Expt. 49. Determining the concentrations of the carbohydrates present in the various isolated fractions.

With the exception of inositol the concentrations of the carbohydrates in the hydrolysates were determined by both colorimetry and G.L.C. The lengthy isolation procedure necessary for colorimetric determinations of inositol and glycerol was omitted and inositol was determined only by G.L.C.; thus the concentration of glycerol determined colorimetrically would have been slightly inaccurate since there were other carbohydrates present to interfere (G.M. 9-2h). Peak 2 contained very little organic phosphate and chromatography had revealed the predominant presence of myo-inositol and glucose with very faint traces of fructose arising from hydrolysed sucrose. It was assumed that of the 0.07 mμmoles of organic phosphate present in this fraction per g. of starch extracted, 0.05 mμmoles was inositol phosphate and the 0.02 mμmoles which were acid labile were G-1-P. Fraction 3 was a complex mixture containing predominantly glycerol and myo-inositol together with glucose, sucrose and traces of fructose. It was hoped that the inositol could have been determined from the difference between the organic phosphate which was present in this fraction and the total concentration of the other carbohydrate phosphates, but the difference between this figure and the value

arrived at by G.L.C. was too great to be acceptable. For the G.L.C. determinations ribose was added as an internal standard, being added to the hydrolysates in such a concentration that 5 µlitres of the TMS-ether solutions, equivalent to 10 g. of starch, also contained 0.25 µg. of ribose. The remaining peaks were estimated in the same manner, determining fructose by G.M. 9-2e, glucose by G.M. 9-2d and glycerol by G.M. 9-2h. The results are summarised in Table 10, page 122. Of the 31.4 mµmoles of organic phosphate which was present in Peaks 2 to 7, 10.54 mµmoles was estimated to belong to the sugar phosphates. A small proportion of the remainder would have been AMP.

Expt. 50. The reduction of sugar phosphates with potassium borohydride and the assignment of the position of the phosphate ester upon the carbohydrate moiety.

Ribose and R-5-P (5 mg. each), dissolved in water (1 ml.) were incubated with KBH_4 (5 mg.) for 1 hour and then the borohydride destroyed by addition of a few drops of dilute acetic acid. The esters were hydrolysed with acid phosphatase, the solutions deionised, and small portions chromatographed (solvent a) to demonstrate that in both cases the reduction of the ribose was quantitative. This experiment could not be repeated directly with glucose, G-6-P and G-1-P since the only solvent capable of resolving glucose and sorbitol (g) inhibits the reduction of alkaline silver nitrate.

Recourse was made to G.L.C. In the course of this it was found that borate ions completely prevent the silanization reaction from taking place and so extra precautions were taken to remove them; this was done as usual by co-evaporation with methanol. G-1-P and G-6-P hydrolysed by acid phosphatase gave the same two G.L.C. peaks as glucose upon silanization. However, reduction with borohydride before hydrolysis formed sorbitol-6-phosphate from G-6-P and left G-1-P unaffected. Thus G.L.C. of reduced G-6-P showed no trace of glucose whilst "reduced" G-1-P showed no trace of sorbitol.

After repeating this upon Peak 3 (Expt. 47) (1.0 ml., 5 mg. KBH_4), glucose was present in the hydrolysate with only very slight traces of sorbitol, less than 5% by relative peak areas. The sugar phosphates of Peak 4 upon reduction and hydrolysis produced sorbitol and mannitol, showing that originally G-6-P was present. (Both F-1-P and F-6-P should be reducible with borohydride).

Expt. 51: The acid hydrolysis of sucrose phosphate.

An aliquot of Peak 3 (Expt. 47, 5.0 ml.) was hydrolysed in 1.0M HCl at 100° for 10 mins., neutralised with NaOH, deionised and the TMS-ethers prepared. G.L.C. of aliquots of this preparation (equivalent to 2.0 g. of starch extracted) revealed predominantly glucose with some fructose. Measurement of the peak areas suggested that there was present 1.15 mμmoles of glucose and 0.11 mμmoles of fructose/g. of starch. Of this glucose, 0.52 mμmoles would have

arisen from the hydrolysis of the G-1-P present (Table 11). Thus 0.63 mμmoles of glucose arose by acid hydrolysis of the sucrose phosphate which suggests that the sucrose was phosphorylated upon carbon six of the fructose moiety. Acid hydrolysis of this ester would produce glucose and F-6-P which would not normally be regarded as an acid labile phosphate.

Expt. 52. To determine whether phytic acid is present in starch granules.

Reagent: 0.167M HCl, containing $FeCl_3$ (0.083 mg/ml). Potato starch (100 g.) was mixed with a little cold reagent and added to 800 ml. of boiling reagent. The mobile starch solution was boiled for 30 mins. to disrupt the granules and then allowed to precipitate for three days. The precipitate was collected by centrifuging, washed 3 times with cold reagent (50 ml.) and then dissolved in 0.1M NaOH (10 ml.) by heating it at 100° for 15 mins. The precipitate ($Fe(OH)_3$) was removed by centrifuging and the supernatant found to contain 2 μmoles of organic phosphate. Both this supernatant and the material insoluble in 0.1M NaOH were hydrolysed with 2.5M HCl at 100° for 6 hours. TMS-ethers were prepared from the carbohydrates in the hydrolysates and G.L.C. used to reveal the presence of glucose in both preparations, but no myo-inositol. From this it was concluded that there is no phytic acid in starch.

Expt. 53. A brief investigation of the feasibility of analysing the free sugars by G.L.C.

The exhaustive extraction of starch (1 kg.) with 50% aqueous methanol was described in Expt. 5. This solution when concentrated (250 ml.) was adjusted to pH 10.0 and transferred entire to a column of Dowex-1x4, Cl^-, (1.5x15 cms), which was eluted with 10^{-4}M LiOH (3 litres) which completely removed the free sugars and also 1.9% of the phosphate from the column (15 µmoles of 800). The column was then eluted successively with 0.03M LiCl, 0.04M H_3BO_3, pH 9.0 which eluted the Pi etc., and then 0.005M HCl which eluted AMP together with the borate-complexing sugar phosphates. These fractions were not examined further (Fig. 21, page 129). The TMS-ethers of the free sugars in the initial solution were examined by G.L.C., which demonstrated the presence of glucose, fructose, ribose, maltose and sucrose and also traces of myo-inositol and two carbohydrates having retention times similar to α- and β-xylose. However, since the supposed α-anomer was consistently more abundant than the β-anomer, whereas with xylose the converse obtains, it was decided that these two peaks could not be xylose and they remained unidentified.. Myo-inositol was added as an internal standard to determine the concentrations of the sugars, the amount which was already present being no more than about 1% of that which was added,

so that it would have been only a minor source of error. By this means the concentrations of sucrose and β-glucose were determined, the other peaks being too small for accurate estimation by comparison with myo-inositol. However they were estimated by preparation of a fresh sample and comparison with β-glucose, the concentration of which was already known. Maltose was determined after removal of the sucrose by mild acid hydrolysis (0.3M HCl, room temperature, 18 hours).

EXPERIMENTS TO SECTION IV

Expt. 54. The isolation of potato starch and the extraction of the metabolites therefrom.

Two batches of potatoes, after 10 and 18 weeks' growth, were dug manually and washed free of dirt in $\frac{1}{2}$ cwt. batches. The younger ones were used immediately, the others dried in air for 3 days and then stored in peat fibre for 3 weeks. Parts of each crop were taken, peeled, de-eyed, cut into segments and found to weigh 21.35 and 24.92 kg. respectively. The starch was extracted from these as rapidly as possible, taking about 15 mins. between the initial disruption of the tuber cells and separation of the starch from the cell fluid. Batches of 1 kg. were macerated with 35% glycerol (1 litre) by pressing the potato segments against a rapidly rotating abrasive wheel. This wheel was held in a vertical position and the glycerol solution was directed onto it so that it

mixed intimately with the potato during homogenization and the combined homogenate fell clear of the machine into a beaker. The fibrous pulp was sieved through 60 and 120 mesh sieves and further washed with 20% glycerol (500 ml.), after which the starch suspension collected in the bottom was poured into a stainless steel beaker. The suspension from 1 kg. batches was centrifuged for 1 min. at 1,100 r.p.m., the supernatant discarded and the starch suspension from a further kg. of potatoes added to the centrifuge bottles to repeat the centrifugation. The starch in each bottle was resuspended in 20% glycerol (200 ml.) and again centrifuged (5 min at 2,500 r.p.m.). The supernatant was discarded and the starch washed out of the bottles with 50% aqueous methanol. Aliquots of the supernatant cell fluid were also retained, there being approximately 1900 ml. per kg. of potatoes. These were sealed into airtight flasks and stored at -20° until required.

Further 50% methanol was added to the starch to a total volume of 8 litres. The suspension was stirred for 4 hours and then allowed to settle overnight before decanting the solution. Altogether 3 extractions with 50% and 2 with 10% methanol were done. Finally the starch was dried in a vacuum oven and weighed. The process is summarised in Table 21, page 137.

Expt. 55. To determine the extent to which cell fluid contaminated the potato starch preparations.

The starch from 1 kg. of potatoes was extracted under conditions identical to those used in the previous experiment, except that a dye, Blue Dextran 2000 (Pharmacia), was added to the 35% glycerol solution which was mixed with the potatoes during homogenization. After the first centrifugation an aliquot of the supernatant homogenate was taken and further centrifuged. (20 mins. at 3,000 r.p.m.) then filtered (Whatman No. 50), diluted 3 times and the colour intensity measured at 600 mμ, from which it was determined that there were 2.1×10^5 O.D.U. in the entire solution. The starch was washed once with 20% glycerol as described and centrifuged, discarding the washings. Then the starch was washed from the bottles with 50% methanol (2 litres) and in this medium it was suspended and stirred for 3 hours before taking an aliquot, filtering it, concentrating the filtrate ten times and measuring the colour intensity. There were 800 O.D.U. present in the entire solution. This was 0.38% of that present originally in the homogenate and this value was taken as the proportion of the cell fluid which remained to contaminate the starch preparations.

Expt. 56. The separation of the free sugars and the organic phosphates.

There were four separate preparations to be analysed, the cell fluid from young potatoes (I) and the starch extract (II),

and also the cell fluid (III) and starch extract from mature potatoes (IV). The volumes of cell fluid taken represented about 500 g. and 320 g. of potatoes respectively. The solutions were adjusted to pH 9.0, diluted to about 2 litres with water, and passed through columns of anion exchange resin (Dowex-1x4 Cl^-, 2.5x20 cms.). Some difficulties arose at this stage. The flow of solution I was stopped for several hours and some of the solution lost. Meanwhile the remainder became very dark and a precipitate formed. The separation was discontinued after about half the solution had passed through the column and no attempt was made to gain quantitative results from it.

Difficulty was also experienced in maintaining the rate of percolation of solutions II and IV through their respective columns and several times it was necessary to resuspend the upper layers of the resin with a glass rod, in order to break up the crust of impacted resin which appeared to form.

The columns were then washed with water (3 litres, pH 9.0) and the total eluate bulked, neutralised and examined for free sugars. These solutions contained no phosphate at all. Organic phosphates were recovered from the resin with 0.02M LiCl, 0.02M HCl (6 litres), which from Expt. 35 was known to elute mono- and diphosphates from Dowex-1. During the elution of solution III the fraction collector

broke down so that some phosphate was lost. However, by determining that the recovery of the inorganic phosphate was 82%, this loss could be compensated for. Because of the hazards involved, fraction collectors were not used for solutions II and IV. The phosphate esters were recovered in the usual manner by anhydrous methanol-acetone precipitation, washing the precipitate twice with anhydrous acetone (250 ml.) and dissolving the residue in water.

Expt. 57: Fractionation of the phosphate esters.

The solutions I, II, III (250 mls. each) and IV (125 mls.), designated as before, were adjusted to pH 9.5 and applied to columns of Dowex-1x4 Cl^- (2x11.6 cms.), which were then washed with water (2 litres, pH 9.5). The removal of the free sugars, hypothetically absent, was followed by noting the falling acetone concentration with the u/v absorption at 260 mμ. The columns were then eluted with the solvents listed in Fig. 22, pages 141, 142, and monitored by following the elution of phosphate (G.M.8 -2) and carbohydrate (G.M.8 -1b). Finally the appropriate fractions were bulked together, the borate removed and the phosphates recovered by anhydrous methanol-acetone precipitation, washing the precipitate twice with anhydrous acetone.

Thus each of the original solutions had been subdivided several times, the fractions of particular interest being solution A,

the free sugars; B, the non-complexing sugar phosphates; C, the complexing sugar phosphates and monophosphonucleotides; and E, the sugar diphosphates.

Expt. 58: Estimating the phosphate concentration of the various solutions and determining the recovery of the inorganic phosphate.

Having isolated and subdivided the potato preparations, their total phosphate contents were determined (G.M.9 -1). These are summarised in Table 15, page 153. At the same time phosphate was estimated in aliquots of the original solutions which had been retained. Comparing the inorganic phosphate in the original solutions and in solution B of the previous experiment gave an indication of the percentage recoveries.

The original extract of young potato starch, solution II, contained 148 mμmoles of Porg. and 212 mμmoles of Pi, whilst solution II(B) contained 204 mμmoles of Pi, so a 96.5% recovery of the inorganic phosphate was effected and this recovery was assumed to obtain for all the other metabolites so that subsequently corrections could be made to the values obtained.

Solution III contained 412 mμmoles of Porg. and 515 mμmoles of Pi; since solution III(B) contained 425 mμmoles of Pi, the

recovery was only 82%.

Solution IV contained 143 mμmoles of Porg. and 315 mμmoles of Pi, of which 305 mμmoles were recovered in solution IV(B), or 97.5%.

Expt. 59: The separation of glycerol from the monosaccharides by charcoal adsorption chromatography.

Charcoal columns were prepared in the manner described (G.M.7 -1), mixing Ultrasorb SC 120/240 (10 g.) and celite 535 (10 g.). Through these was percolated the free sugar solution, A (50 ml., 5% of total volume), followed by water (100 ml.). The sugars were eluted with 20% ethanol (250 ml.), which was allowed to fall onto $BaCO_3$ (about 10 g.). This was afterwards removed by filtration, the eluate deionised and concentrated to a smaller volume (0.5 ml.) for examination by chromatography. Paper chromatography showed that most of the glycerol and some of the monosaccharides remained in the aqueous eluate, whilst some of the glycerol and most of the sugars were retained by the charcoal to be eluted with the ethanol solution.

Expt. 60: Chromatographic identification of the free sugars.

The free sugar solutions the preparation of which was described in the previous experiment, were examined by paper

chromatography using solvents a, b, and c (G.M.1). There was still a substantial amount of glycerol present, but it had been reduced to such a level that it no longer interfered with the separation of the other sugars. Solution II(A) (as designated in Expts.56 and 58) contained glucose and sucrose as the predominant sugars, together with traces of maltose, fructose and ribose, and a slow running spot which remained unidentified (R_G solvent a, 36; myo-inositol, 38; solvent b, 38; myo-inositol, 56). There was no apparent difference between the free sugars of solutions III(A) and IV(A) and they were both on the whole similar to young potato starch. As before, glycerol was still present, whilst glucose and sucrose were the major carbohydrates, together with traces of fructose, but apparently no ribose. There were also several slow running carbohydrates which were not well separated from each other and tended to form a continuous streak between R_G 35 and the origin (solvent a). Solvent a resolved a carbohydrate whose mobility was slightly greater than glycerol - R_G 168, cf. glycerol, 157. This was not glyceraldehyde, dihydroxyacetone or glycolaldehyde, and it only appeared in chromatograms using solvent a.

Gas liquid chromatography.

Gas chromatograms of the solutions purified with charcoal showed the presence of glucose, sucrose, fructose, maltose, and

definite traces of myo-inositol. The presence of ribose was not confirmed (TMS-ethers in hexane, upon a column of 2.5% SE 30, 1.5 metres long, temperature programmed from 100° to 250° at 6°/min.; and other conditions (G.M.5 -3)).

TMS-ethers of the original solutions were prepared by taking an aliquot (0.1 ml.), removing the water by co-evaporation with pyridine, dissolving the residual glycerol (about 20 µlitres) in anhydrous pyridine (1.0 ml.), adding hexamethyl disilazane (0.5 ml.) and trimethyl chlorosilane (0.5 ml.). The silanizing reagents were removed in the usual manner (G.M.5, 3) and the TMS-ethers heated at about 50° in a current of dry air for 20 mins., which removed most of the glycerol. G.L.C. of these preparations revealed a complex pattern of peaks (Fig. 23, page 148). Amongst these were prominently sucrose, α- and β-glucose, fructose, α- and β-maltose, myo-inositol, and, of course, glycerol. The presence of ribose could not be confirmed because the ribose peak, if any, was obscured by a large neighbouring peak. By the use of a wide range of standard compounds, it was demonstrated that most of the unidentified peaks were not commonly occurring sugars, and so were extraneous to the present problem. The sugars eliminated were erythrose, arabinose, fucose, xylose, mannose, galactose, sorbose, sorbitol, sedoheptulose, lactose, trehalose, cellobiose, gentiobiose and raffinose.

Expt. 61: Investigating the effects of potassium borohydride upon various carbohydrates.

1. Ketohexoses.

Fructose (100 µg.) and sucrose (100 µg.) in solution (1.0 ml.) were incubated with KBH_4 (5 mg.) for 1 hour and the remaining ketohexose estimated (G.M.9 -2e). These were compared with similar samples untreated with borohydride, and it was observed that fructose had been completely destroyed, whilst sucrose remained unaffected (resorcinol reag. G.M.9 -2f).

2. Glucose.

Glucose (100 µg.) in solution (1.0 ml.) gave no reaction with GADH-peroxidase-o-tolidine reagent (G.M.9 -2d), after incubation with KBH_4 (5 mg.).

3. Sucrose and maltose with invertase and maltase.

Sucrose (100 µg. in 1.0 ml.) gives no reaction with G.M.9 -2d with or without KBH_4 (5.0 mg.). The residuum after destruction of the KBH_4 (with dil. acetic acid) has no effect upon the action of invertase and the glucose liberated reacts stochiometrically with G.M.9 - 2d.

Maltose (100 µg. in 1.0 ml.) was observed to react very slowly with G.M.9 -2d, prior to reduction with KBH_4 (5.0 mg.), but not at all afterwards. It was also noted that equine maltase either

could not hydrolyse maltitol to glucose and sorbitol, or that the residuum after destruction of borohydride inhibited the catalysis of maltase, for no reaction with G.M.9 - 2d could be observed under these circumstances.

Expt. 62: Determining the concentrations of glucose, fructose, sucrose and maltose.

Samples of each solution (1.0 ml.) were submitted to various treatments and then diluted to a standard volume (10.0 ml.), following which the fructose and glucose concentrations were estimated.

(a) was hydrolysed with invertase and maltase, and then reduced with borohydride. This was regarded as a blank solution.

(b) was estimated directly, giving a measure of the glucose concentration (provided the incubation with G.M.9 -2d was not unduly prolonged beyond 2 hrs.), and the total fructose.

(c) was hydrolysed with maltase so that the estimated glucose was the sum of the original glucose and maltose concentrations. Ketose would be the same as (b).

(d) was hydrolysed with invertase, after which estimation of the glucose concentration gave the sum of the glucose and sucrose concentrations. Ketose was again the same as in (b).

(e) was hydrolysed with both invertase and maltase, following which the estimated glucose concentration would have been the total of all the glucose present.

(f) The sample was reduced with borohydride (5.0 mg.) and then hydrolysed with invertase. The estimated glucose and fructose concentrations were both due solely to sucrose.

Thus glucose concentrations were estimated from (b) - (a) and (d) - (f); fructose from (b) - (a) and (b, c or d) - (f); maltose from (c) - (b) and (e) - (d), and finally sucrose from both the glucose and fructose of (f), and from (e) - (c) by glucose estimation. There was a satisfactory agreement between the values obtained by the various methods, the greatest discrepancy being 5%. The results are summarised on page 151.

Expt. 63: Chromatographic identification of the carbohydrate moieties of the sugar phosphates.

Preparation I, the juice from young potatoes (Expt. 58),

was not examined further because a substantial proportion of the inorganic phosphate appeared in fraction C, instead of being confined to fraction B (Table 15). Aliquots of fractions B and C of the other preparations were hydrolysed with acid and alkaline phosphatases. The carbohydrates were isolated as usual (G.M.13) and identified by paper chromatography in solvents a and b. Fraction II(B), the non-borate complexing sugar phosphates of young potato starch contained predominantly myo-inositol and glycerol, together with traces of sucrose, but no glucose. Fractions II(B) and IV(B) contained these carbohydrates together with traces of glucose. Fractions II(C), III(C) and IV(C) all contained myo-inositol, glycerol, glucose and fructose.

Comparative solutions were deionised (G.M.13), and chromatographed directly without prior hydrolysis in order to demonstrate that the free sugars were absent. There were faint traces of glycerol present in all of them, barely discernible in solution C. Visual comparison of the intensities of the spots suggested that free glycerol contributed no more than a very small percentage of the glycerol present after enzymatic hydrolysis of the glycerophosphate.

Gas chromatography.

Aliquots (5.0 ml.) of the sugar phosphate solutions were

hydrolysed enzymatically, the carbohydrates isolated (G.M.13) and TMS-ethers prepared (G.M.5 -3). These were examined by G.L.C. upon 2.5% SE 30 columns 1.5 metres long under a variety of conditions. The formation of TMS-ethers was problematical; often no peaks at all appeared or only a single asymmetrical peak with a retention time between those of α- and β-glucose (retention time cf. α-glucose=1.17). When recognizable peaks appeared, their size suggested that derivative formation was far from quantitative as compared with the concentrations suggested by paper chromatography and organic phosphate concentrations. However, the chromatograms on the whole suggested the same results as those gained by paper chromatography - that myo-inositol, glycerol, glucose, sucrose and fructose were present in the hydrolysates.

Variations were made in the hydrolysis and isolation procedures in an attempt to overcome the inhibition of silanization. It was ascertained that hydrolysis of the phosphates was complete. Lesser amounts of enzyme were used, 1.0 mg. for 5.0 ml. of substrate instead of 2.5 mg., and the enzymes combined. Both mild and strong acid hydrolysis were tried, 1N HCl for 24 hours at room temperature and at 100°. The deproteinization and the deionization steps of the isolation procedure were alternatively omitted and repeated upon separate hydrolyses and an attempt made to isolate the carbohydrates

by dialysis of the hydrolysates. However, the results of these modifications were as unpredictably variable as those of the conventional procedure, and attempts to gain meaningful results by G.L.C. were necessarily abandoned.

Expt. 64: Colorimetric estimation of the carbohydrate moieties of the sugar phosphates after hydrolysis.

Aliquots of each solution were hydrolysed with alkaline phosphatase until the concentration of inorganic phosphate was the same as that of the total phosphate. Glucose, fructose and glycerol were all estimated in the usual manner (G.M.9—2d, e, f, h).

Glycerol was estimated in the presence of the other carbohydrates; it was believed that the comparatively low levels present would contribute but little formaldehyde to interfere with the estimation of glycerol. The same result was obtained from samples deproteinized with the Somogyi reagents (G.M.13—2a), and those in which the protein was still present. The apparent levels of glycerol in similar unhydrolysed solutions were subtracted before arriving at the final concentrations.

Glucose-1-phosphate was assumed to be the concentration of acid labile phosphate present in solutions B. This was not compared with glucose concentrations after hydrolysis, since high

levels of inorganic phosphate such as were present in these solutions can interfere with the action of the GADH-peroxidase-o-tolidine reagent. Sucrose phosphate concentration was assumed to be equivalent to the ketose present in solutions B, and fructose-6-phosphate equivalent to the ketose present in solutions C. Similarly, G-6-P concentrations were taken as being equivalent to the glucose concentrations of solutions C.

No attempt was made to equate the myo-inositol monophosphate concentrations to organic phosphate which had not been accounted for by being assigned to the other sugar phosphates. The results are summarised in Table 16.

Expt. 65: Examination of the sugar diphosphate fractions.

The fractions referred to are III(E) and IV(E), which were eluted from Dowex 1x4 Cl^- under conditions consistent with their being analogues of F-1:6-diP. Aliquots of each solution were hydrolysed with acid phosphatase at 37° for 72 hours and the carbohydrates isolated so that they could be identified by paper chromatography (solvent a). Myo-inositol was by far the most intense spot upon the chromatograms and there were also present well defined traces of glucose and fructose, but no other carbohydrates.

Once again attempts to confirm these identifications by

G.L.C. were futile. It was therefore tentatively decided that myo-inositol diphosphate, fructose-1:6-diphosphate and glucose-1:6-diphosphate are present in both the cell fluid and the starch granules of mature potatoes.

Since myo-inositol was apparently by far the most abundant carbohydrate in the hydrolysates, it was though that the concentration of this diphosphate would be approximately the same as that of the total organic phosphate present, which was 29 mμmoles/g. of potatoes in III(E) and 16 mμmoles/g. of starch in IV(E). This is equivalent to 14.5 and 8 mμmoles of myo-inositol diphosphate respectively.

Expt. 66: Correcting the estimated concentrations of the starch metabolites to eliminate the contribution made by contaminating cell fluid.

1. The mature potato starch which was isolated consituted 10.7% of the wet weight of the tubers. Therefore to convert the estimated concentrations from mμmoles/g. of starch to mμmoles/g. of potatoes, the values were divided by 9.35.
2. In Expt. 55 it was found that 0.38% of the cell fluid metabolites were contaminating the isolated starch preparation. So 0.38% of the estimated concentrations in mature potato juice were

subtracted from the results obtained in paragraph (1).

3. To compare the levels of the metabolites in the starch granule and the cell fluid, the concentrations in starch were converted back to a relationship with starch weight by multiplying the results of paragraph (2) by 9.35.

4. For this same comparison, it was assumed that the cell fluid constituted 85% of tuber weight, so the estimated concentrations of the metabolites in mature potato juice were divided by 1.18.

The results of these manipulations are summarised in Table 18.

Expt. 67. To examine the possibility that glycerol was being phosphorylated during the isolation of starch.

Starch was extracted from potatoes (1 kg.) in a manner identical to that described in Expt. 54. The starch collected in the centrifuge bottles was suspended in 20% glycerol solution (500 ml) and an aliquot (100 ml) immediately withdrawn. This was poured into hot methanol (50°, 300 ml.) in which medium it was stirred for 30 min. whilst allowing the suspension to cool. The time between maceration of the tuber and the isolated starch suspension being poured into methanol was 16 min. Aliquots (100 ml) of the 20% glycerol suspension of starch were also taken after 46 min. (30 min. stirring in the suspension) and 106 min.(90 min). The 46 min. sample was

poured into methanol and stirred for 5 min. and the 106 min. sample used directly. All three samples were, separately, filtered and the methanol removed by rotary evaporation. The aqueous solutions (about 200 ml) were adjusted to pH 8.5 with LiOH and percolated through three identical columns of Dowex-1x4, Cl^- (1.8 x 15 cms). The resin was washed with water (1 litre) and then with 0.01M HCl (500 ml) to elute the phosphate esters. These solutions were neutralised and then evaporated to dryness and redissolved in 25.0 ml. of water. The organic and inorganic phosphate content of these three solutions were determined. Aliquots of them were also hydrolysed with alkaline phosphatase in standard digests (G.M.13-1b). Similar aliquots were deionised without prior hydrolysis. The glycerol concentration of each of these solutions was determined so that the amount of glycerol arising from glycerophosphate could be determined. The results, seen below, suggested that there was no phosphorylation of glycerol during the incubation of starch with 20% glycerol solution.

Sample	Pi	Porg	glycerophosphate
16 minutes	0.31	0.11	0.05
46 minutes	0.22	0.12	0.08
106 minutes	0.18	0.11	0.06

values expressed as μmoles per ml. of solution.

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