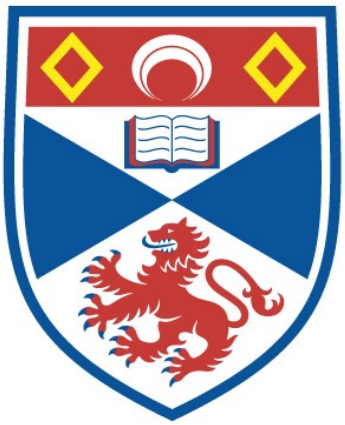
THE PHOSPHORYLATION OF SOME ANALOGUES OF FRUCTOSE BY YEAST HEXOKINASE

James R. Burt

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



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BY

YEAST HEXOKINASE

by

JAMES R. BURT, B.Sc.

A Thesis presented to the

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DEGREE of DOCTOR of PHILOSOPHY

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DECLARATION

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

The research was carried out in the Department of Physiology and Biochemistry in the United College of St Salvator and St Leonard, St Andrews, under the direction of Stephen Bayne B.Sc., M.B., Ch.B.

James R. Burt.

CERTIFICATE

I hereby certify that JAMES ROBERTSON BURT has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance 16 (St Andrews), and is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

Stephen Bayne.

CAREER

I first matriculated in the University of St Andrews in October, 1951. I graduated in June, 1955 with 2nd Class Honours in Chemistry and was accepted as a research student in October, 1955. In that year I was awarded an Andrew Bell Postgraduate Scholarship by the Trustees of the Madras College Endowment and I held this until September, 1958.

JAMES R. BURT.

PREFACE

Since Meyerhof separated hexokinase in a purified form from bakers' yeast a great deal of information on the properties of this enzyme has been obtained by numerous workers. In particular, the question of the specificity of yeast hexokinase has attracted much attention. This state of affairs arose probably because hexokinase was believed to initiate hexose metabolism in the living organism, and hence the enzyme would be expected to play an important part in determining whether certain sugars could be utilized or not by yeast. Certain cases of inhibition of various metabolic processes in the living cell were found to have been caused by the specific inhibition of hexokinase.

The pattern of specificity of the substrates of hexokinase falls into two different classes. Some sugars are phosphorylated in the pyranose form and others in the furanose form. The specific requirements for pyranoses have been investigated extensively, but there is relatively little information in the literature on the furanose aspect of hexokinase specificity. The object of this research was to

investigate the specificity of the enzyme in respect of analogues of fructose, and in particular those sugars which differ only slightly from fructofuranose at carbon-1 and carbon-2.

To this end, the chemical syntheses of the following compounds were undertaken:-

1-amino-1-deoxy-D-fructose

1-deoxy-D-fructose

2,5-anhydro-D-mannitol

2,5-anhydro-D-sorbitol.

Preparations of 1-amino-1-deoxy-D-fructose and 2,5-anhydro-D-sorbitol have been published, but the other two sugars are new compounds.

Part I of this thesis contains a review of the chemistry of 1-amino-1-deoxy-D-fructose, 1-deoxyketoses, and 2-deoxyketoses, a report of the experimental work carried out by the author in synthesizing the above-mentioned compounds, and a discussion of the results obtained.

Part II contains a review of the knowledge of yeast hexokinase, a report of the experimental work carried out in investigating the effects of the synthesized fructose analogues in the hexokinase reaction, and a discussion of these results in the light of other

information on the specificity of the enzyme. A systematic investigation of a spectrophotometric assay method for following hexokinase activity is included in Part II. The appendix contains the data from which the graphs and tables, shown in the thesis, have been compounded.

The author is greatly indebted to Dr S. Bayne for supervision and advice, to Mr J. Doyle for continued encouragement and help, to Drs G. R. Tristram, D. Calvert, C. Horrex, and D. H. Reid for discussions on and assistance with certain aspects of the work, to his father for proof reading of the manuscript and to Mr B. A. Carstairs and Miss E. Buttercase for photography.

NOTE

Throughout this thesis, the D-isomers of carbohydrates will be named without using the configurational prefix, while the names of L-isomers will always include it.

In addition, the following abbreviations will be used:-

AMP, adenosine monophosphate.

ADP, adenosine diphosphate.

ATP, adenosine triphosphate.

DPN, diphosphopyridine nucleotide.

TPN, triphosphopyridine nucleotide.

ITP, inosine triphosphate.

UTP, uridine triphosphate.

DNA, deoxypentosenucleic acid.

The enzyme referred to as hexokinase is specifically that present in, or obtained from, yeast. Other hexokinases are identified according to their origins.

Micro-analyses were carried out by Drs Weiler and Strauss, Oxford.

THE PHOSPHORYLATION OF SOME ANALOGUES OF FRUCTOSE BY YEAST HEXOKINASE

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PART I

SYNTHESIS OF THE FRUCTOSE ANALOGUES

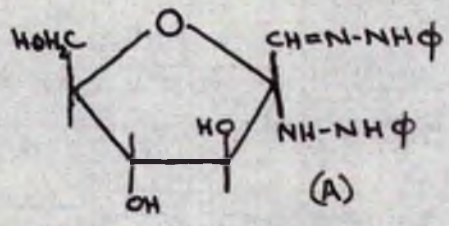
CONTENTS

1. A REVIEW OF THE CHEMISTRY OF 1-AMINO-1-DEOXYFRUCTOSE.

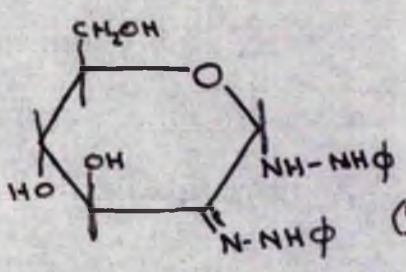
1(a). Preparations.

Since Fischer first prepared 1-amino-1-deoxyfructose (fructosamine, isoglucosamine) in 10-12 % yield, by the action of zinc and acetic acid on glucose phenylosazone, vastly improved methods for its synthesis have been published.

Maurer and Schiedt (1935) obtained a 60 % yield of the acetic acid salt of this aminosugar by the hydrogenation of glucosazone using palladium acetate as catalyst. Since fructosamine and not glucosamine is the product of this reaction, Druey and Huber (1957) have concluded that glucosazone, under these reducing conditions, exists in a furanose form with an external double bond (A) and not in a pyranose form with an internal double bond (B).



(A)



(B).

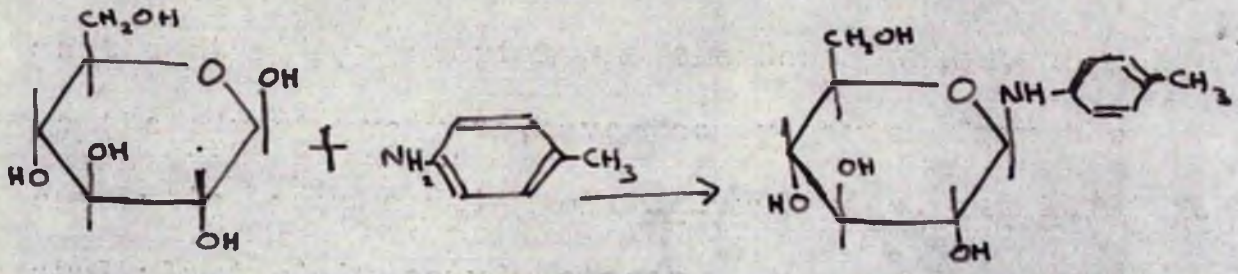
However, since most other evidence points to the fact that glucosazone exists in the open chain form with a chelate ring (Mester, 1955; Mester and Major, 1957), this result can hardly be taken as conclusive.

More recent workers have abandonded glucosazone

as the starting material and the best yields, as well as the purest products,have been reported from the catalytic hydrogenation of 1-*N*-arylamino- and 1-(di-*N*-aralkylamino)-1-deoxyfructoses.

These *N*-substituted fructosamines are prepared from glucose *via* the Amadori rearrangement. The Amadori rearrangement was first so called by Kuhn and Weygand (1937) after the Italian chemist Mario Amadori, who, from 1925 to 1931, published several papers on the condensation products of glucose with certain aromatic amines, *viz* *o*- and *p*- anisidine, *o*-and *p*-phenetidine and *o*- and *p*- toluidine (Amadori, 1925, 1929a, 1929b, 1931a, 1931b). Amadori showed that each of these reactions could yield, according to experimental conditions, two structurally different isomers which are not members of an $\alpha\beta$-anomeric pair; one of these isomers being more labile than the other toward hydrolysis, and more susceptible to decomposition on standing in air. He identified the labile isomers correctly as being *N*-substituted glycosylamines, but thought (mistakenly) that the stable isomer was of the Schiff-base type.

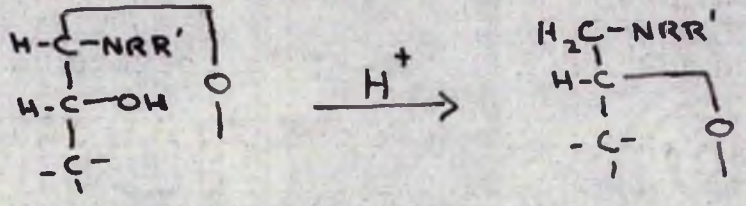
Kuhn and Dansi (1936) proved that Amadori's labile isomer obtained from the condensation of glucose and *p*-toluidine is in fact *N*-*p*-tolyl-β- glucopyranosylamine.



glucose p-toluidine N-p-tolyl-β-gluco-pyranosylamine

They also determined that the stable isomer is not a Schiff-base, but the result of a molecular rearrangement, and thought (again incorrectly) that it contained a branched-chain carbon skeleton.

Kuhn and Weygand (1937) reported the true structure of the rearranged product. Amadori's stable isomers are unbranched N-substituted 1-amino-1-deoxy-2-ketoses. On this basis, the term Amadori rearrangement is now used to describe the complete conversion of a N-substituted aldosylamine to a N-substituted 1-amino-1-deoxy-2-ketose.



This rearrangement is catalysed by acid (Weygand, 1940) but, as shown by Hodge and Rist (1953), it can take place, albeit slowly, in the solid state at 25°, and more rapidly in hot alcoholic solution in the presence of compounds containing active methylene groups. Druey and Huber (1957) prepared di-N-benzylfructosamine

and di-N-benzyltagatosamine from glucose and galactose respectively using ammonium chloride in hot ethanol as the rearrangement catalyst.

Many aldoses have been shown (Hodge, 1955) to give rise to 'Amadori-Compounds', as Kuhn and Haas (1956) term the products of Amadori rearrangements, while the rearrangement has been demonstrated with mono-N-substituted glycosylamines derived from aromatic amines and aralkyl- and alkylamines, and with di-N-substituted glycosylamines containing dialkyl-, alkylaryl-, aralkyl-, isocyclic-, and heterocyclic-alkyl radicals. If the sugar moiety of a N-substituted glycoside is substituted so that a pyranose ring cannot form, the tendency for the Amadori rearrangement to take place is increased remarkably (Micheel, 1957).

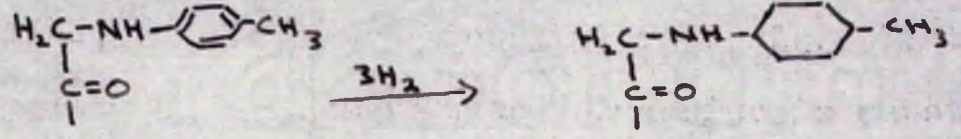
The rearrangement has been reported for N-glycosyl derivatives of amino acids (e.g. Gottschalk, 1952; Hodge and Rist, 1953; Anderson, 1954; Lowey and Borsook, 1956), but not as yet for any nucleoside. Hodge (1953) and Hodge and Rist (1953) have indicated the importance of the Amadori rearrangement in the series of reactions producing non-enzymatic browning of foodstuffs.

The mechanism of the Amadori rearrangement has not yet been satisfactorily explained. The scheme suggested by Kuhn and Weygand (1937) and Weygand (1940) appears

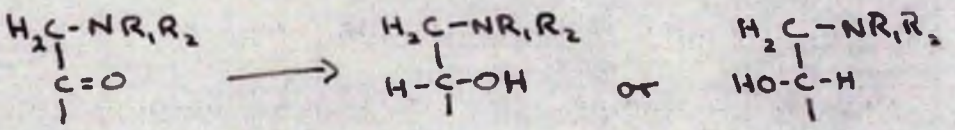
to lie closest to the truth, but has as yet not received any corroberation other than that of analogy with the acid-catalysed sugar enolisation (Petuely, 1953). Hodge (1955) has reviewed the this question of mechanism.

Amadori compounds can be hydrogenated to give three different types of compounds:-

(i) Weygand (1940), using Adams platinum catalyst in acid solution, observed the uptake of three moles of hydrogen by 1-deoxy-1-p-tolylaminofructose. He concluded that the aromatic nucleus alone was being hydrogenated.

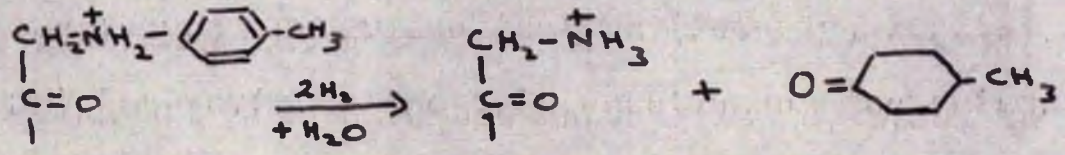


(ii) In weakly alkaline solution, hydrogenation with a platinum catalyst is limited to the sugar radical, and, depending on the experimental conditions, one of the two epimeric 1-arylamino-1-deoxy-2-ketoses is obtained (Kuhn and Weygand, 1937; Weygand, 1940; Hodge, 1955).



(iii) Kuhn and Haas (1956) used their palladium hydroxide - barium sulphate catalyst in acid solution to convert N-arylfructosamines to fructosamine. Two moles of hydrogen are taken up and a substituted cyclo-

hexanone is the other product of the reaction. The yield of fructosamine claimed is 80-85 %.



Druey and Huber obtained fructosamine and 1-amino-1-deoxy-tagatose in almost quantitative yields from their respective di-N-benzyl derivatives using a 10 % palladium - charcoal catalyst.

Table 1 summarises the purity and percentage yield of fructosamine prepared by various authors from glucos-azone and from Amadori compounds.

TABLE 1.

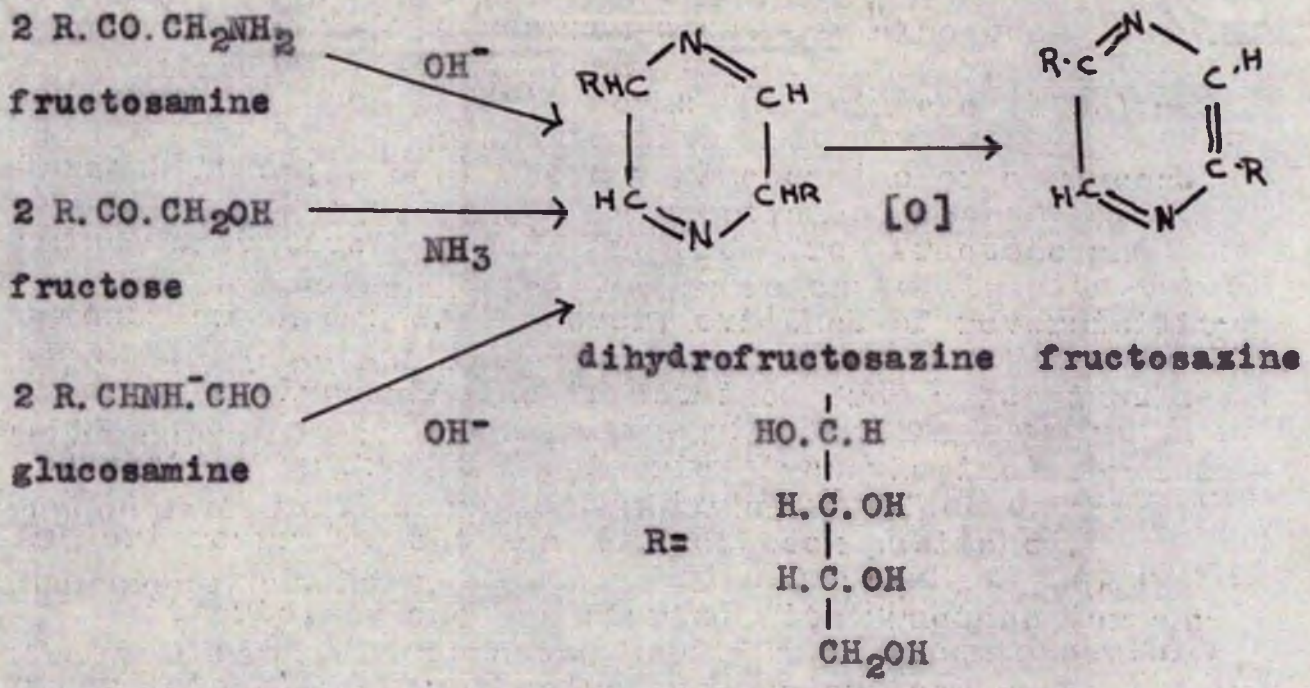
Author	m.p.	yield %	$[\alpha]_D^0$
Fischer (1886)	135	11	--
Maurer & Schiedt (1935)	137	60	-63.7 (w)
Kuhn & Haas (1956)	136-7	67	-66 (w)
Kuhn & Haas (1956)	137	82	-69.8 (w)
Kuhn & Haas(1956)	138	83	-68.9 (w)
Kuhn & Haas (1956)	132	40	-69.3 (w)
Kuhn & Haas (1956)	137	79	-69.3 (w)
Druey & Huber (1957)	135-7	64	--
Druey & Huber (1957)	145-6	94	-63 (pyr)

An Amadori-type rearrangement has also been reported by Heyns and Koch (1952), Heyns and Meinecke (1953) and Carson (1955a; 1955b) whereby glucosamine or a glucosamine derivative has been prepared from fructose and ammonia or an amine. However, evidence of reversibility (whereby a glycosylamine is obtained from a 1-amino-1-deoxyketose or from the epimeric glycosylamine) for the Amadori rearrangement has not yet been published.

Hitherto, no one has started with fructose (or any other ketose) and synthesized an Amadori compound. Hodge (1955) has suggested that this could be done from tetra-O-acetyl-1-chloro-1-deoxyketofructose which has been synthesized by Wolfrom et al. (1942) and Bredereck and Protzer (1954). Addition of the correct amine to this chloro compound at low temperatures, followed by deacetylation, should produce the desired Amadori compound.

1(b). Properties.

Maurer and Schiedt (1935) attempted to isolate the free aminoketose (1-amino-1-deoxyfructose) by treating its acetic acid salt with diethylamine. This treatment led to the ready formation of 2,5-bis-(D-arabotetrahydroxy-n-butyl)-pyrazine (fructosazine),



which is also formed by the intermolecular condensation of two molecules of glucosamine (Lobry de Bruyn and van Ekenstein, 1899) or by the direct action of ammonia on fructose, via fructosimine (Newbold and Spring, 1945). Because of the unavailability of the free base of fructosamine, which differs in this respect from glucosamine (Breuer, 1898) which has even been crystallized (Allison and Hixon, 1928), the properties discussed in this section, except when specifically mentioned to the contrary, apply to the acetic acid salt of fructosamine.

Melting points of fructosamine, reported in the literature, lie between 135 and 146°. As fructosamine melts it evolves nitrogen quite vigorously, and turns dark brown. Specific rotations quoted vary from -63 to

-70°. (See Table 1). No mutarotation has been observed though N-substituted fructosamines are known to mutarotate quite markedly (Amadori, 1929a).

The molecular configuration of fructosamine has not as yet been elucdated, although Druey and Huber (1957) depict its hexa-O-acetyl derivative in the furanose form, while Micheel and Klemer (1957) show a fructosamine molecule as a pyranose derivative. Neither set of authors quotes any evidence for the particular structure chosen.

Fructosamine is very soluble in water, slightly so in ethanol,and insoluble in ether. It crystallizes readily from an aqueous solution that has been saturated with ethanol. Fructosamine has also been isolated as salts of other acids. Fischer (1886) prepared crystalline picrate and oxalate; the latter melts at 140-150° with decomposition, and is soluble in water and virtually insoluble in ethanol. He also prepared a sulphate and a hydrochloride but these salts were too soluble in water and ethanol to be crystallized. This is again in contrast with glucosamine which forms a crystalline hydrochloride very easily. Chloroplatinate solutions give a flocculent, yellow, very hygroscopic precipitate with the acetic acid salt.

Apart from the above-mentioned salts, and the Amadori compounds, (which are N-substituted fructosamines), there are very few derivatives of fructosamine described in the literature. Treatment of fructosamine with phenylhydrazine produces glucosazone (Fischer, 1886), and on reduction fructosamine yields a mixture of the two isomeric 1-amino-1-deoxyhexitols (1-amino-1-deoxymannitol and 1-amino-1-deoxysorbitol) (Kent and Whitehouse, 1955). Glucosamine is formed by the addition of liquid ammonia to fructosamine (Druey and Huber, 1957) and the action of nitrous acid on fructosamine results in the formation of fructose (Fischer and Tafel, 1887). The latter reaction is another difference between fructosamine and glucosamine. With nitrous acid, glucosamine forms an anhydrosugar, 2,5-anhydromannose (chitose) (Fischer and Tiemann, 1894 ; Fischer and Andreae, 1903).

Fructosamine reduces Fehlings solution and methylene blue.

2. A REVIEW OF THE CHEMISTRY OF DEOXYSUGARS.

2(a). Introduction.

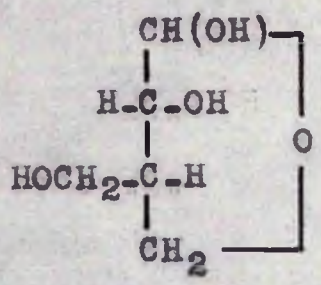
Deoxysugars are of widespread natural occurrence and are of considerable biological importance. The commonest deoxysugar is 2-deoxyribose, which occurs naturally as the carbohydrate component of DNA, and which is the only 2-deoxypentose found so far to occur in DNA. It has been isolated in fair yields from natural sources by Levene and his co-workers (1929, 1930) and by Laland *et al.* (reported by Overend and Stacey, 1953).

Many other deoxysugars occur as glycosides in nature. L-Rhamnose (6-deoxy-L-mannose) is a constituent of gums and mucilages and is also found in combination with flavones and anthocyanins. L-Fucose (6-deoxy-L-galactose) occurs in gum tragacanth and the blood group polysaccharides, while fucose and quinovose (6-deoxy-glucose) occur together in Jalap resin.

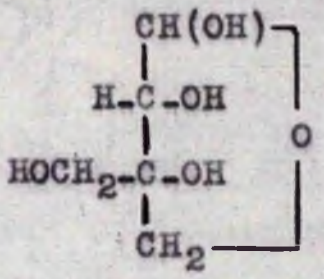
Several other methylpentoses (6-deoxyhexoses) occur as methoxy- or deoxy-derivatives in cardiac glycosides (Elderfield, 1945), the methoxy- and deoxy-group invariably being situated at carbon-3 and carbon-2 respectively.

A unique 3-deoxysugar has been isolated and characterised by Cunningham *et al.* (1951) from cordycepin, a

metabolic product isolated from culture solutions of Cordyceps militaris (Linn.) Link. It is structurally related to apiose, a sugar which occurs in parsley, and has been called cordycepose. (Bentley et al., 1951;



cordycepose



apiose

Hudson, 1949)

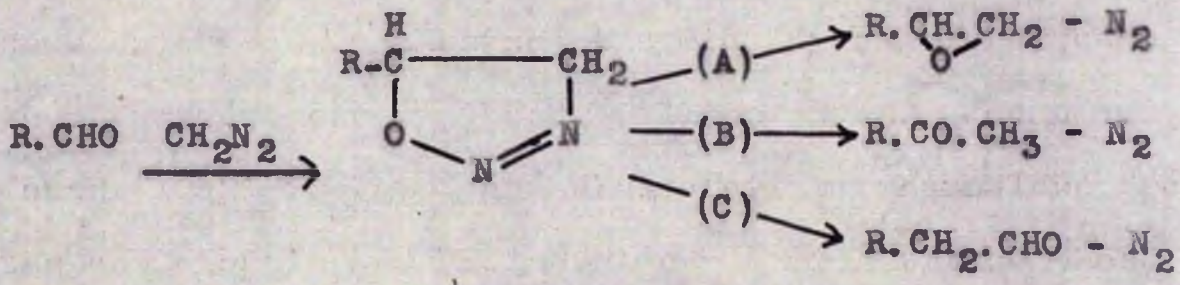
Srinavasen et al. (1955) have shown that a precursor in the biosynthesis of shikimic acid is 3-deoxy-2-keto-7-phosphoglucoheptonic acid.

Apart from those deoxy sugars that occur naturally, some have been synthesized enzymically and referrence to a few of these syntheses will be given. Hough and Jones (1952) isolated and characterized rhamnulose from the condensation of DL-lactaldehyde with triose phosphate in the presence of an enzyme preparation from peas. Rhamnulose has also been prepared from rhamnose (Palleroni and Doudoroff, 1956) by the action of a 'mannose isomerase' preparation on the latter, and the former has been isolated as a glucosyl rhamnuloside after the action of sucrose phosphorylase on it.

For the purposes of the biological aspect of the work reported here, the chemical synthesis of three deoxyfructoses (1-deoxyfructose, 2,5-mannitan and 2,5-sorbitan) was attempted. Consequently the chemical synthesis of 1- and 2-deoxyketoses will be reviewed in rather greater detail than that of other deoxysugars.

2(b). Synthesis of 1-deoxy-2-ketoses.

The reaction of diazomethane with aldehydes to give the corresponding methylketone was discovered by Meyer in 1905, and was more closely investigated by Schlotterbeck (1907). The type of product resulting from this reaction depends on the chemical constitution of the reacting aldehyde as well as on the nature of the solvent used.

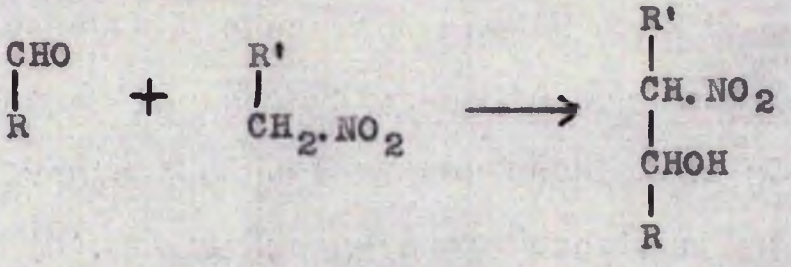


If the group 'R-' is electrophilic, reaction (A) takes place and an epoxide results. If it is nucleophilic, and the reaction is carried out in organic solvents, the corresponding methylketone is produced (B), and in ionic solvents the next higher homologous aldehyde is produced (C). The products of reactions B and C still

contain carbonyl groups and can therefore react further with diazomethane. Consequently a mixture of products can result in each of these two cases, and the addition of diazomethane to aldehydes containing nucleophilic groups is generally not a very satisfactory method of production of ketones.

The diazomethane synthesis of ketones has been applied to the carbohydrate field. Brigl et al. (1931) obtained a crystalline methyl ketone from aldehydoglucose 3,4,5,6-tetrabenzoate. No further characterization of this product was reported by them. Wolfrom et al. (1941) prepared a 1-deoxyketofructose tetraacetate by treating aldehydoarabinose tetraacetate with diazomethane. However these workers did not isolate the free 1-deoxyketose from this product. Hyslop (1956) treated 2,3:4,5-di-O-isopropylidenearabinose with diazomethane, hydrolysed off the isopropylidene groups from the product and obtained an impure sample of 1-deoxyfructose.

Henry (1895) showed that nitroparaffins will react with aldehydes, in the presence of a basic catalyst, to produce nitroalcohols.

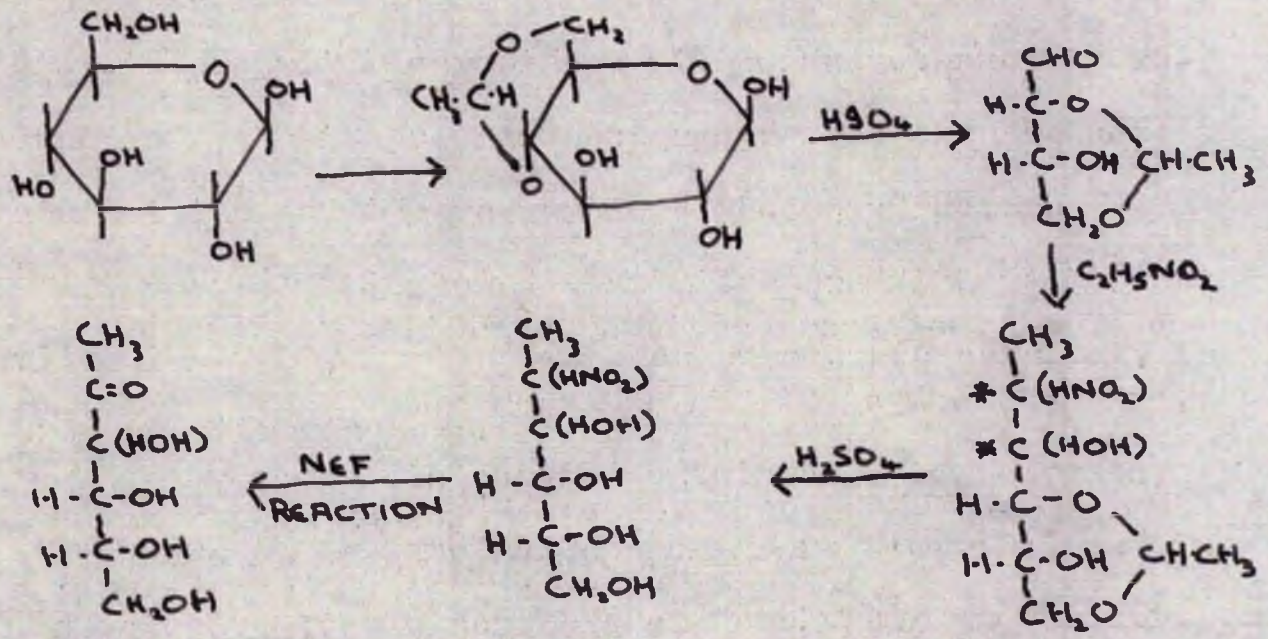


Pictet and Barbier (1921) attempted to produce

higher-carbon sugar alcohols from aldoses by condensation with nitromethane, but their experiments did not demonstrate unequivocally that the condensation had taken place.

Sowden (1951) prepared several sugar nitroalcohols from benzylidene-substituted aldoses containing a free aldehyde group, and he also prepared ketoses by treating aldoses with 2-nitroethanol (1950).

Hyslop (1956) prepared an impure sample of 1-deoxyfructose by condensing nitroethane with 2,4-ethylidene-erythrose, hydrolysing the resultant 1-deoxy-4,6-ethylidene-2-nitrofructose with dilute sulphuric acid and the carrying out a Nef reaction on the product. The starting compound in this synthesis was glucose.



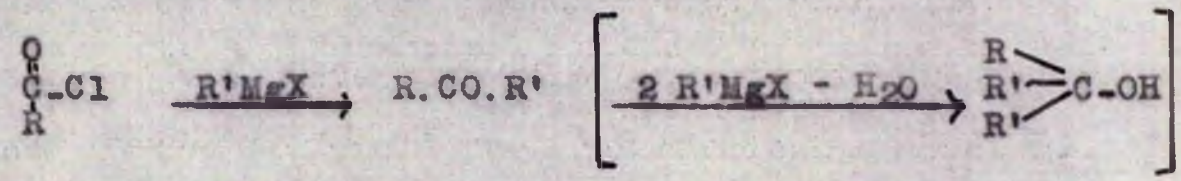
Two new asymmetric centres are created at the nitroethane condensation stage (C2 and C3), but as in most of these reactions, only two of the four isomers predominate, and since the asymmetry at C2 is destroyed by the Nef reaction, only two anomeric products (1-deoxyfructose and 1-deoxypsicose) are to be expected, with one of them predominating.

The 1-deoxyfructose synthesized by Hyslop (1956) by either of the above two methods was too impure and produced in too low yield for much chemical characterization to be carried out. However, quite extensive chromatographic analysis of the products was done by her. She reported that the triphenyl tetrazolium bromide reagent of Trevelyan et al. (1950) was much more sensitive for the detection of 1-deoxyfructose than were certain phenolic colour sprays. Rf values of 0.3 to 0.45 are quoted for this compound in chromatograms run on Whatman No 1 paper in n-butanol - acetic acid - water (4:1:5) at room temperature. Hyslop also found that 1-deoxyfructose was not phosphorylated by hexokinase, nor did it inhibit the phosphorylation of fructose by this enzyme.

Other workers have not reported the the preparation of 1-deoxyfructose in the free state, although, as

stated above, Wolfrom et al. (1941) prepared the tetraacetate of it and of its enantiomorph. They also crystallized the racemate from a mixture of these two compounds, obtained iodoform from 1-deoxyketofructose tetraacetate and prepared the oxime of the latter.

A general type of reaction that may be utilized for the preparation of a 1-deoxyketose is the Grignard synthesis of ketones from acyl chlorides:

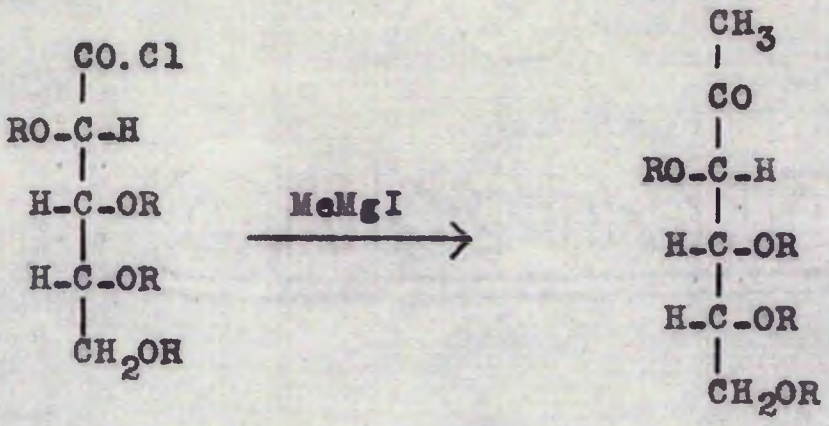


The initial step is more rapid than the formation of the tertiary alcohol, and consequently, by using equimolar proportions of the acyl chloride and Grignard reagent, the reaction can be stopped at the ketone stage.

Ohle and Hecht (1930) treated di-O-isopropylidene-2-ketogluconic acid with four moles of methyl magnesium iodide and obtained, in the ratio of seven to one, 1-deoxy-2,3:4,5-di-O-isopropylidenefructose and 2,3:4,5-di-O-isopropylidene-1-di-C-methylfructose. Further treatment of the first product gave the second one.

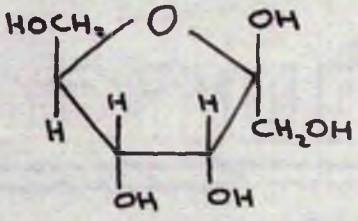
Although the reaction has not been reported, this method can lead to the formation of 1-deoxyfructose starting from a substituted arabonic acid or arabonyl

chloride:

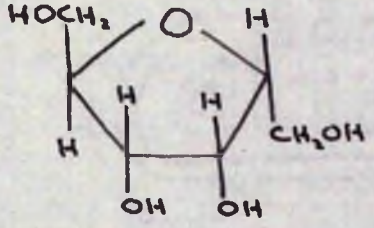


2(c) Synthesis of 2-deoxy-2-ketoses.

Technically, 2-deoxy-2-ketoses are anhydrosugar alcohols in which C2 is involved in the formation of the anhydro-ring, but for uniformity they are classified here under the heading of deoxysugars.



2-ketohexose



2,5-anhydrohexitol

A number of anhydrohexitols, in which C2 has been involved in the anhydro-ring, has been described in the literature.

Brigl and Grüner (1933, 1934) obtained several anhydrohexitol derivatives when they heated 1,6-dibenzoyl-mannitol with p-toluene sulphonic acid in sym-tetra-chloroethane. One of these products was described as

2,5-anhydro-1,6-dibenzoylmannitol. Hockett et al. (1946), however, reinvestigated this reaction, and on the result of the oxidation of this compound with lead tetraacetate, proved that a Walden inversion had taken place at C2 and that the compound was in fact 2,5-anhydro-1,6-dibenzoylsorbitol. Treatment of this with p-toluene sulphonyl chloride gave a ditosyl derivative identical with the compound formed by the tosylation of 1,6-dibenzoylmannitol (Müller and Vargha, 1933). Wiggins (1950) has postulated the temporary formation of 1,6-dibenzoyl-2-tosylmannitol in the latter reaction, from which the tosyl group is split off with simultaneous Walden inversion and anhydro-ring formation between C2 and C5 giving 2,5-anhydro-1,6-dibenzoylsorbitol. Complete tosylation of the latter gives 2,5-anhydro-1,6-dibenzoyl-3,4-ditosylsorbitol.

2,5-Anhydrosorbitol is the only analogue of fructofuranose that has been shown to be phosphorylated under the action of a hexokinase (Sols and Crane, 1954; for brain hexokinase).

Chitose has been shown by Akiya and Osawa (1954) and Grant (1951,1956) to be 2,5-anhydromannose, thus confirming the opinions of Levene and his co-workers (Levene and La Forge, 1915; Levene, 1918). Its forma-

tion by the deamination of glucosamine must therefore involve a Walden inversion at C2. Akiya and Osawa claimed that they had formed 2,5-anhydromannitol from chitose by reducing it with Raney nickel at 80 atmospheres and 100°. Although the compound was not crystallized, they did prepare crystalline dityrityl- and ditosyl- derivatives from it. Mitra and Karrer (1955) prepared 2,5-anhydro-l.3:4,6-dimethylenemannitol from 1,3:4,6-dimethylene-2,5-di-O-tosylmannitol. 2,5-Anhydro-1-O-tosyl-L-iditol has been obtained by Vargha et al. (1935) from 2,4-benzylidene-1,6-di-O-tosylsorbitol. Vargha et al. (1948) obtained 2,5-anhydro-L-iditol itself by treatment of its 1-O-tosyl derivative with sodium amalgam.

2(d) Synthesis of other deoxysugars.

Many general methods for synthesizing deoxysugars (apart from 1- and 2-deoxy-2-ketoses) have been described. A large number of these methods refer to the preparation of 2-deoxyaldoses and these will be mentioned first. As these syntheses have been reviewed recently by Overend and Stacey (1953) only a general account will be given in each case.

(i) The Fishher glycal method.

Since 1920, when Fischer first described this reaction,

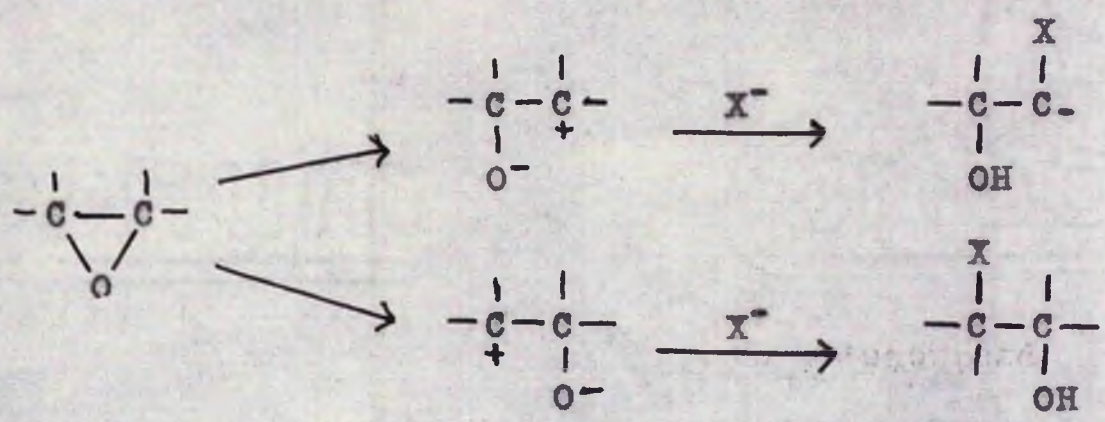
many 2-deoxysugars have been prepared by the addition of the elements of water to the olefinic link in a 2,3-glycal by treatment with dilute sulphuric acid at a low temperature.

(ii) The Fischer and Sowden method.

This method was initially used successfully for the preparation of 2-deoxyglucose by Fischer and Sowden (1947), who treated arabinose with nitromethane in the presence of sodium methoxide to get a mixture of 1-deoxy-1-nitromannitol and 1-deoxy-1-nitroglucitol. The mixture was not resolved, but was acetylated and subsequently treated with bicarbonate to convert it to arabo-3,4,5,6-tetra-O-acetyl-1-nitro-1-hexene, reduction of which with hydrogen in the presence of a palladium catalyst yielded 3,4,5,6-tetra-O-acetyl-1,2-dideoxy-1-nitroglucitol. Treatment of this with sulphuric acid readily afforded 2-deoxyglucose.

(iii) From 2,3-anhydrosugars.

A number of methods available for the synthesis of 2- and 3-deoxysugars depend on the cleavage by various reagents of 2,3-anhydro-rings in alkyl aldosides. This change can theoretically give rise to two different products, i.e.



The product that is obtained experimentally depends on the structure of the molecule containing the epoxide ring and on the nature of the cleaving agent.

(iv) Miscellaneous syntheses.

2-Deoxyribose has been prepared by condensation of 2,3-O-isopropylideneglyceraldehyde with allyl magnesium bromide (Hough, 1951) or with acetaldehyde (Overend and Stacey, 1952), and by a degradation of Nef's mixed glucometasaccharinic acids (Richards, 1954).

The latest and best method for preparing this compound is based on the treatment of α-glucose monohydrate with solid calcium hydroxide (Diehl and Fletcher, 1958a, 1958b).

Reductive desulphurization of deoxythiosugars with Raney nickel affords a useful pathway for preparing deoxysugars, the thiol being prepared by one of several methods. Baker (1955) replaced the tosyl group of

2,3:4,5-di-O-isopropylidene-1-O-tosylfructose with an ethanethiol group. However he did not take the synthesis any further. This reaction is of especial interest since the tosyl group of a 1-O-tosylketose is extremely resistant to replacement by iodine (Tipson, 1953).

Many tosylated sugars react with sodium or potassium iodide in acetone whereby desulphonyloxylation takes place and the deoxyiodo-derivative is formed (Tipson, 1953). Reichstein and his colleagues (Barnett and Reichstein, 1937; Morgan and Reichstein, 1938; Müller and Reichstein, 1938) prepared 6-deoxyfructose, 6-deoxy-tagatose and 6-deoxy-L-sorbose by reduction of their corresponding 6-deoxy-6-iodo-derivatives.

3. DISCUSSION OF EXPERIMENTAL RESULTS. THE CHEMISTRY OF 1-AMINO-1-DEOXY-D-FRUCTOSE.

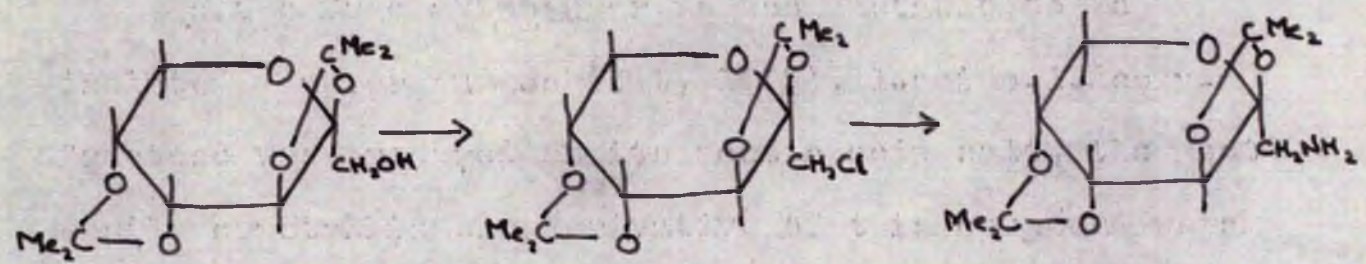
3(a) Preparation.

As the free aminosugar is too unstable to be isolated (Maurer and Schiedt, 1935), 1-amino-1-deoxy-fructose was prepared as its acetic acid salt, the most easily crystallizable derivative of this compound which has been reported. All the work reported here was carried out on the acetic acid salt, and, except in certain instances where ambiguity might result, this compound will be referred to as fructosamine acetate.

(i) Fructosamine acetate was prepared by the method of Fischer (1886) and by that of Kuhn and Haas (1956). The method of Maurer and Scheidt (1935) was modified, but this modified procedure gave a yield of only 15 % (compared with 60% as published, and 70 % from Kuhn and Haas's procedure). The product obtained by the reduction of glucosazone with zinc and acetic acid (Fischer, 1886) would not recrystallize and was considered unsuitable for further chemical and biological tests. The products of the other two preparations were identical and chromatographically pure, and were used for the later investigations, reported in this thesis, into the physical, chemical and biological properties of this

compound.

(ii) The preparaion of fructosamine acetate was attempted from 2,3:4,5-di-O-isopropylidenefructose by the following method:



The chlorination was attempted with phosphorus pentachloride and with thionyl chloride after the method of Haworth and Wiggins (1944), but only in the latter case was any visible reaction observed. However, even in this instance only the unchanged starting material was recovered and the further stages of the synthesis had to be abandoned. It was thought that thionyl chloride reacted with the free hydroxyl group to form an unstable sulphite, thus releasing hydrogen chloride, and that the ester decomposed during the working up to give back the starting material.

(iii) Fructosamine was shown to be formed by the following series of reactions:

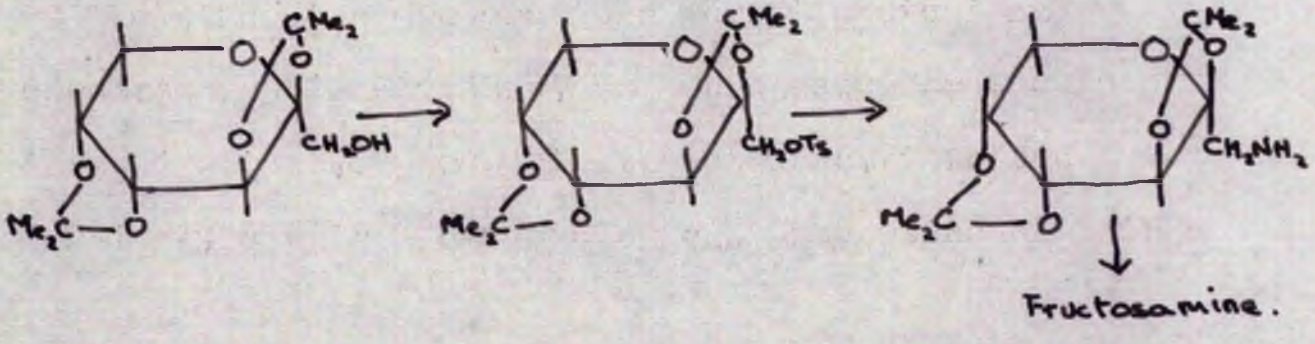
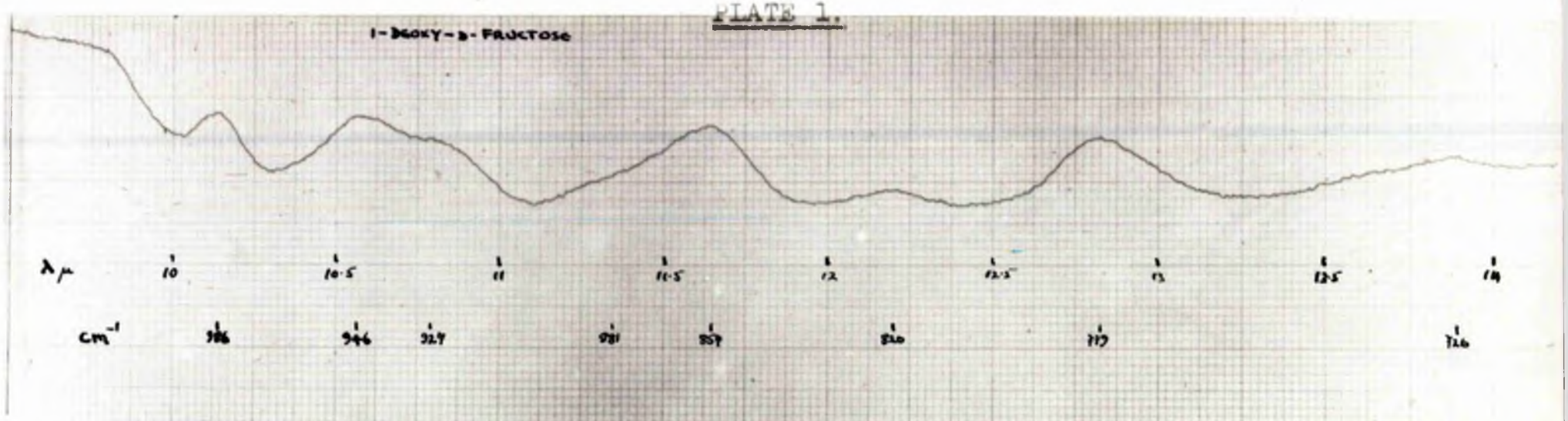
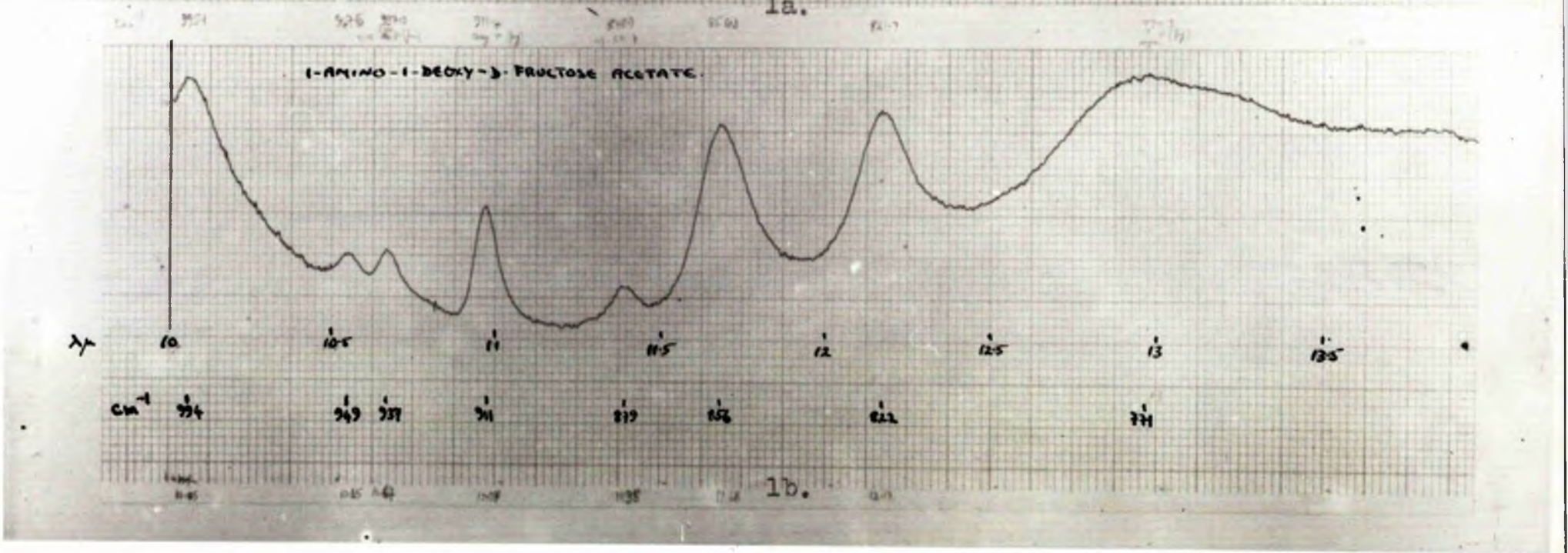


PLATE 1.


1-DEOXY-D-FRUCTOSE
λμ
10
10·5
11
11·5
12
12·5
13
13·5
14
cm⁻¹
986
946
927
881
857
820
779
726

1a.


1-AMINO-1-DEOXY-D-FRUCTOSE ACETATE.
λμ
10
10·5
11
11·5
12
12·5
13
13·5
cm⁻¹
994
949
937
911
879
856
822
771

1b.

PLATE 1.

The infrared absorption spectra of (a) 1-deoxy-fructose and (b) 1-amino-1-deoxyfructose in a Nujol mull between 10 and 14 u. The frequencies of the absorption maxima are given.

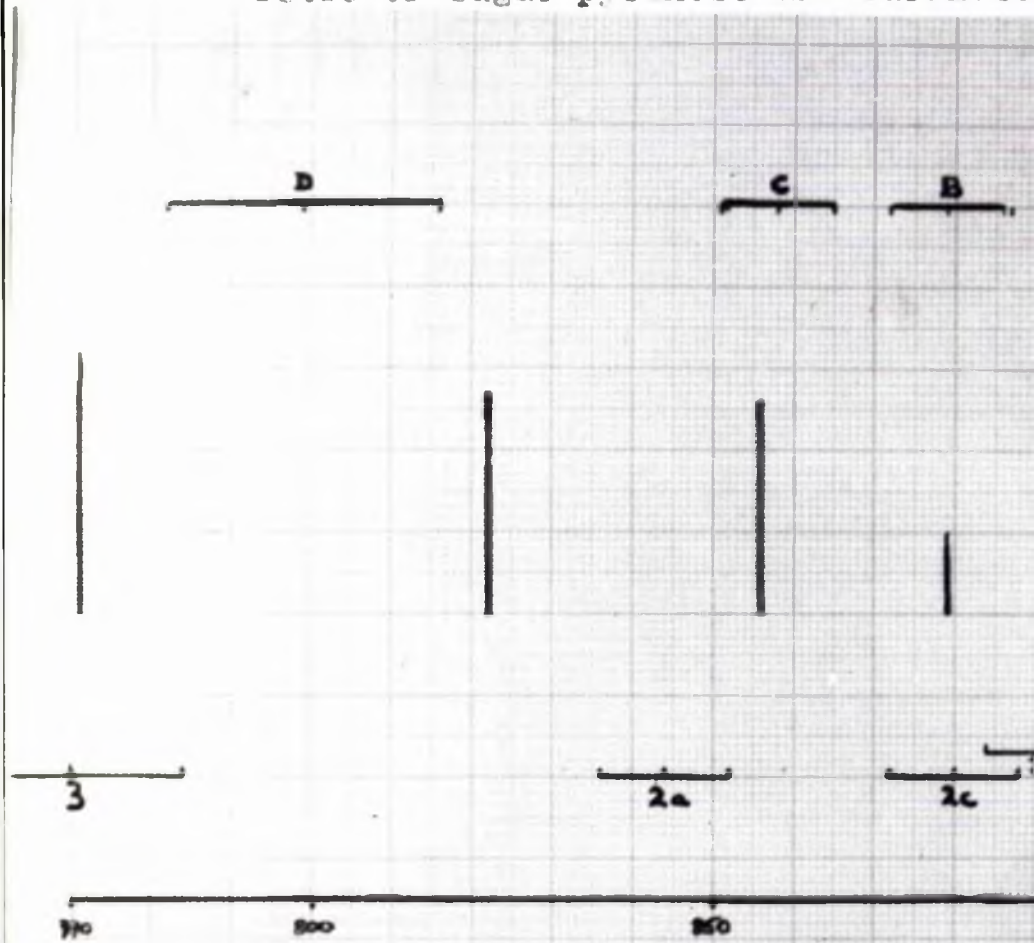
TABLE 2.

Solvent system	Whatman paper grade	Temperature °C	Rf
methyl cellosolve/ pyridine/ acetic acid. (8:4:1)	1	18 (A)	0.40
phenol/ water. (4:1)	1	18 (D)	0.55
n-butanol/ acetic acid/ water. (4:1:5)	1	26 (D)	0.33
ethyl acetate/ amyl alcohol/ formamide. (1:2:3)	2	40 (A)	0.14
butanol saturated with a saturated aqueous solution of oxalic.	2	18 (D)	0.04
collidine saturated with water.	4	40 (A)	0.13

(A) ascending. (D) descending.

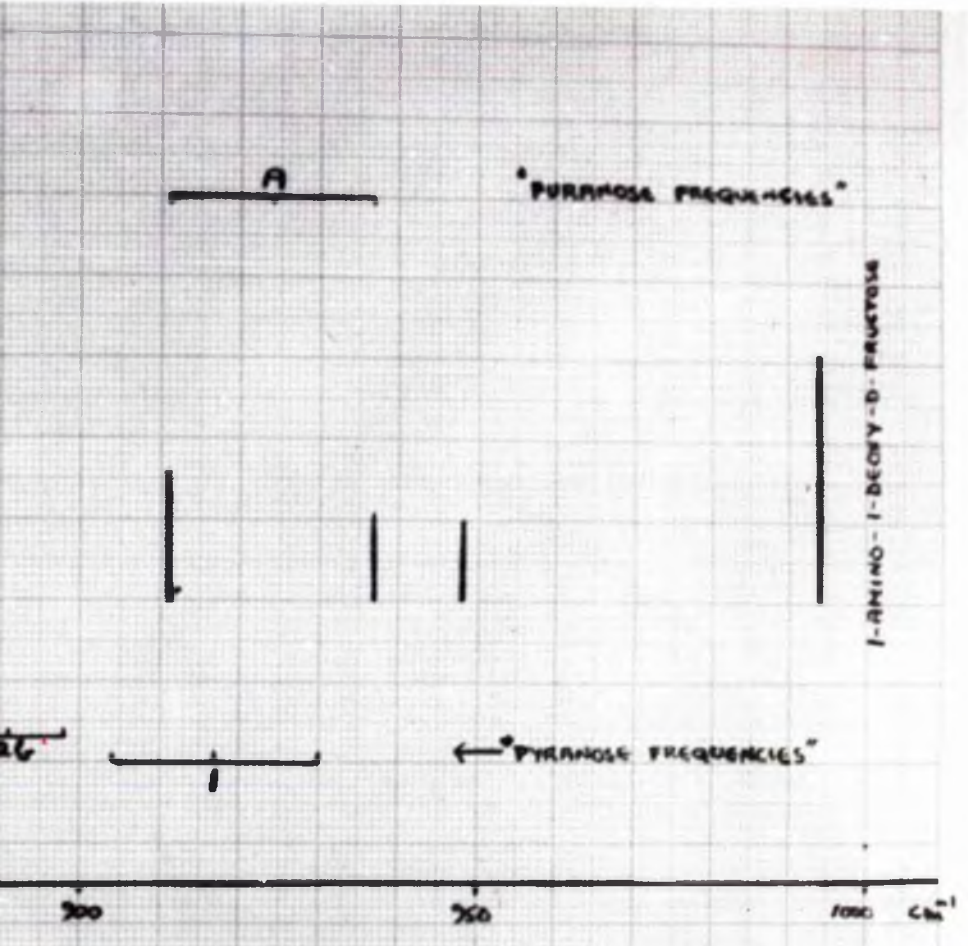
PLAT

Infrared absorption frequencies
indication of relative intensities)
istic of sugar pyranose and furanose



E 2.

of 1-amino-1-deoxyfructose (with
compared with the frequencies character-
rings.



The final product was shown to be present in trace amounts by paper chromatography, but the yield obtained by this ammonolysis is too small to have any preparative value.

3(b) Properties.

(i) Paper chromatography.

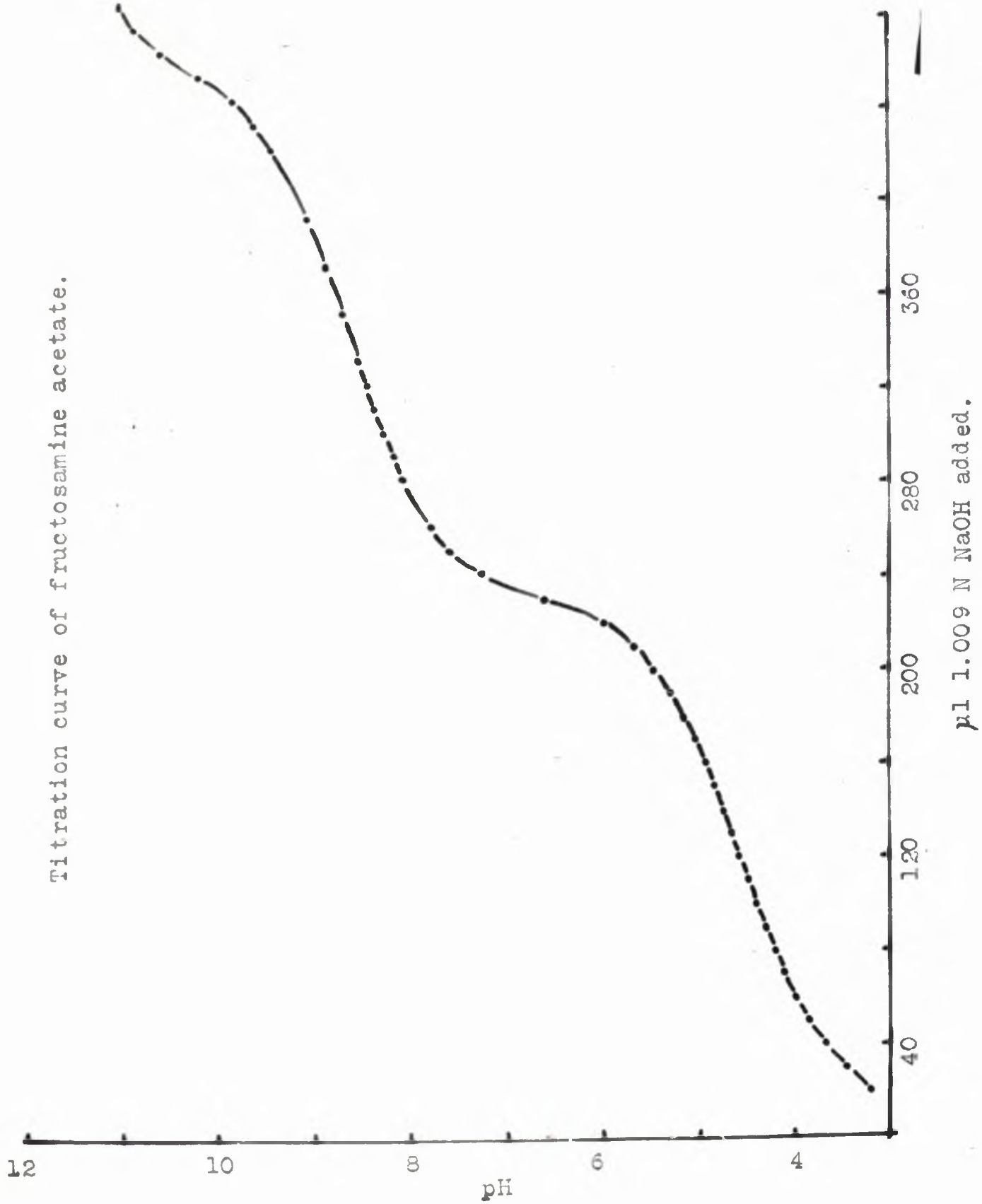
The Rf values obtained for fructosamine acetate under various eluting conditions are listed in Table 2.

(ii) Infrared absorption.

The infrared absorption spectrum in the region 10-14u of fructosamine acetate is reproduced in Plate 1b. Plate 2 contains an abstract of the frequencies of the absorption maxima on this trace, along with the absorption frequencies associated with pyranose and furanose ring systems (Barker, Bourne, Stacey and Whiffen, 1954; Barker and Stephens, 1954). From this evidence alone it is not possible to assign to fructosamine acetate a pyranose or a furanose structure, although a tentative assignment of a pyranose one has been made for the following reasons.

The peak at 911 cm^{-1} corresponds to the pyranose type 1 absorption more than it does to the furanose type A. These type 1 absorptions have been ascribed to antisymmetrical ring vibrations in the pyranose ring.

PLATE 3.


Titration curve of fructosamine acetate.
12
10
8
6
4
pH
40
120
200
280
360
µl 1.009 N NaOH added.

Similarly the peak at 771 cm^{-1} has been ascribed to the pyranose type 3 (symmetrical ring brething vibration) absorption, rather than to the furanose type D. The other frequencies are even less easily assigned, and this prevents a firmer conclusion from being drawn.

(iii) Titration curve.

The titration curve of fructosamine acetate from pH 3 to pH 11 is shown in plate 3. pK values of 4.7 and 8.6 can be derived from it.

(iv) Mutarotation.

It had been hoped that studies of the mutarotation of fructosamine acetate in water and in dimethylformamide would lead to some information on the ring configuration of this compound (Kuhn and Grassner, 1957). Unfortunately mutarotation was not detected in water or in water/ dimethylformamide (1:4) (fructosamine is insoluble in anhydrous dimethylformamide), but a striking difference was observed between the two specific rotations. In water it was -72.8°, and in the water/ dimethylformamide mixture it was -28.0°. Without further information it is impossible to draw any conclusions from this difference, or even to comment on its significance.

TABLE 3.

Solvent system	Temp. °C.	Rf values			Plate No.
		Product	Fructosamine	Glucosone	
n-butanol/ acetic acid/ water. (4:1:5).	26	0.38	0.33	0.38	-
phenol/ water. (4:1)	18	0.21	0.55	0.22	4
collidine saturated with water.	40	0.28	0.13	0.27	5
ethyl acetate/amyl alcohol/ formamide. (1:2:3)	40	0.41	0.14	0.39	6
n-butanol saturated with a saturated aqueous solution of oxalic acid.	26	0.16	0.03	0.16	7

PLATE 4.

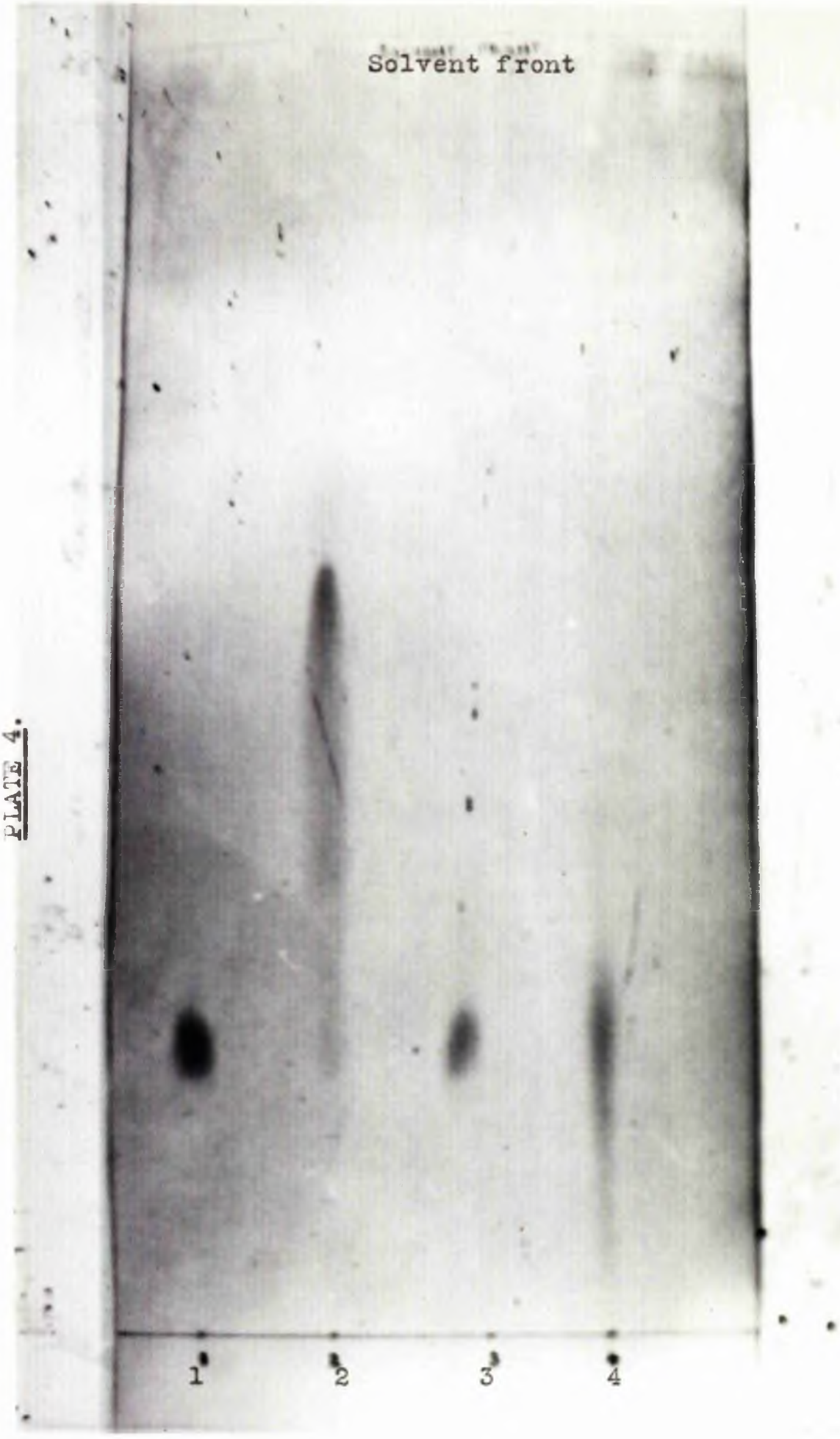


PLATE 4.

1. and 3. Product from reaction of ninhydrin with fructosamine acetate.

2. Fructosamine.

4. Glucosone.

The chromatogram was run on Whatman No 1 paper in phenol/ water (4:1) at 18°.

Spray: Trevelyan's (1950) triphenyltetrazolium reagent.

PLATE 5.

PLATE 5.

1 and 3. Product from reaction of ninhydrin with fructosamine acetate.

2. Fructosamine.

4. Glucosone.

The chromatogram was run in collidine saturated with water, on Whatman No 4 paper, at 40°.

Spray: Triphenyltetrazolium reagent (Trevelyan *et al.*, 1950).

PLATE 6.

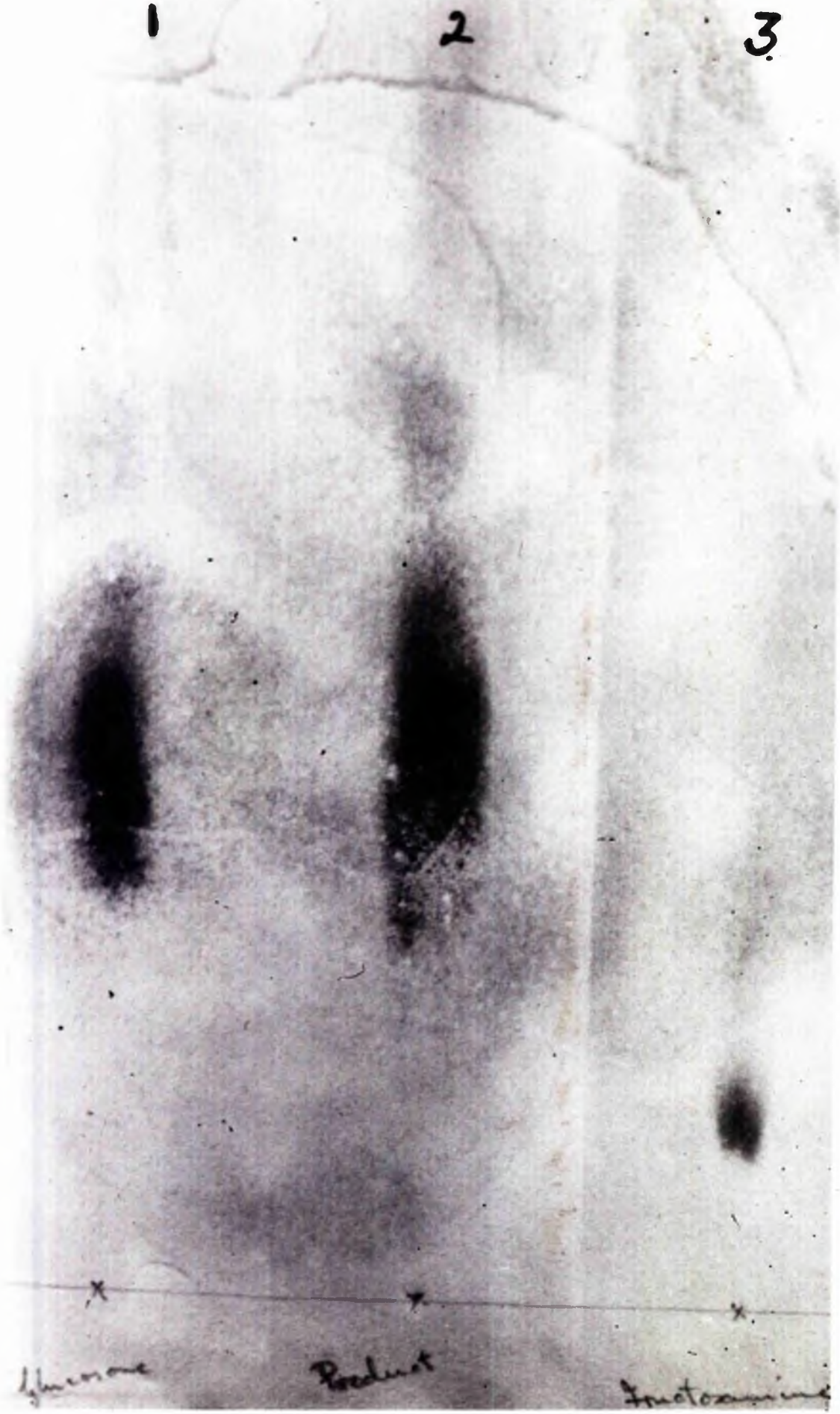


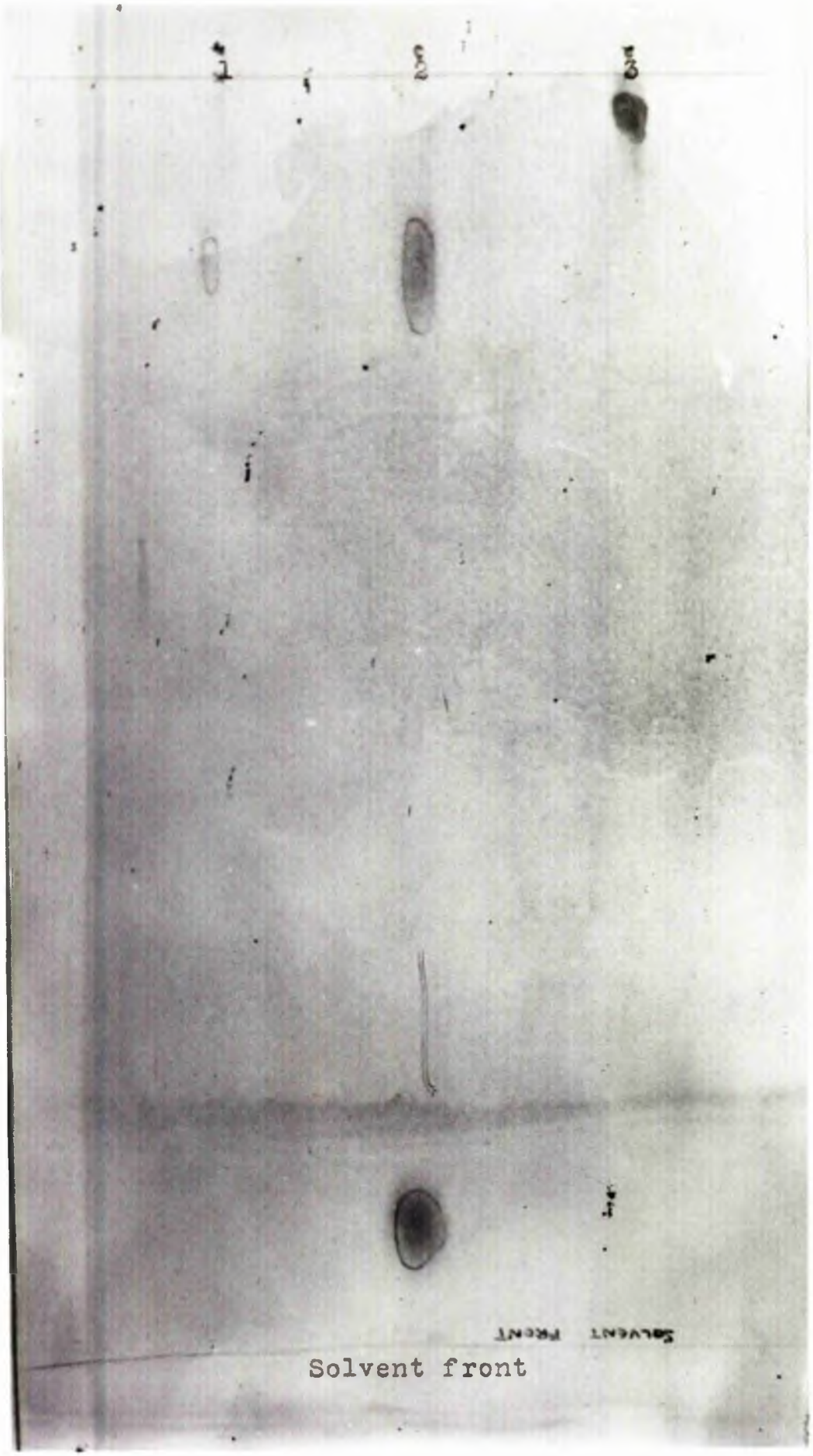
PLATE 6.

1. Glucosone.
2. Product from reaction of ninhydrin with fructosamine acetate.
3. Fructosamine acetate.

The chromatogram was run on Whatman No 4 paper in ethyl acetate/ amyl alcohol/ formamide (1:2:3) at 40°.

Spray: Arsenotungstic acid - NaOH/ KCN.

PLATE 7.



Solvent front

PLATE 7.

1. Glucosone.
2. Product from reaction of ninhydrin with fructosamine acetate.
3. Fructosamine.

The chromatogram was run on Whatman No 2 paper in n-butanol saturated with a saturated aqueous solution of oxalic acid, at 26°.

Spray: Triphenyltetrazolium reagent (Trevelyan *et al.*, 1950).

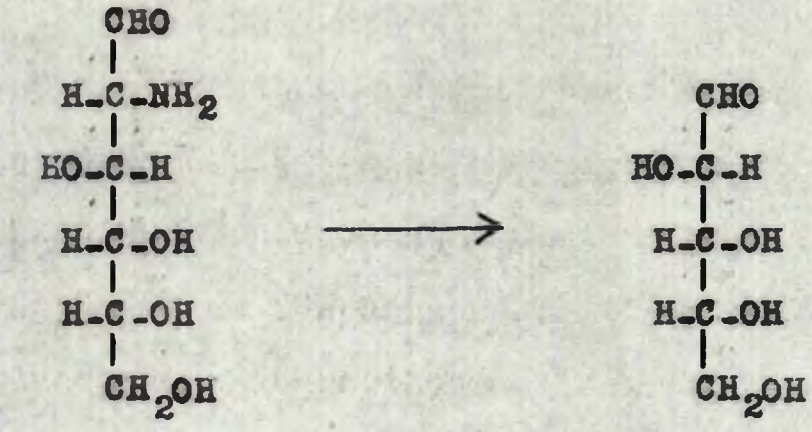
(v) Reducing properties.

Fructosamine was shown to be a strongly reducing compound in so far as it reacted with Fehling's solution without heating, and as it also gave a blue colour with Benedict's arsenotungstic reagent. It did not reduce 2,6-dichlorphenolindophenol, nor react with Seliwanoff's resorcinol reagent for ketoses.

(vi) Reaction with ninhydrin.

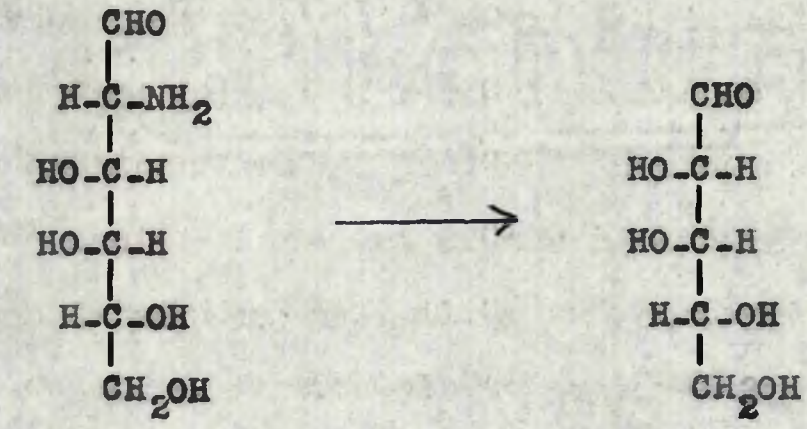
With ninhydrin fructosamine formed the blue colour characteristic of amines and amino-acids. From paper chromatographic analysis (results in Table 3 opposite), fructosamine was shown fairly conclusively to be oxidatively deaminated by ninhydrin, the product of this reaction being glucosone.

A further indication of the nature of the reaction product was furnished by the behaviour of the various spots with the arsenotungstic acid spray. The glucosone marker and the reaction product, on chromatograms run in acidic or neutral solvents, appeared as deep blue spots as soon as the chromatogram was placed in the alkali-cyanide solution. Fructosamine appeared as a paler blue after the paper was dried. On chromatograms run in collidine/ water the fructosamine spot showed up as soon as it was sprayed with arseno-tungstic acid,



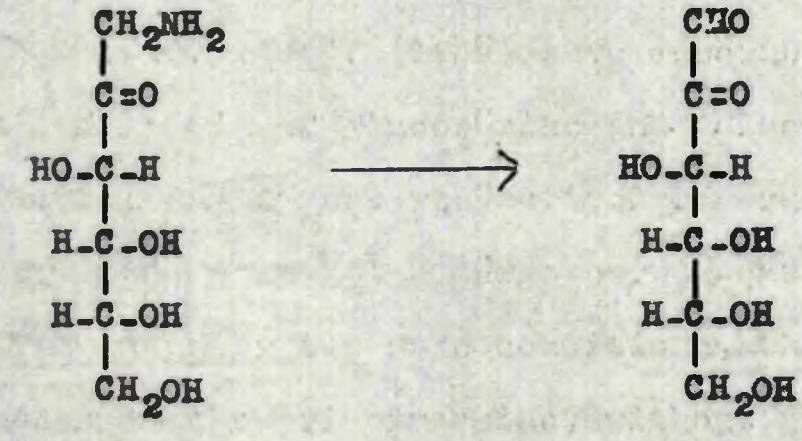
glucosamine

arabinose



galactosamine

lyxose



fructosamine

glucosone

whereas the glucosone and reaction product spots did not appear till after alkali-cyanide treatment.

The reaction product formed an osazone which was shown by melting point and mixed-melting point determinations to be glucosazone, but no osotriazole could be prepared from the osazone. Attempts to form glucosone dibenzyl dithioacetal from the reaction product also failed.

The above results tend to show that glucosone is formed by the reaction of ninhydrin on fructosamine acetate, although the reaction has no preparative value since the glucosone produced is contaminated by the decomposition product of the ninhydrin used. This appears to have similar solubility and chemical reactivity to glucosone, and attempts to purify the latter by extraction with various solvents or by making derivatives were unsuccessful.

Gardell *et al.* (1950) have described the oxidation with ninhydrin of glucosamine and galactosamine to arabinose and lyxose respectively. Fructosamine, on the other hand, does not undergo carbon-carbon scission. It forms, under the same conditions, the aldehyde corresponding to the primary amine, without any shortening of the carbon chain.

(vii) Fructosamine acetate does not appear to form an O-isopropylidene derivative by direct acetonation under the conditions employed. The product obtained was shown to be the sulphuric acid salt of the aminosugar.

(viii) As described in section 3(a).(iii) (page 25), small amounts of what is presumably 1-amino-1-deoxy-2,3:4,5-di-O-isopropylidenefructose were formed by the ammonolysis of 2,3:4,5-di-O-isopropylidene-1-O-tosyl-fructose at 100°. The yield was too small, however, for any characterization of the product to be carried out.

(ix) The failure of attempts to produce 1-amino-1-deoxy-O-isopropylidenefructose by hydrogenation of 1-deoxy-O-isopropylidene-1-p-tolylaminofructose in the presence of Kuhn and Haas's (1955) catalyst, can probably be ascribed to the hydrolysis, by the acid present and necessary for the hydrogenation, of the starting material and of any product that might have been formed from it.

1-Deoxy-O-isopropylidene-1-p-tolylaminofructose does not mutarotate in water (Douglas and Honeyman, 1955). The change in optical rotation observed for this compound in acetic acid solution may be due either to mutarotation or to hydrolysis of the O-isopropylidene group. As some O-isopropylidene compounds are known to be acid labile, and as the presence of fructosamine was shown in the

resultant syrup, the latter is probably the correct explanation of the change in optical rotation.

Removal of the acetic acid by evaporation would increase the rate of hydrolysis because of the rise in temperature involved, and the concomitant rise in acid strength as evaporation proceeded.

To try to avoid this hydrolysis the solution was neutralized with sodium hydroxide, but the product could not be crystallized. This was probably due to the large amounts of sodium acetate with which the product was contaminated.

4. DISCUSSION OF EXPERIMENTAL RESULTS. THE CHEMISTRY OF 1-DEOXY-D-FRUCTOSE.

4(a). Synthesis.

When this work was started, no synthesis of 1-deoxyfructose had been described in the literature, but concurrently with the experiments reported here Hyslop (1956) synthesized this compound by two methods which gave rather low yields. The results to be presented describe a third synthesis of 1-deoxyfructose by an unambiguous scheme. The yields obtained were all high, (greater than 60 %) for each step in the synthesis, and as each step is of an essentially simple nature, it affords a productive method of preparing this compound.

Starting from fructose, the outline of the scheme is as follows:

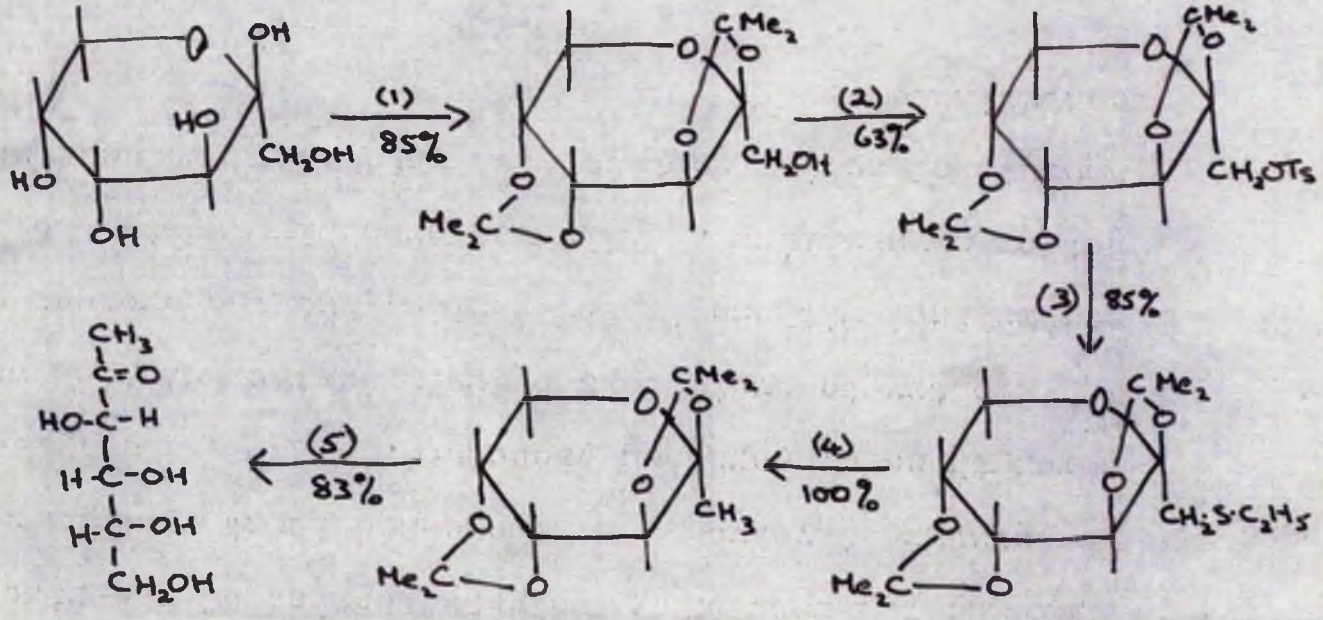


PLATE 8.

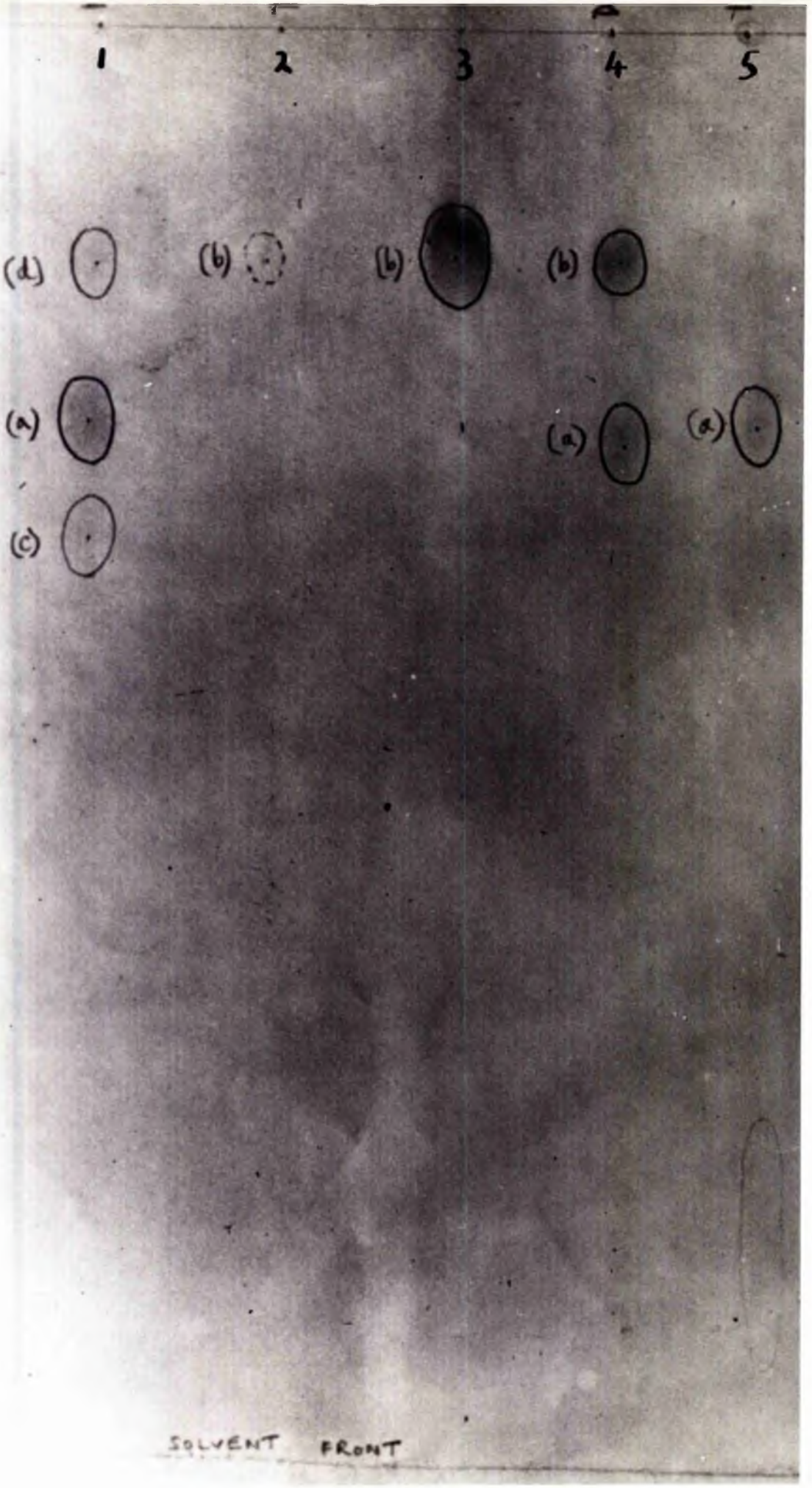


PLATE 8.

1. Nitroethane preparation of 1-deoxyfructose (Hyslop).
   (a) 1-deoxyfructose.
   (c) and (d) impurities.
2. Diazomethane preparation of 1-deoxyfructose (Hyslop) shows (b) arabinose.
3. Arabinose.
4. Diazomethane preparation of 1-deoxyfructose (Hyslop).
   (a) 1-deoxyfructose.
   (b) arabinose.
5. (a) 1-deoxyfructose (author).

The chromatogram was run on Whatman No 1 paper in n-butanol/ acetic acid/ water (4:1:5) at 18°.

Spray: Triphenyltetrazolium reagent of Trevelyan *et al.* (1950).

Steps (1), (2) and (3) were carried out according to the methods of Pacsu *et al.* (1939), Levene and Tipson (1937) and Baker (1955) respectively. Step (4) involved reductive desulphurization by Raney nickel and step (5) was a straightforward acid hydrolysis.

Characterizing derivatives were not obtained for either the free deoxysugar nor its di-*O*-isopropylidene derivative, although an elementary analysis of the latter agreed with the theoretical values. A chromatogram of the final product is shown (Plate 8) comparing it with Hyslop's two products. From this it is seen that the products of the three syntheses are identical and that the product of the above synthesis is uncontaminated by any other reducing compound.

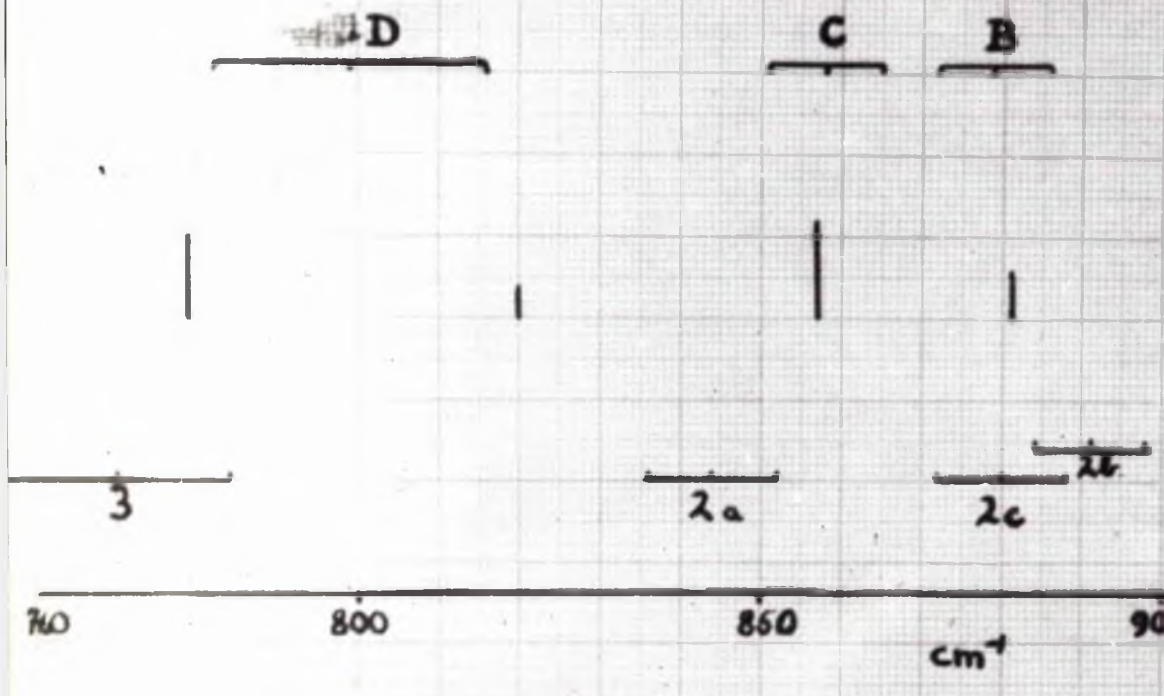
4(b). Properties.

(i) Chemical properties.

Reducing properties are quite strong in 1-deoxyfructose as is shown by its reaction with Fehling's solution and Benedict's arsenotungstic acid reagent. It also gives a positive reaction in Seliwanoff's test for ketoses, but it does not reduce 2,6-dichlorphenolindophenol. It will not act as a substrate in the glucose oxidase catalysed reaction.

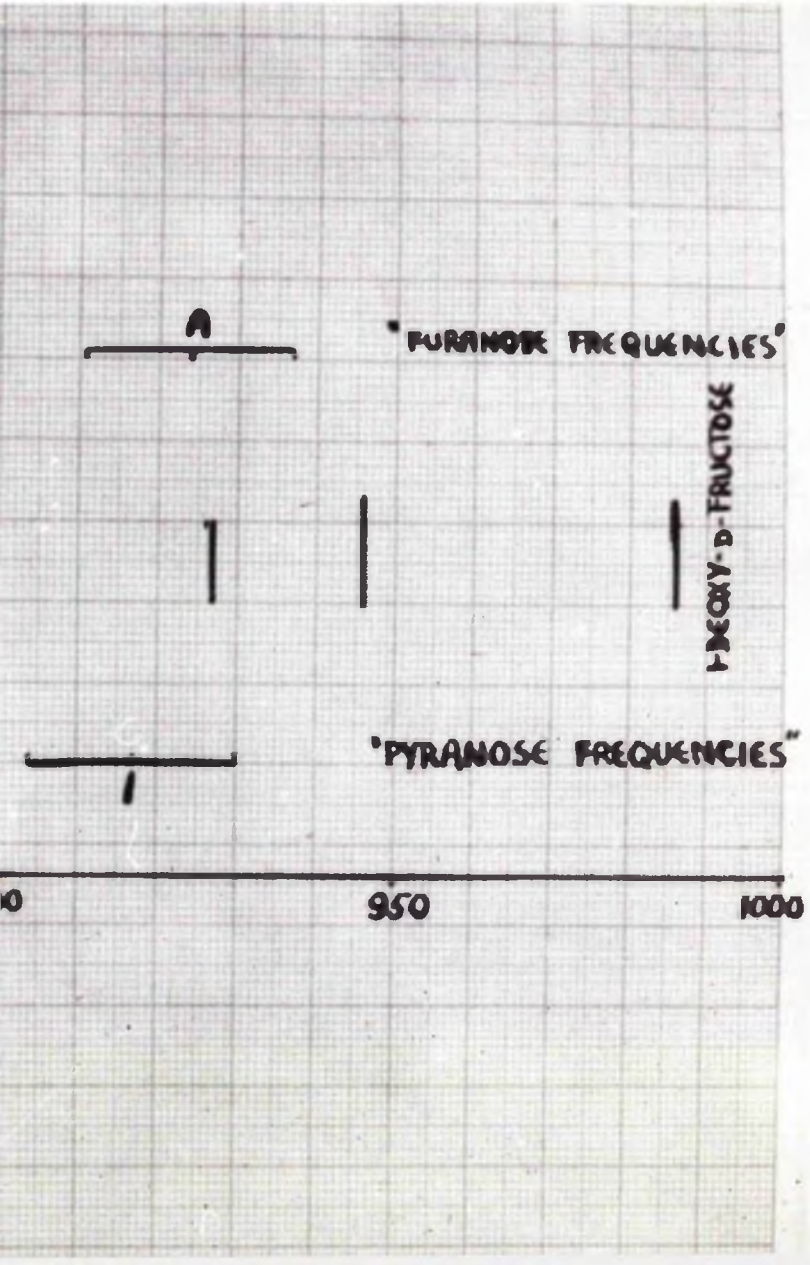
PLATE 9.

Infrared absorption frequencies of
of relative intensities) compared with t
of sugar pyranose and furanose rings.



l-deoxyfructose (with indication

he frequencies characteristic



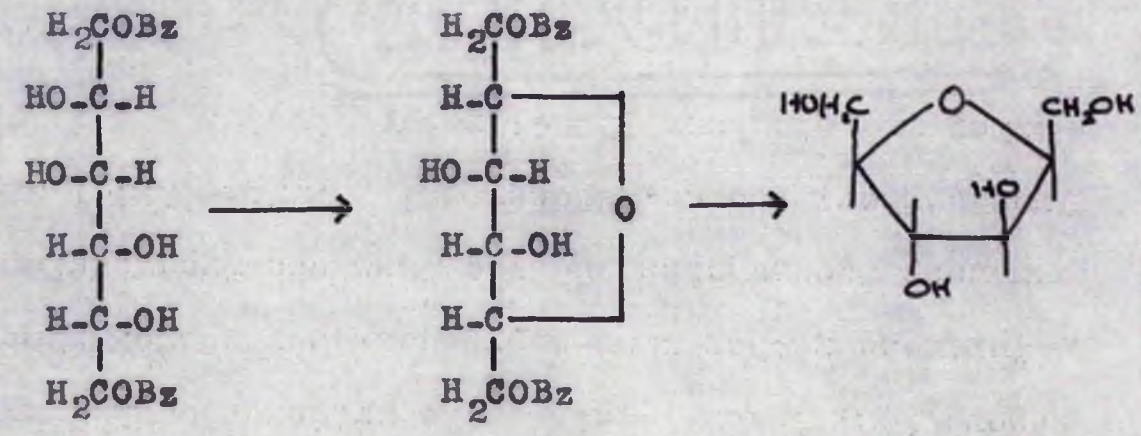
(ii) Infrared absorption.

Plate 1 shows the infrared absorption spectrum of 1-deoxyfructose. From the analysis of the absorption peaks' frequencies (Plate 9), a tentative assignment of a furanose ring has been made to the deoxysugar in the amorphous, anhydrous state for the following reasons.

The absorption at 927 cm^{-1} could correspond either to the pyranose type 1 absorption or to the furanose type A absorption. However, the peaks at 857 cm^{-1} and 881 cm^{-1} appear to correspond well with the furanose types B and C absorptions respectively, and, although 779 cm^{-1} is slightly outside the usual range of type D absorption, taken together the evidence would indicate a furanose rather than a pyranose structure. The pyranose frequencies and the furanose frequencies are from Barker, Bourne, Stacey and Whiffen (1954) and Barker and Stephens (1954) respectively.

5. CHEMISTRY OF 2,5-ANHYDROSORBITOL.

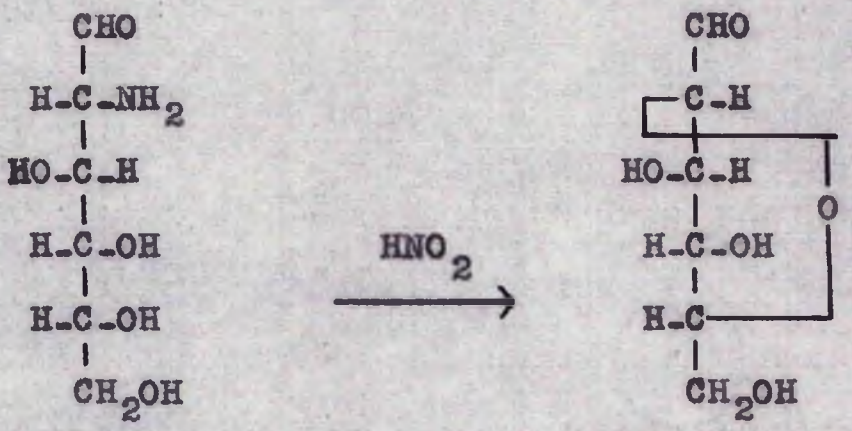
Hockett *et al.* (1946) proved that the dibenzoyl 'anhydromannitol' prepared from the reaction between 1,6-dibenzoylmannitol and p-toluene sulphonic acid by Brigl and Grüner (1933, 1934) was in fact a sorbitol derivative. They showed that an inversion of configuration occurs during detosylation and anhydro-ring formation:



The sample of 2,5-anhydrosorbitol prepared by the author was obtained by following the procedure exactly as described by these two groups of workers.

6. CHEMISTRY OF 2,5-ANHYDROMANNITOL.

Several workers have confirmed the findings of Levene and La Forge (1915) (Akiya and Osawa, 1954; Grant, 1956) that the deamination of glucosamine with nitrous acid brings about anhydro-ring formation between C2 and C5 with concomitant Walden inversion at C2. Thus, the product of this reaction is 2,5-anhydromannose (chitose).

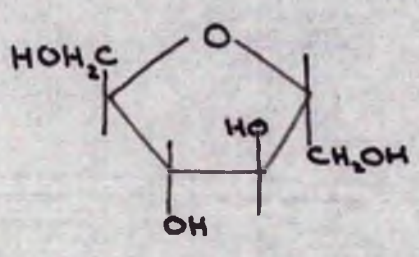


glucosamine chitose

Grant (1956) has published a method of preparing chitose in excellent yield and of a high degree of purity.

It can be seen that reduction of the aldehyde group to a primary alcohol group can lead to only one product as an asymmetric centre is not involved. This reduction was effected both by Raney nickel and by sodium borohydride, the product in each case being isolated as its tetraacetyl derivative. Hydrolysis of the latter gave an uncrystallizable syrup as product. Since none of

the conditions employed in the series of reactions was likely to cause the ring to open, and although no charactrization was possible through shortage of time, it was assumed that the final product was substantially 2,5-anhydromannitol.



7. EXPERIMENTAL DETAILS.

7(a) Preparation and properties of 1-amino-1-deoxyfructose.

(i) By the method of Fischer (1886), a crude sample of fructosamine acetate was obtained in 9 % yield. This would not recrystallize and was considered unsuitable for further chemical and biological experiments.

(ii) After Maurer and Schiedt (1935), finely pulverized glucosazone (20g) (Fewster, 1953) was covered with a mixture of glacial acetic acid (100 ml) and water (20 ml), and hydrogenated at room temperature and atmospheric pressure using 5 % palladium as catalyst (Cheronis and Levin, 1944). The hydrogenation was stopped after three days by which time the hydrogen uptake had virtually ceased and about half the glucosone had been used up. After filtration, the filtrate was processed by Maurer and Schiedt's procedure, and yielded chromatographically pure fructosamine acetate (1 g, 15 %), m.p. 136-7°(d). At 129° the salt started to char, and at its melting point evolved nitrogen. After recrystallizing the salt thrice, the melting point remained unchanged at 136-7°. $[\alpha]_D^{18}$ -72.8° (c, 1.706 in water); $[\alpha]_D^{18}$ -28.0° (c, 1.21 water/ dimethylformamide, 1/ 4). No mutarotation was observed in either case.

Elementary analysis gave:- C, 40.4; H, 7.5; N, 5.71 %.

Calculated for $C_8H_{17}NO_7$:- C, 40.15; H, 7.2; N, 5.9 %.

(iii) By the method of Kuhn and Haas (1956), fructosamine acetate was obtained in 70 % yield by hydrogenation of 1-deoxy-1-p-tolylaminofructose (Weygand, 1940) using the "brown palladium hydroxide - barium sulphate" catalyst of Kuhn and Haas (1955). A mixed-melting point determination with the product of method (ii) above gave no depression of the melting point.

(iv) Attempted preparation from 2,3:4,5-di-O-isopropylidene fructose.

2,3:4,5-di-O-isopropylidenefructose, m.p. 95°, (Pacsu, Wilson and Graaf, 1939) was treated with thionyl chloride after the method of Haworth and Wiggins,(1944). On adding it to thionyl chloride, the crystalline diacetone compound dissolved with a slow evolution of a gas. After refluxing the solution for one hour, the thionyl chloride was distilled off leaving a pasty mass which was taken up in dry chloroform and re-evaporated under reduced pressure. After allowing the solution to stand, dirty white crystals were obtained, m.p. 94° after recrystallization from 80/100 petroleum ether. A Lassaigne test on this product gave negative results for chlorineand sulphur.

(v) Formation fwom 2,3:4,5-di-O-isopropylidene-1-O-tosylfructose.

2,3:4,5-Di-O-isopropylidene-1-O-tosylfructose (0.5 g) (Müller and Reichstein, 1938) was heated in a sealed tube with liquid ammonia (10 ml) for 24 hours at 100°. For this step, a narrow tube of annealed soda-glass was used. Pyrex and lead-glass were found to be too brittle, while tubes of soda-glass of internal diameter greater than 1.3 cm and thickness greater than 1.0 mm would also explode under the pressures generated by the conditions used. After cooling it to room temperature, the tube was immersed in liquid air and opened. The ammonia was then allowed to evaporate gradually and the residue was extracted with chloroform. About 50 mg of insoluble material remained and this was treated with 0.1 N hydrochloric acid (5 ml) for 10 minutes in a boiling water bath. The presence of fructosamine was shown on a paper chromatogram.

(vi) Paper chromatography.

The Rf values obtained for this salt (fructosamine acetate) from paper chromatograms run in various solvents are given in Section 3(b) (i), Table 2.

(vii) Infrared spectrophotometry.

The infrared spectrum of fructosamine acetate in the region 10-14 u was obtained using the "Nujol" mull technique with a Grubb-Parsons Double-Beam Recording

Infrared Spectrometer (Plate 1).

(vii) Titration curve of fructosamine acetate.

A 0.01 M solution (25 ml) of fructosamine acetate (pH 6.6 initially) was titrated with 1.1 N hydrochloric acid using a micrometer syringe pipette. The pH of the solution was measured with a glass electrode after the addition of each 0.01 ml acid until the pH reached 2.8 (0.2 ml added). The resultant acid solution was then titrated in a similar manner with 1.009 N sodium hydroxide until the pH was 11.4 (0.53 ml added). The titration curve is shown in Plate 3, Section 3(b)(iii).

(ix) General chemical reactions.

Fructosamine acetate reduces Fehling's solution in the cold, but gives a negative result in Seliwanoff's test for ketoses. It does not reduce 2,6-dichlorphenol-indophenol, and gives a slowly developing blue colour with Benedict's arsenotungstate reagent.

(x) Reaction with ninhydrin.

Fructosamine acetate (1 g) and ninhydrin (0.9 g) were dissolved in water (750 ml) (pH 7.5) and the solution was heated on a boiling water bath for 1 hour. The purple coloured solution was allowed to stand for 2 days at room temperature by which time a greenish yellow precipitate had appeared and the purple colour in the

supernatant had disappeared. After filtering, the solution was evaporated nearly to dryness under reduced pressure, the residue extracted twice with cold water (5 ml) and the combined extracts evaporated under reduced pressure to about 2 ml. An osazone was prepared from 0.5 ml of this extract, m.p. 205-6°. Under a microscope, these crystals appeared to be glucosazone, but attempts to prepare glucosotriazole from them (Hann and Hudson, 1944) were unsuccessful. A mixed-melting point with pure glucosazone (m.p. 204°) was 202-3°.

Further 0.5 ml aliquots of the solution were used in unsuccessful attempts to prepare the dibenzyl dithioacetal of glucosone (Bayne, 1958), the 2,4-dinitrophenylosazone and the 3,5-dichlorophenylosazone.

Paper chromatograms were run in a variety of solvents in order to compare the reaction product with fructosamine acetate and glucosone. The results from these are tabulated in Section 3(b)(vi), Table 3. The spots were shown up either by treating the chromatograms with triphenyltetrazolium bromide and alkali (Trevelyan *et al.*, 1950) or with the following modification of Benedict's arsenotungstate reagent.

Arsenotungstic acid was dissolved in acetone containing the minimum amount of water necessary to produce a clear solution, so that the final concentration of

As_2O_3 was 1 %. After drying, the chromatogram was sprayed lightly in a fume cupboard with this solution and dried immediately. It was then dipped into 0.05 N sodium hydroxide in ethanol saturated with potassium cyanide (ca 3 %) and dried again. Compounds reacting with this spray appear as deep blue spots on a white background. This colour fades on standing.

(xi) Attempted preparations of O-isopropylidene derivatives.

Fructosamine acetate (1 g) suspended in dry acetone (20 ml), was treated with concentrated sulphuric acid (0.5ml). The mixture was shaken for 4½ hours at room temperature, by which time the liquid phase had turned yellow in colour and the solid had altered its appearance. The suspension was then filtered, and the residue was washed free of acid with acetone (50 ml) and resuspended immediately in acetone (50 ml). An aliquot of this suspension was filtered, the residue dissolved in water, neutralized to Congo Red with sodium hydroxide and evaporated under reduced pressure to a syrup. On adding ethanol (10 ml), a white solid (Na_2SO_4) and a syrup were thrown out. All attempts to crystallize this syrup failed. Further portions of crystalline material removed from the suspension were found to be very soluble in

water and insoluble in methanol, ethanol, acetone, ether, chloroform and benzene. Another portion was filtered off, boiled up with methanol, filtered again, washed with cold methanol and dried over phosphorus pentoxide under under reduced pressure. m.p. 115-9° with decomposition and evolution of a gas. An aqueous solution of the product gives a white precipitate with barium chloride solution. After treatment of the product with strong, cold hydrochloric acid, no acetone was detected by Rothera's test.

As previously described, (Section 7(a)(v), page 39), 1-amino-1-deoxy-2,3:4,5-di-O-isopropylidenefructose was formed in low yield by the action of liquid ammonia on 2,3:4,5-di-O-isopropylidene-1-O-tosylfructose at 100°.

Prehydrogenated "brown palladium hydroxide-barium sulphate" catalyst (2 g) (Kuhn and Haas, 1955), and 1-deoxy-O-isopropylidene-1-p-tolylaminofructose (2 g) (Douglas and Honeyman, 1955) in 5 N acetic acid (30 ml) were shaken for three hours in a hydrogenator at 18° and atmospheric pressure. After uptake of 300 ml hydrogen had occurred, the catalyst was centrifuged off at 5° and the supernatant evaporated under reduced pressure to a thick syrup. No crystals were obtained. The presence of fructosamine acetate was shown by paper chromatography.

The above preparation was repeated as far as the removal of the catalyst. The supernatant was neutralized with sodium hydroxide and evaporated to a thick syrup. Acetone (10 ml) was added, whereupon crystals of sodium acetate appeared and an oil was thrown out. The oil was separated off and filtered with acetone when it solidified to a semi-crystalline mass, m.p. 58-62°. This solid was soluble in water and warm ethanol, but insoluble in benzene, acetone, chloroform and cold ethanol. All attempts to crystallize it failed, and the product was not characterized further.

The change in optical rotation of 1-deoxy-O-isopropylidene-1-p-tolylaminofructose (0.2257 g) in acetic acid (10 ml, 5 N) solution was followed in a polarimeter at 18°. In 6 hours the optical rotation changed from -1.01° to -0.875°.

7(b) Preparation and properties of 1-deoxyfructose.

(i) 2,3:4,5-di-O-isopropylidenefructose.

By the method of Pacsu, Wilson and Graaf (1939) (a modification of the method of Ohle and Koller, 1924), 2,3:4,5-di-O-isopropylidenefructose was prepared in 85% yield; m.p. 95°; $[\alpha]_D^{18}$ -32.6° (c, 0.98 in water).

(ii) 2,3:4,5-di-O-isopropylidene-1-O-tosylfructose.

2,3:4,5-Di-O-isopropylidene-1-O-tosylfructose was prepared from 2,3:4,5-di-O-isopropylidenefructose by the method of Levene and Tipson (1937) in 63 % yield; m.p. 83°.

(iii) 1-Deoxy-2,3:4,5-di-O-isopropylidene-1-thioethyl-fructose.

This compound was prepared in 85 % yield from the diacetonemonotosyl derivative by the method of Baker (1955); b.p. 140° (0.5 mm mercury pressure)

(iv) 1-Deoxy-2,3:4,5-di-O-isopropylidenefructose.

1-Deoxy-2,3:4,5-di-O-isopropylidene-1-thioethyl-fructose (5 g) was dissolved in ethanol (250 ml) and refluxed for 2½ hours with the nickel catalyst prepared from 75g Raney alloy (Mozingo et al., 1943). After filtering off the nickel, ethanol was distilled off at 40° under reduced pressure. A mobile oil (4 g; 100 %) was obtained at this stage. This oil was insoluble in hot and cold water. Ethanol was added dropwise to a hot mixture of the oil with water (10 ml) until the mixture clarified. On cooling the solution, a turbid suspension was obtained, but crystals did not appear even after the suspension had stood for 7 days at 0°. After re-evaporating it to dryness, the oil was distilled

at 70° (0.2 mm mercury pressure). $[\alpha]_D^{18}$ -21.3° (c, 1.509 in ethanol). A redistilled sample gave the following elementary analysis:- C, 58.8; H, 8.3 %.
Calculated for $C_{12}H_{20}O_5$:- C, 59.0; H, 8.2 %.

The product gave a negative Seliwanoff test, a negative Benedict reaction, and showed no sulphur to be present in a Lassaigne test. After boiling a small amount with 50 % hydrochloric acid for 5 minutes, a positive Benedict reaction was obtained. A chromatogram of this hydrolysate gave a single spot that was not the same as one obtained from fructose.

After treating another sample of the product with 0.1 N sulphuric acid for 6 hours at 100°, the resulting solution was neutralized with barium carbonate, filtered and the filtrate concentrated under reduced pressure. Treatment of this hydrolysate with 3,5-dichlorphenylhydrazine in ethanol did not give a crystalline hydrazone.

(v) 1-Deoxyfructose.

1-Deoxy-2,3:4,5-di-O-isopropylidenefructose (1.8 g) was dissolved in ethanol (1 ml), N sulphuric acid solution (10 ml) was added and the solution heated for 140 hours at 40°. After neutralisation with barium carbonate, the solution was filtered, the residue washed with distilled water , and the combined filtrate

and washings evaporated to a thick syrup under reduced pressure at the lowest possible temperature; 1 g, 83 %; $[\alpha]_D^{17}$ -77.5° (c, 0.0775 in water). This syrup was soluble in water, methanol and hot ethanol, but insoluble in cold ethanol and isopropanol. Attempts to crystallize the product from ethanol failed. Plate 8 shows a paper chromatogram of this compound, confirming it to be 1-deoxyfructose.

(vi) Infrared spectrum.

The infrared absorption spectrum of 1-deoxyfructose from 10-14 u was obtained on a Grubb-Parsons Double-Beam Recording Spectrometer after subjecting the compound to the following treatment. After repeated solution in methanol and evaporation under reduced pressure, a few milligrams of the deoxysugar were dissolved in dry methanol (0.5 ml) and the solution poured into anhydrous ether (20 ml), thus obtaining a flocculent, white amorphous precipitate. This was found to be extremely hygroscopic. The suspension was centrifuged, and the bulk of the ether decanted. Isooctane (10 ml) was then added, the precipitate resuspended and the suspension centrifuged. The decantation, resuspension in isooctane and centrifugation were repeated five times, and to the final suspension, 1 drop of "Nujol" was added. The isooctane was then evaporated off under reduced pressure at

room temperature. When all volatile material had distilled off, the "Nujol" was found to be covering the surface of the sample of 1-deoxyfructose. A mull was then made and the spectrum obtained.

7(c). Preparation of 2,5-anhydrosorbitol.

(i) 2,5-Anhydro-1,6-dibenzoylsorbitol.

This was prepared by Mrs F.S.Steven (to whom thanks are given for the gift of a sample) by the method of Brigl and Grüner (1933).

7(ii) 2,5-Anhydrosorbitol.

2,5-Anhydrosorbitol was prepared from its dibenzoyl derivative in 97 % yield by the method of Hockett, Zief and Goepp (1946). The product would not crystallize, but was used in its semi-solid state.

7(d). Preparation of 2,5-anhydromannitol.

(i) Glucosamine hydrochloride.

This was prepared from chitin by the method of Wolfrom and Cron (1952) in 85 % yield.

(ii) 2,5-Anhydromannose.

A solution of this was obtained by the method of Grant (1956).

(iii) 2,5-Anhydromannitol.

A portion of the above solution (containing 5 g chitose) was evaporated under reduced pressure at 20°

and the residue treated with Raney nickel (75 g) after the method of Karabinos and Ballum (1953). After filtering off the catalyst, the resulting solution was evaporated under reduced pressure to 20 ml. Crystals of sodium acetate that appeared were removed by filtration and the filtrate evaporated down to dryness.

Pyridine (150 ml) and acetic anhydride (150 ml) were added and the mixture shaken for 48 hours at room temperature. The reaction mixture was poured into water (2 l) and a homogeneous solution obtained. This was extracted twice with chloroform (250 ml), the combined extracts washed free of acid with 5 % sodium carbonate and then washed till neutral with distilled water. The chloroform solution was dried over sodium sulphate and evaporated to a thick syrup which was found to be soluble in ether, ethanol, chloroform and carbon tetrachloride but insoluble in petroleum ether, water and ethyl acetate. Crystals were not obtained from an ethanol-water or an ether-petroleum ether system.

Alternative hydrogenation of chitose.

Chitose (15 g) was hydrogenated in aqueous solution with sodium borohydride by the method of Abdel-Akher, Hamilton and Smith (1951). The product was acetylated and worked up as before, but still crystals of acetyl-

ated 2,5-anhydromannitol were not obtained. The syrupy acetate was dissolved in ethanol (50 ml) and aliquots (10ml) removed for hydrolysis. On evaporating one of these aliquots, 1.8 g of acetate were obtained, and this was heated on a boiling water bath for 15-20 minutes in 30 % sulphuric acid. The solution was then cooled and neutralized with excess barium carbonate. The precipitated barium sulphate was removed by filtration and dilute sulphuric acid added dropwise to remove traces of barium. The refiltered solution was evaporated under reduced pressure to a thick syrup. Yield 0.8 g (97 %); $[\alpha]_D^{18}$ +41.6°.

2,5-Anhydromannitol does not reduce Benedict's reagent, and gives a negative Mohlisch reaction.

PART II

PHOSPHORYLATIONS BY YEAST HEXOKINASE

CONTENTS

1. A REVIEW OF THE PROPERTIES OF YEAST HEXOKINASE.

1(a) Historical introduction.

It has been known for over fifty years that phosphate esters are formed at some primary stage in glycolysis. Harden and Robison (1914) demonstrated that a mixture of hexose monophosphates, in addition to hexose diphosphate, is contained in yeast juices, and von Euler (1914), in early work on the chemistry of yeast and alcoholic fermentation, indicated that glucose is phosphorylated by a yeast enzyme.

Thirteen years later, Meyerhof (1927) separated from bakers' yeast a protein fraction that was shown to enable aged extracts of skeletal muscle to ferment glucose. He called this partially purified enzyme 'hexokinase' to indicate that it initiates hexose metabolism. In 1930 he pointed out the similarities between muscle glycolysis and yeast fermentation, and emphasized the importance of pyrophosphates, of adenylic acid and of phosphoric esters of carbohydrates in these metabolic systems.

The actual mode by which hexokinase initiates hexose utilization was not immediately understood, and the three fermentable sugars (glucose, mannose and fructose)

were thought to be changed to an easily fermentable form, possibly the enol, by the enzyme (Harden, 1932).

In 1935, von Euler and Adler reported that the hexose monophosphate-dehydrogenase system contains an enzyme, heterophosphatese, which transfers phosphate from ATP to glucose and fructose, and which is activated by magnesium ions. In the same year Parnas, Lutwak-Mann and Mann published further elucidations of the enzyme steps involved in the fermentation of sugars by yeast.

In a review written the following year Quastel (1936) suggested that, as the enzyme phosphorylates a molecule at the expense of an already phosphorylated molecule, free phosphate not being involved, a better name for it would be ' phosphorylase'. This term is now applied to enzymes catalyzing a different type of reaction, and heterophosphatese is no longer widely used. The generic name of 'phosphokinase' was coined by Dixon and Needham in 1940 (see Dixon and Needham, 1946) to describe the type of enzyme which catalyzes the reaction:-

$$ATP + X = ADP + XP$$

by transfer of the terminal phosphate from ATP to the organic molecule, X.

Meyerhof and Lohman (1931) were the first to suggest that ATP is concerned in the primary esterification of

carbohydrates, labile phosphoric acid groups being transferred to the carbohydrate during glycolysis, the ATP being re-synthesized during a later stage. Meyerhof later (1935) suggested that the initiating reaction in yeast and muscle glycolysis is the phosphorylation of hexoses by ATP, the phosphate being transferred according to the equation:-

ATP + 2 glucose $\longrightarrow$ adenylic acid + 2 glucose 6-phosphate.

He also showed that hexokinase and heterophosphatese behave in an identical fashion. An electrophoretic study of the hexokinase of Meyerhof and the heterophosphatese of von Euler, by Meyerhof and Mohle (1937) would appear to indicate that these two enzymes are not the same. However, the overwhelming bulk of later evidence has shown that yeast contains but one enzyme with the properties shown by both these fractions.

When Colowick and Kalckar (1941) demonstrated that, in yeast, only one phosphate group is transferred to glucose or fructose from ATP, and not two as had been previously thought, the catalytic action of hexokinase could be defined as:-

ATP + hexose $\longrightarrow$ ADP + hexose 6-phosphate.

A number of workers (Gamble and Najjar, 1954, 1955; Kaufmann, 1955; Ågren and Engström, 1956) have since

proved the reaction to be reversible, and Gamble and Najjar (1955) demonstrated that the reverse reaction proceeds at one-fiftieth the rate of the forward one, although this reverse reaction has no significance in the normal glycolytic scheme.

The location of the hexokinase molecules within the whole yeast cell has been shown to be just inside the cell-wall. The evidence for this, albeit somewhat circumstantial, has been accumulated by several workers using widely different techniques. (Barron et al., 1948; Cramer and Woodward, 1952; Derrick et al., 1953; Rothstein, 1954; Blackley and Boyer, 1955; Sols, 1956). For a time, hexokinase was thought to be involved in the permeability of the cell-wall towards sugars, but this view is no longer held (Sols, 1956; Burger et al., 1959).

The high sensitivity of the enzyme to vesicants and lachrymators was responsible for the interest taken in it by Dixon and his colleagues (Dixon and Needham, 1946) during the last war, and this in turn led to the crystallization of the enzyme by three groups of workers (Bailey and Webb, 1944, 1948; Berger, Slein, Colowick and Cori, 1946; Kunitz and MacDonald, 1946a).

With better availability of more purified preparations of the enzyme, hexokinase has been used to good effect in elucidating other biochemical problems. With its aid

Strehler and Totter (1952) have demonstrated the ATP-dependence of firefly luminescence, and sensitive techniques for the micro-estimation of glucose and hexokinase have been evolved by measuring the depression of luminescence produced in their presence. The existence of a non-phosphorylating pathway of glucose metabolism in chick embryos has been shown by Needham and Lehmann (1937) using added hexokinase and fluoride ions, while the addition of glucose and hexokinase to rat hearts by Rabinowitz et al. (1951) stimulated pyruvate and oxalacetate oxidation, thus proving that the latter are dependent on ADP. Similar additions to mammary gland suspensions by Terner (1954) showed a Pasteur effect in them. Analytically, hexokinase has been used to fractionate adenosine pyrophosphates in human blood (Mackler et al., 1954), and Rosenberg (1955) used it in conjunction with other pure enzyme systems to incorporate labelled phosphorus into ATP and phosphocreatine.

During the past twenty years the presence of hexokinase has been demonstrated in a large variety of bacteria and in the tissues of animals and plants of many different species. Its mode of action in initiating glycolysis by phosphorylation is identical in all of these organisms, although its substrate specificity in them appears to vary considerably.

2. Preparation and physico-chemical properties.

The crystalline enzyme has been prepared by a number of workers (Bailey and Webb, 1944, 1948; Berger et al., 1946; Kunitz and MacDonald, 1946). The various purification steps involved in these procedures are autolysis of yeast, followed by fractionation of the proteins with ammonium sulphate, with ethanol, and by adsorption on alumina or calcium phosphate. The enzyme is finally crystallised at 5° in the presence of ammonium sulphate and dilute phosphate buffer at pH 7.

Crystalline hexokinase has been shown by electrophoretic, solubility and ultracentrifugal studies to be a pure, single protein (Kunitz and MacDonald, 1946), although Boser (1955) obtained two fractions by electrophoresis at pH 8.6. The electrophoresis in the former case was carried out at pH 5.6 and 6.8. Its molecular weight, calculated from sedimentation and diffusion data obtained at 1° in pH 5.5 acetate buffer, is 96,600 (Kunitz and MacDonald, 1946), and the same figure was obtained by Bailey and Webb (1948). Northrop et al. (1943) stated that the crystalline hexokinase obtained by them was found to be homogeneous by electrophoresis, and was also homogeneous in the ultracentrifuge at pH 5.5. At pH 6.8 two boundaries appeared, and when the components were

separated, both were found to be active. Recombination of these two components took place on dropping the pH to 5.6.

Bailey and Webb, and Kunitz and MacDonald have reported that the iso-electric point of the enzyme lies between pH 4.5 and pH 4.8, while its maximum stability is around pH 5.0. Sols et al. (1958), investigating the effect of pH on hexokinase activity in greater detail, have found that the maximum in the pH-activity curve is at pH 7.5, and that the enzyme is stable in the range of pH 5-8.

Evidence for a special prosthetic group was not obtained (Berger et al., 1946) from its ultraviolet absorption spectrum which shows a maximum molar extinction coefficient of 125,000 near 278 mμ and a minimum of 66,000 at 250 mμ.

Boser (1955) has published a qualitative amino acid assay of the pure enzyme and has shown it to be a glycoprotein with mannose, as sole sugar constituent, present to the extent of 52 %. An elementary analysis by Kunitz and MacDonald (1946) gives the following values expressed in percent dry weight: C, 52.2; H, 7.08; P, 0.11; S, 0.91; ash, 0.36. The phosphorus value indicates a minimum molecular weight of 30,000.

3. Specificity.

Kunitz and MacDonald (1946) showed that the pure enzyme acts on glucose, fructose and mannose, but not on xylose, galactose, L-rhamnose, sucrose, lactose, maltose, trehalose or raffinose.

The phosphorylation of glucosamine by ATP using crystalline hexokinase has been described by Brown (1951) and by Grant and Long (1952), while Brown showed that N-acetylglucosamine would not act as a substrate. Distler et al. (1958) prepared crystalline glucosamine 6-phosphate using ATP and hexokinase. Cramer and Woodward (1952), using a partially purified yeast hexokinase preparation, have shown that 2-deoxyglucose is phosphorylated; and Johnstone and Mitchell (1953), using a similar enzyme preparation, found that glucosone is phosphorylated at about the same rate as glucose. Hudson and Woodward (1958) verified the latter findings. Gluconic acid has been shown not to act as a substrate of this enzyme (Barkhash and Demyanovskaya, 1951).

Gottschalk (1943) proved that glucose can be phosphorylated in both the α- and β-configurations, and in a later paper (1947) showed that the same was true for mannose. He also indicated in 1943, following on from the work of Hopkins and Roberts (1935) on the fermentation of fructose by yeast, that fructose is phosphorylated in the furanose

form and confirmed it later (1945), but his conclusion that there are two hexokinases present in yeast was later proved false by Slein, Cori and Cori (1950) who, incidentally, substantiated Gottschalk's conclusions about the phosphorylation of fructose. Scharff and Rothstein (1956) also supported the view that there are two hexokinases in yeast with pH optima of 8.4 and 5-6, while Sols et al. (1958) contradicted this.

This does not take into account the galactokinase present in galactose-adapted yeast, which differs from the usual yeast hexokinase in that it appears to be more specific and that phosphorylation takes place at carbon-1 of the sugar and not at carbon-6 (Kosterlitz, 1943).

Slein et al. (1950) also showed, from studies of the mutual inhibition of substrates, that in yeast hexokinase there is only one active site that combines with all the substrates they tested.

Mitchell (1954), using a partially purified hexokinase preparation, showed by means of the relatively insensitive manometric method of assay that none of the following compounds is phosphorylated;-

arabinose, galactose, L-sorbose, galactosone, L-glucosone, galactosamine, N-acetylglucosamine, 1,5-anhydroglucitol, 1,5-anhydromannitol, α-methyl glucoside, β-methyl glucoside, glucose 1-phosphate, glucose 6-phosphate,

and fructose 1-phosphate,

while Wildy (1953) showed that 3-O-methylglucose, 3-O-methylfructose, 1-O-methylfructose and 6-O-methylglucose were also not phosphorylated by hexokinase. Grieve (1954) found that 4-O-methylfructose is not a substrate of this enzyme either.

Recently, Sols et al. (1958) have shown that yeast hexokinase can phosphorylate a much wider range of compounds than was previously thought to be the case. These workers showed that the following compounds can act as substrates:-

glucose, 1,5-sorbitan, fructose, arabinose, mannose, 2-deoxyglucose, glucosamine, glucosone, allose and galactose,

and that these compounds:-

methyl α-glucoside, 1-O-methylfructose, 3-O-methylglucose, 3-O-methylfructose, 1,4-sorbitan, L-sorbose and sorbitol have no measurable effect on the hexokinase reaction. They conclude that 'the pattern of substrate specificity (of yeast hexokinase) is broadly similar to that of brain hexokinase (Sols and Crane, 1954), although there are several significant differences'.

Kleinzeller (1942) demonstrated that ITP is one-fifth as effective as ATP in phosphorylations catalyzed by hexokinase, and Kornberg (1951) found that UTP and hexokinase can phosphorylate glucose. Apart from these two

compounds, other phosphate donors have not been found for the reaction, although many have been tried. Von Euler and Adler (1935) reported that AMP, phosphagen, sodium pyrophosphate and hexose diphosphate are not active in the hexokinase catalyzed reaction, and Colowick and Kalckar (1943) showed that ATP is not dephosphorylated beyond ADP.

4. Activation.

Von Euler and Adler (1935) found that heterophosphatese requires magnesium ions to activate it, a fact confirmed by Colowick and Kalckar (1941), Berger et al. (1946) and Bailey and Webb (1948) for hexokinase. The last mentioned workers stated that calcium ions cannot replace magnesium ions, while Dische and Ashwell (1955) stated that barium, calcium and lanthanum ions do not inhibit hexokinase, as they might be expected to by competition with magnesium. Van Heyningen (1942) found that calcium and zinc are inactive in the reaction. Manganese also activates hexokinase, according to von Euler, Adler and Vestin (1937) and Ohlmeyer and Ochoa (1937), but details concerning the dissociation constant of the manganese-enzyme complex are not known.

A specific protein activator has been extracted from muscle by Weil-Malherbe (1951) which causes a two- to four-fold increas in hexokinase activity. This activator

is not associated with any other enzyme, is fairly heat stable and is specific for the phosphorylation of glucose and fructose. Long and Thomson (1955) reported the presence of a similar activator in erythrocyte lysates and in rat-muscle extracts, and showed that it gives a direct activation of yeast hexokinase when glucose or 2-deoxy-glucose is present as substrate.

Williams et al. (1957) discovered another example of an activity potentiator. Glycolysis is reduced in biotin-deficient yeast cells, and this reduction has been demonstrated to occur at the hexokinase level. This effect differs from that of magnesium in that it is not co-enzymatic, but is probably due to the fact that biotin is involved in the synthesis of the enzyme. Strauss and Moat (1957) also reported this activation of yeast glycolysis by biotin and confirm that it acts on hexokinase.

5. Stabilisation and inhibition.

Mackworth (1941) conducted a comprehensive investigation into the effect of lachrymators on enzymes, and from her findings she was able to conclude that the enzymes that are poisoned by the small amounts of lachrymators used, are all -SH enzymes. (i.e. enzymes which contain -SH groups, the intactnes of which is essential to their activity). Hexokinase was included by her in the group

of -SH enzymes.

Van Heyningen (1942) investigated the mode of action of lachrymators and vesicants on hexokinase more fully, and concluded that this inactivation by the former class of compounds is caused by an irreversible combination of the inhibitor with the -SH groups present in the active enzyme. The inactivation caused by mustard gas and other vesicants is also irreversible, but not thought to be due to an -SH attack. Lewisite differs from the other compounds tested, in that it inhibits hexokinase by combining reversibly with -SH groups and therefore protects the enzyme from the action of lachrymators, but not, however, of mustard gas.

Another type of inactivation investigated by van Heyningen was the spontaneous inactivation of hexokinase obsefved when the enzyme is incubated for a few minutes at 38°. Half of the total inactivation is due to heat denaturation, while the other half is caused by oxidation of -SH groups. The latter can be reversed by treatment with cysteine or cyanide, and it can be prevented by the presence, during incubation, of cysteine, reduced glutathione, thioglycollic acid or cyanide. Glucose was found to stabilize the enzyme against both heat denaturation and oxidation (but not to reverse inactivation that had already taken place), and to protect the enzyme from

vesicant poisoning. At neutral pH, Sols *et al*. (1958) found that hexokinase is rapidly inactivated in the range 55-60°, and that glucose produces a definite, but slight, protection of the enzyme at these elevated temperatures. They also found that the enzyme rapidly loses its activity below pH 4 and above pH 9. No effect of glucose was reported in this case. Dixon *et al*. (1942) developed a test for vesicants using hexokinase, but stipulated that the glucose concentration in the system must not be higher than that present in normal tissues, and in the following year, Dixon (1943) formulated his phosphokinase theory of vesicant action.

A second type of spontaneous inactivation takes place on diluting the enzyme with water in the absence of glucose or of high salt concentrations. Berger *et al*. (1946) found that this could be counteracted by insulin, haemoglobin or serum albumin; insulin being the most effective in this respect. The protective power of insulin was shown to be separate from its physiological activity. Heat-inactivated samples of crude hexokinase also afforded protection from dilution-inactivation, whereas heat-inactivated crystalline hexokinase gave very little protection.

Trypsin, but not chymotrypsin, was found to inactivate

hexokinase (Kunitz and MacDonald, 1946; Berger et al.,1946) The inactivation by trypsin proceeds more rapidly than digestion by it, and glucose again affords protection.

Berger et al. (1946) reported the absence of any stimulatory or protective effect on hexokinase from cysteine or glutathione, and found that the enzyme was optimally active when these compounds were absent. They therefore concluded that hexokinase is not an -SH enzyme.

Hexokinase solutions exhibit maximum stability around pH 5. The rate of inactivation increases with temperature and varies with pH, this inactivation being accompanied by denaturation and not hydrolysis. At pH 7 and 27°, the enzyme retains most of its activity for several hours in the presence of glucose, mannose or glycine (Kunitz and MacDonald, 1946). The addition of cysteine or neutralized sodium sulphide to autolysing yeast stabilizes hexokinase, accelerates autolysis and increases the final yield of the pure enzyme. The addition of glucose at this stage stabilizes hexokinase but retards autolysis (Bailey and Webb, 1948).

Although fluoride is known to be a potent inhibitor of magnesium-containing enzymes, on account of its magnesium-binding properties, Berger et al. (1946) reported that hexokinase was unaffected by fluoride concentrations of up to 0.125 M when the magnesium ion concentration was

TABLE 4

Substrate	Inhibitor	Source
Fructose	2-deoxyglucose[2], 6-deoxy-6-fluoroglucose[2], N-acetylglucosamine	Sols (1956)
	glucose[2], mannose[2]	Slein et al. (1950)
	glucosone[2]	Hudson & Woodward (1958)
	glucose 6-phosphate[1]	Wajzer (1953) Najjar & McCoy (1958)
	glucose[2], 1,5-sorbitan[2], mannoheptulose, xylose, arabinose, mannose[2], N-acetylglucosamine, 2-C-hydroxymethylglucose 6-deoxy-6-fluoroglucose[2]	Sols et al. (1958)
	3-O-methylfructose[1]	Wildy (1953)
Glucose	2-deoxyglucose[2]	Woodward & Hudson (1955)
	glucosone[2]	Mitchell (1954) Hudson & Woodward (1958)
	glucose 6-phosphate[1]	Wajzer (1953)
	3-O-methylglucose[1]	Wildy (1953)
Glucosone	N-acetylglucosamine	Sols et al. (1958)
Arabinose	N-acetylglucosamine	
2-Deoxyglucose	glucose[2]	Woodward & Hudson (1958)

[1] Also claimed not to be an inhibitor.

[2] Also a substrate.

TABLE 5.

L-sorbose 1-phosphate	Lardy *et al.* (1950)
glucose 1-phosphate, glucose 6-phosphate galactose 1-phosphate, galactose[2] mannose 6-phosphate	Slein *et al.* (1950)
6-deoxy-6-fluoroglucose[1]	Blackley & Boyer (1955)
glucose 6-phosphite	Robertson & Boyer (1956)
fructose 6-phosphate	Wajzer (1953)
glucose 6-phosphate	Weil-Malherbe & Bone (1951) Sols & Crane (1953) Crane & Sols (1953)
glucosone phosphate	Bauchop (1956)
methyl α-glucoside, 1-O-methylfructose 3-O-methylfructose, β-O-methylglucose 1,4-sorbitan, L-sorbose, sorbitol	Sols *et al.* (1958)
galactose[2], L-arabinose, xylose[1] L-rhamnose, sucrose, lactose maltose, trehalose, raffinose	Kunitz & MacDonald (1946)
arabinose[2], galactose[2], galactosamine L-sorbose, galactosone, L-glucosone N-acetylglucosamine[1], 1,5-sorbitan[2] 1,5-mannitan, glucose 1-phosphate glucose 6-phosphate, fructose 1-phosphate methyl α-glucoside, methyl β-glucoside	Mitchell (1954)
1-O-methylfructose, 6-O-methylglucose	Wildy (1953)
4-O-methylfructose	Grieve (1954)
gluconic acid	Barkhash & Demyanovskaya (1951)

[1] Later shown to be an inhibitor.

[2] Later shown to be a substrate.

6.5×10^{-3} M. Bailey and Webb on the other hand, report a 46 % inactivation of hexokinase by 0.04 M sodium fluoride in the presence of 1.1×10^{-3} M magnesium chloride. Mitchell (1954), after Warburg and Christian (1942), thought that these differences were due to variations in the concentration of orthophosphate present. However Melchior and Melchior (1956) have published a study on the inhibition of hexokinase by fluoride ions, and showed that a finite time was required for the inhibition to manifest itself, and that it could be minimised by attending to the order of mixing the reagents.

Sols et al. (1958) have reported inhibition of hexokinase activity by ethylenediaminetetraacetic acid, which acts specifically by making magnesium ions unavailable for the reaction.

Inhibition of phosphorylation by hexokinase has been demonstrated with a large number of substrate analogues. A list of these is tabulated opposite (Table 4).

The compounds listed in Table 5 were not found to have any inhibitory activity on hexokinase phosphorylations at the concentrations.

The adenine nucleotides were thought not to have any regulatory effect on hexokinase, as it had been reported by Colowick and Kalckar (1943) that ADP and AMP were not inhibitory to its activity, a finding confirmed by Weil-

Malherbe and Bone (1951) with a similar partially purified hexokinase preparation. However Gamble and Najjar (1955) stated that 5×10^{-4} M ADP produces a non-competitive inhibition of 50 % at all levels of ATP concentration, and that their findings confirm some unpublished data of Handler.

The activity of partially purified hexokinase is not affected by DL-glyceraldehyde (Adler, Calvet and Günther, 1937; Lardy, Wiebelhaus and Mann, 1950), although glucose fermentation by yeast extracts is inhibited by it (Adler et al., 1937; Boyland and Boyland, 1938). Sols (1956), after considering evidence of his own and other workers, postulated the existence of a transferring agent prior to hexokinase in the cell's metabolic sequence, while the findings of Burger et al. (1959) also seem to confirm the existence of a transferrence system for sugars across the cell-wall. Consequently, the fact that yeast takes up a certain sugar from its suspension medium cannot be construed as proof of its phosphorylation by hexokinase, and vice versa. Errors have been made in this respect in the past.

Hexokinase activity has been shown to be reduced by a large number of other compounds. In addition, both ultra-violet- and X-irradiation depress hexokinase activity in live yeast cells to the same extent, although these two types of rays are believed to bring about their biological

effects by different mechanisms (Stewart and Aldous, 1950; Aldous and Long, 1952; Aldous and Stewart, 1952).

Sodium N-lauryl sarcosinate inhibits yeast glycolysis and hexokinase, but the inhibition of the latter can be abolished by raising the concentration of magnesium ions (Carbon et al., 1955). Very low concentrations of suramin have been shown to inhibit hexokinase action and yeast juice glycolysis, but not whole yeast growth. This inhibition is not pH dependent, and is believed to occur through combination of the inhibitor with the active site of the enzyme (Wills and Wormall, 1950). Raiko (1955) showed that trichloroethylamine is toxic to hexokinase, while trimethylamine is not; and Webb and van Heyningen (1947) reported the inhibition, which could be reversed by excess magnesium, of hexokinase by BAL at suboptimal magnesium ion concentrations. In contrast with hexokinases isolated from skeletal muscle and lymphatic cells, yeast hexokinase is not affected by corticosteroids (Bacila and Barron, 1954). Stern (1954) demonstrated the presence in plasma of a heat-stable, non-dialysable inhibitor of glucose phosphorylation by ATP and hexokinase. This effect was not counteracted by insulin, and was shown not to be due to the presence of adenosine triphosphatase. Vishniac (1950) found that sodium tripolyphosphate inhibits hexokinase by competing

TABLE 6

Substrate	Rate	Author
fructose	1.4	Berger et al. (1946)
	1.0	Kunitz & MacDonald (1946)
	1.6	Woodward and Hudson (1955)
	1.0	Mitchell (1954)
	1.4-1.8	Sols et al. (1958)
mannose	0.3	Berger et al. (1946)
	0.5	Kunitz & MacDonald (1946)
	0.82	Mitchell (1954)
	0.75	Slein et al. (1950)
	0.8	Sols et al. (1958)
glucosamine	0.7	Grant & Long (1952)
	0.75	Mitchell (1954)
	0.7	Sols et al. (1958)
2-deoxyglucose	1.3	Woodward & Hudson (1955)
	1.08	Mitchell (1954)
	1.0	Sols et al. (1958)
glucosone	0.87	Mitchell (1954)
	0.2	Sols et al. (1958)
1,5-sorbitan	0.01	Sols et al. (1958)
arabinose	>0.02	Sols et al. (1958)
allose	>0.1	Sols et al. (1958)
galactose	>0.002	Sols et al. (1958)

TABLE 7

Substrate or inhibitor	K_m or K_i ($Mx10^5$)	Authors
glucose	13; 16; 19; 20	Slein et al. (1950)
	111	van Heyningen (1942)
	50	Wajzer (1953)
	18	Woodward and Hudson (1955)
	20	Hudson and Woodward (1958)
	10	Sols et al. (1958)
mannose	10; 8; 7.7	Slein et al. (1950)
	5	Sols et al. (1958)
fructose	120; 170	Slein et al. (1950)
	200	van Heyningen (1942)
	500	Wajzer (1953)
	110	Woodward and Hudson (1955)
	100	Hudson and Woodward (1958)
	70	Sols et al. (1958)
2-deoxyglucose	55	Woodward and Hudson (1955)
	30	Sols et al. (1958)
glucosone	6	Hudson and Woodward (1958)
	2	Sols et al. (1958)
1,5-sorbitan	300	Sols et al. (1958)

TABLE 7 (Continued)

Substrate or inhibitor	K_m or K_i ($M \times 10^5$)	Authors
arabinose	>10,000	Sols et al. (1958)
glucosamine	150	Sols et al. (1958)
allose	>10,000	Sols et al. (1958)
galactose	>5,000	Sols et al. (1958)
ATP (glucose)	9.5	Slein et al. (1950)
	120	Kunitz and MacDonald (1941)
	5; 7	Gamble and Najjar (1955)
	10	Sols et al. (1958)
ATP (fructose)	4.2	Slein et al. (1950)
	10	Sols et al. (1958)
Magnesium ion	260	Berger et al. (1946)
mannoheptulose	15	Sols et al. (1958)
N-acetylglucosamine	100	Sols et al. (1958)
2-C-hydroxymethyl-glucose	150	Sols et al. (1958)
6-deoxy-6-fluoro-glucose	500	Sols et al. (1958)
xylose	1,000	Sols et al. (1958)

with ATP for magnesium ions.

Hexokinase and yeast-cell anti-sera prepared from rabbits (Miller et al., 1949; Pasternak, 1951) were found to inhibit hexokinase activity in vitro, but not when the enzyme was present in whole cells.

Melchior and Melchior (1958) showed that sodium ions inhibit the hexokinase reaction by reacting with an enzyme-ATP complex, and that the inhibition by ATP in excess of the magnesium present is a molecular mechanism by which the metabolism of the cell can be controlled in part by the products of metabolism.

6. Kinetics and reaction mechanism.

The relative rates of phosphorylation of the various substrates depends largely on the actual substrate concentration. At low sugar concentrations, glucose is attacked more rapidly than fructose, while at high sugar concentrations the opposite is the case. As pointed out by Dixon and Needham (1946), this indicates a relatively low affinity of fructose for the enzyme, an observation borne out by later quantitative determinations. Table 6 shows the relative rates of phosphorylation calculated by various workers for different sugars, the rate of glucose phosphorylation being taken as unity in each case.

Table 7 shows the dissociation constants for the

complexes formed, with hexokinase, by various active species in the reaction.

When saturated with substrate, the following turnover numbers for the enzyme have been quoted. All determinations were made at 30°, at pH 7.5. (Except for glucosamine which was determined at pH 7.8).

TABLE 8

Substrate	Turnover Number (moles/10^5g prot./min.)	Authors
glucose	13,000	Berger et al. (1946)
fructose	26,000	Dixon and Needham (1946)
mannose	6,500	Berger et al. (1946)
glucosamine	12,000	Brown (1951)

Colowick and Kalckar (1943) demonstrated that glucose and fructose were both phosphorylated at carbon-6, Brown (1951) showed that the same is true for glucosamine, while Slein et al. (1951) stated that mannose 6-phosphate is the product of the reaction with mannose. Hitherto, proof of the site of phosphorylation in either 2-deoxyglucose or glucosone has not been published, but because of their structural similarity to the other substrates it can be fairly safely assumed that they too are phosphorylated at carbon-6.

Gamble and Najjar (1954, 1955), using ^{14}C-labelled glucose, first demonstrated the reversibility of the reaction. They found that the reverse reaction was one-fiftieth as fast as the forward one, and quote the following dissociation constants for the enzyme complexes formed with glucose 6-phosphate and ADP respectively:- 8×10^{-2} M and 2.8×10^{-3} M. This reversibility has since been confirmed by other workers. Kaufmann (1955) obtained $AT^{32}P$ from $AD^{32}P$ after incubating the diphosphate with hexokinase and glucose 6-phosphate. Robbins and Boyer (1957), using ^{14}C-labelled glucose, calculated the equilibrium constant for the hexokinase reaction from data obtained from the reverse reaction. At 30° and an initial pH of 6.0, they found the equilibrium constants for zero magnesium concentration and excess magnesium respectively to be 386 ± 58 and 155 ± 31.

The latest papers published on the hexokinase reaction all tend to show that the kinetics are not so simple as would appear from the overall equation first proposed by Colowick and Kalckar (1941):-

$$ATP + \text{hexose} \longrightarrow ADP + \text{hexose 6-phosphate.}$$

The temperature coefficient for the reaction (Q_{10}) is in the region of 1.9 to 2.2 over the temperature range

0° to 30°. (Berger et al., 1946; Kunitz and MacDonald, 1946).

Cohn (1956) used $H_2{}^{18}O$ to investigate which bond was split in ATP by the enzyme. She showed that the only possible interpretation of the results obtained, was that the phosphorus-oxygen bond in the terminal phosphate group was split. This places hexokinase in the general group of phosphoryl-transferring enzymes, whose reactions occur by a nucleophilic attack on the phosphorus either by the enzyme or by the acceptor. Ressler (1958) has postulated a general theory for proton transfer mechanisms in enzymatic reactions, and has shown how the hexokinase reaction can fit this theory. This is consistent with other evidence.

Ågren and Engström (1956) isolated phosphoserine from hexokinase that had been incubated with ATP or glucose 6-phosphate. This is of great interest, as it appears to pin-point the position of the active site of the enzyme, and would also show that hexokinase is similar to phosphoglucomutase in this respect (Kennedy and Koshland, 1957; Sidbury and Najjar, 1957). In addition, the hexokinase reaction is shown to be at least a two-stage one:-

(1) hexokinase + ATP $\rightleftharpoons$ hexokinase phosphate + ADP

(2) hexokinase phosphate + glucose $\rightleftharpoons$

glucose 6-phosphate + hexokinase.

Najjar and McCoy (1958) reported that radioactivity is not transferable between ^{14}C-glucose and ^{12}C-glucose 6-phosphate. They therefore proposed the following reaction sequence:-

(1) glucose-enzyme + ATP $\rightleftharpoons$ 6-phosphoglucose-enzyme + ADP

(2) 6-phosphoglucose-enzyme + glucose $\rightleftharpoons$

glucose-enzyme + glucose 6-phosphate,

and claim that it accounts for the following observations;

(a) the lack of exchange of phosphate between glucose and glucose 6-phosphate in the presence of the enzyme.

(b) the labelling of the enzyme with ^{32}P-labelled ATP or glucose 6-phosphate. (The labelled phosphoserine obtained by Ågren and Engström might arise from a transfer of phosphate during acid hydrolysis of the protein).

(c) the marked inhibition of enzyme activity by glucose 6-phosphate. (Sic).

(d) the fact that ^{14}C-labelled glucose labels the enzyme.

Metaphosphate has been described as an intermediate in the reaction by Evert (1958), who showed that, as the metaphosphate concentration increases, orthophosphate and pyrophosphate concentrations decrease, and vice versa.

Melchior and Melchior (1958) carried out a kinetic study of the various ionic species of ATP and complexes of them with different cations, with regard to the hexokinase reaction. An enzyme-ATP complex is shown to be compatible with the experimental results, and an enzyme-magnesium complex is shown to be incompatible with them. Their results are stated to be in agreement with those of Raaflaub and Leupin (1956), although the latter proposed an enzyme-magnesium complex as an intermediate in the reaction.

The unit of hexokinase activity has been defined in different ways. Berger et al. (1946) based their unit on the carbon dioxide released from a bicarbonate buffer during the course of the reaction, and fixed it as the amount of enzyme that would cause an initial rate of carbon dioxide evolution of 1 mm^3 per minute at 30° and pH 7.5. Kunitz and MacDonald (1946), however, defined the unit as the amount of hexokinase which catalyses the formation of 10^{-8} hydrogen equivalents of acid per minute at 5° and pH 7.5. These two units are approximately equivalent.

7. Assay methods.

The hexokinase reaction has been followed by analyzing the system for disappearing reactants, or for appearing

products. The various methods that have been used will be considered under the following heads:-

(i) sugar disappearance.

(ii) labile phosphorus disappearance.

(iii) hexose 6-phosphate formation.

(iv) formation of hydrogen ion.

(i) The disappearance of various sugar substrates can be followed by modifications of standard chemical methods for sugar determinations. Slein *et al.* (1950) analysed the reaction mixture for glucose and fructose, separately and together, but found this method had rather a limited usefulness for the determination of enzyme-substrate dissociation constants. Brown (1951) was able to use a specific reagent for following the utilization of glucosamine, while paper chromatographic procedures were used by Woodward and Hudson (1955) for studying the mutual inhibition of glucose and 2-deoxyglucose.

Sols *et al.* (1958) introduced rather a novel method of assaying hexokinase activity. This method is based on the fact that the phosphorylation of glucose renders it inert for the glucose oxidase reaction. Residual glucose was estimated by direct treatment of the incubation mixture with the glucose oxidase-peroxidase-chromogen reagent of Keston (1956), containing ethylenediaminetetra-

acetic acid in excess.

(ii) As the reaction,

ATP + glucose $\longrightarrow$ ADP + glucose 6-phosphate,

proceeds, there is a reduction of one molar equivalent in the easily hydrolyzable phosphate. Measurements of this labile phosphate disappearance have been used by, among others, Colowick and Kalckar (1943), Bailey and Webb (1948) and Brown (1951) for following the course of phosphorylations by hexokinase.

(iii) Racker (1947) developed a sensitive micro-method for estimating small concentrations of glucose 6-phosphate. In this method, the hexose phosphate is treated with a mixture of enzymes (phosphohexoisomerase, phosphohexokinase, aldolase and -glycerophosphate dehydrogenase) and all necessary coenzymes, activators etc. The change in intensity of optical absorption at 340 mu corresponded to the conversion of DPN to the oxidised form, which was in turn found to be equivalent to the amount of glucose 6-phosphate present originally. This method has also been used by Slater (1953). Slein et al. (1950) used a similar principle when they followed the reduction of TPN at 340 mu in the presence of hexose phosphate, Zwischenferment (glucose 6-phosphate dehydrogenase) and the one or two isomerases needed.

(iv) Colowick and Kalckar (1941) pointed out that one acid equivalent is liberated when one of the pyrophosphate linkages is broken with simultaneous formation of a hexose phosphate. The accompanying pH change can be determined directly with a glass electrode (Colowick and Kalckar, 1943), but this method can be applied only to the assessing of relative velocities for various substrates and is not amenable to quantitative interpretation. Kunitz and Mac Donald (1946) used a direct titration of the acid formed to follow the reaction.

Colowick and Kalckar (1943) developed a useful method for following the hexokinase reaction in Warburg manometers. The reaction is carried out in pH 7.5 bicarbonate/carbonic acid buffer, and the acid produced in the course of the reaction releases carbon dioxide which is measured manometrically.

Wajzer (1949) followed the change in pH of the reaction medium by measuring the change in optical density of phenol red indicator at 558 mμ. Sols and Crane (1954) adapted this method, using bromothymol blue as indicator and 560 mμ as the absorption wavelength, for following brain hexokinase phosphorylations. They state,(Crane and Sols, 1955), that this method is particularly useful for the estimations of K_m values. Melchior and Melchior (1956)

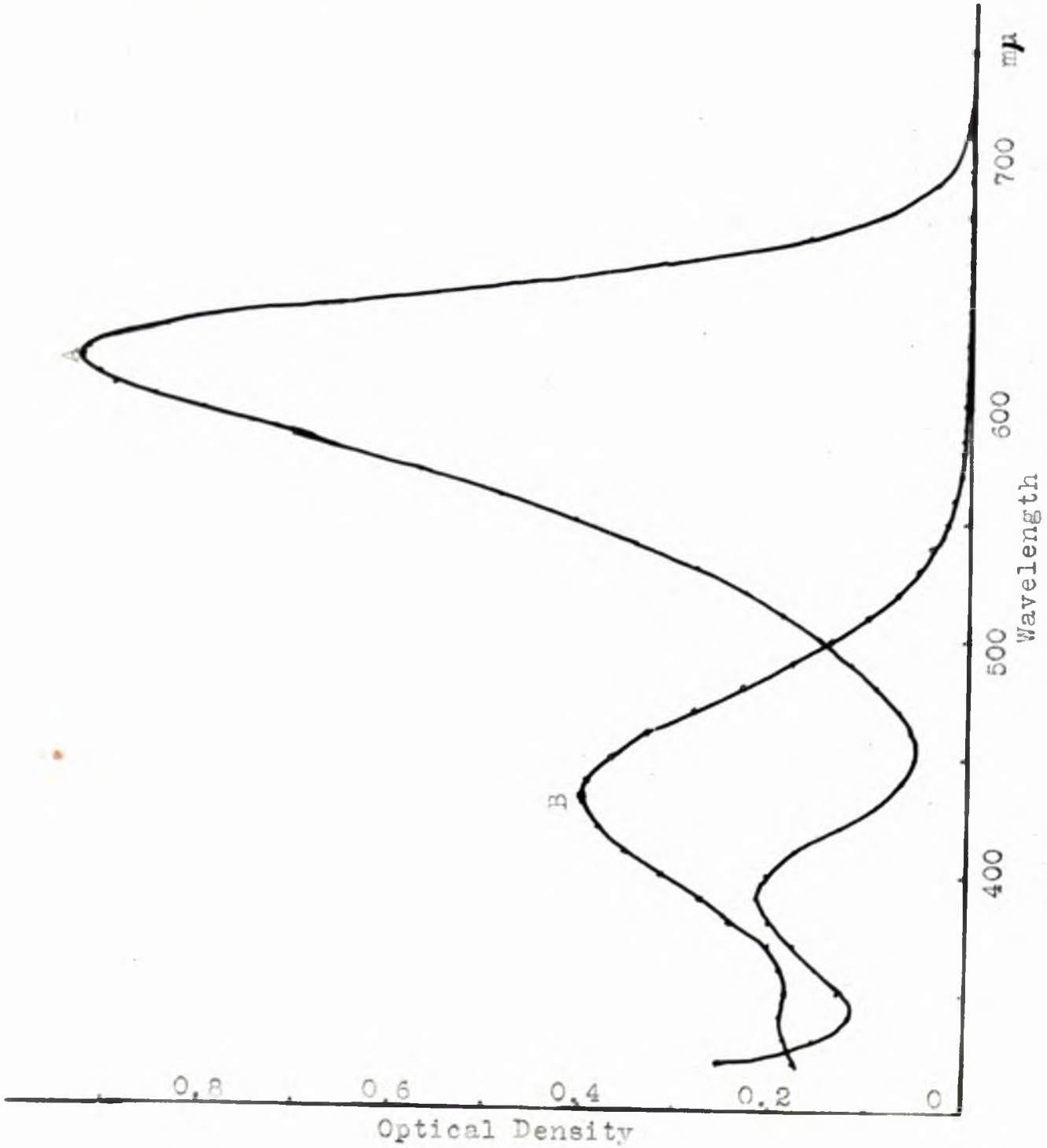
used yet another indicator (cresol red) and followed the reaction in triethanolamine buffer (starting pH, 8.4), at 571 mμ.

PLATE 10

A 1 ml 0.015 % bromothymol blue plus 10 ml 0.1 N sodium hydroxide.

B 1 ml 0.015 % bromothymol blue plus 10 ml 0.1 N sulphuric acid.

The spectra were obtained in a Unicam SP 500 spectrophotometer.



2. EXPERIMENTS WITH YEAST HEXOKINASE.

1 An investigation of the spectrophotometric indicator method of assay.

(a) Introduction.

Wajzer (1949) described a simple, neat method for following phosphorylations catalyzed by hexokinase. In this method, the reactants are placed in an unbuffered medium containing phenol red indicator. The acid produced by the action of the enzyme causes the optical density at 558 mμ of the indicator to change. This change can be followed in a spectrophotometer. Crane and Sols (1955) modified this method by using bromothymol blue as the indicator, and they claimed that this method is particularly useful for the determination of K_m values. Since their limited instructions refer to the Klett-Summerson photometer, a fuller investigation of this system, with expression of the results in more general terms, was deemed advisable.

(b) Absorption spectra of bromothymol blue.

The absorption spectra of bromothymol blue in acidic and basic solutions are shown opposite (Plate 10). Curves A and B were obtained from mixtures of bromothymol blue with sodium hydroxide and sulphuric acid respectively. It

PLATE 11a

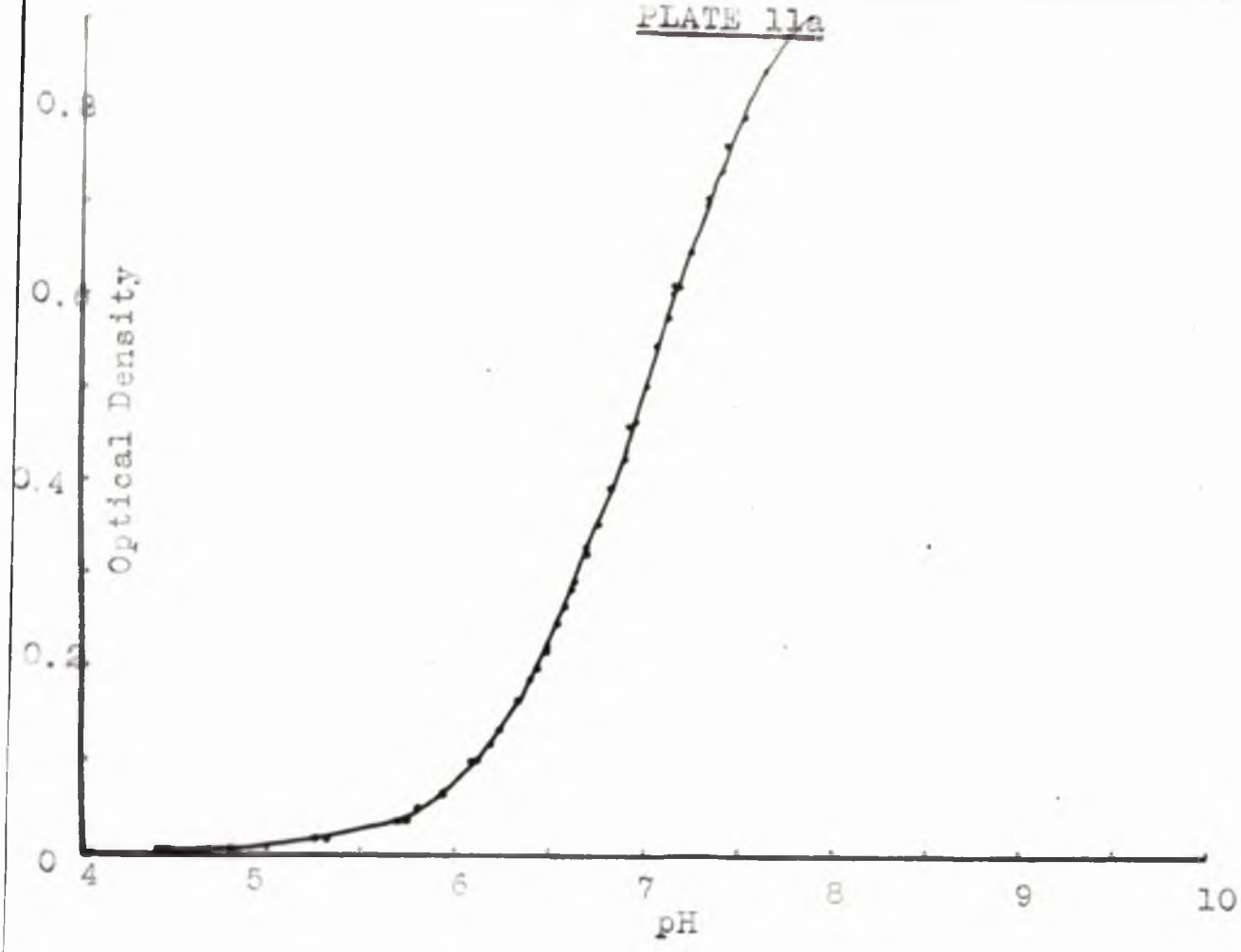


PLATE 11b

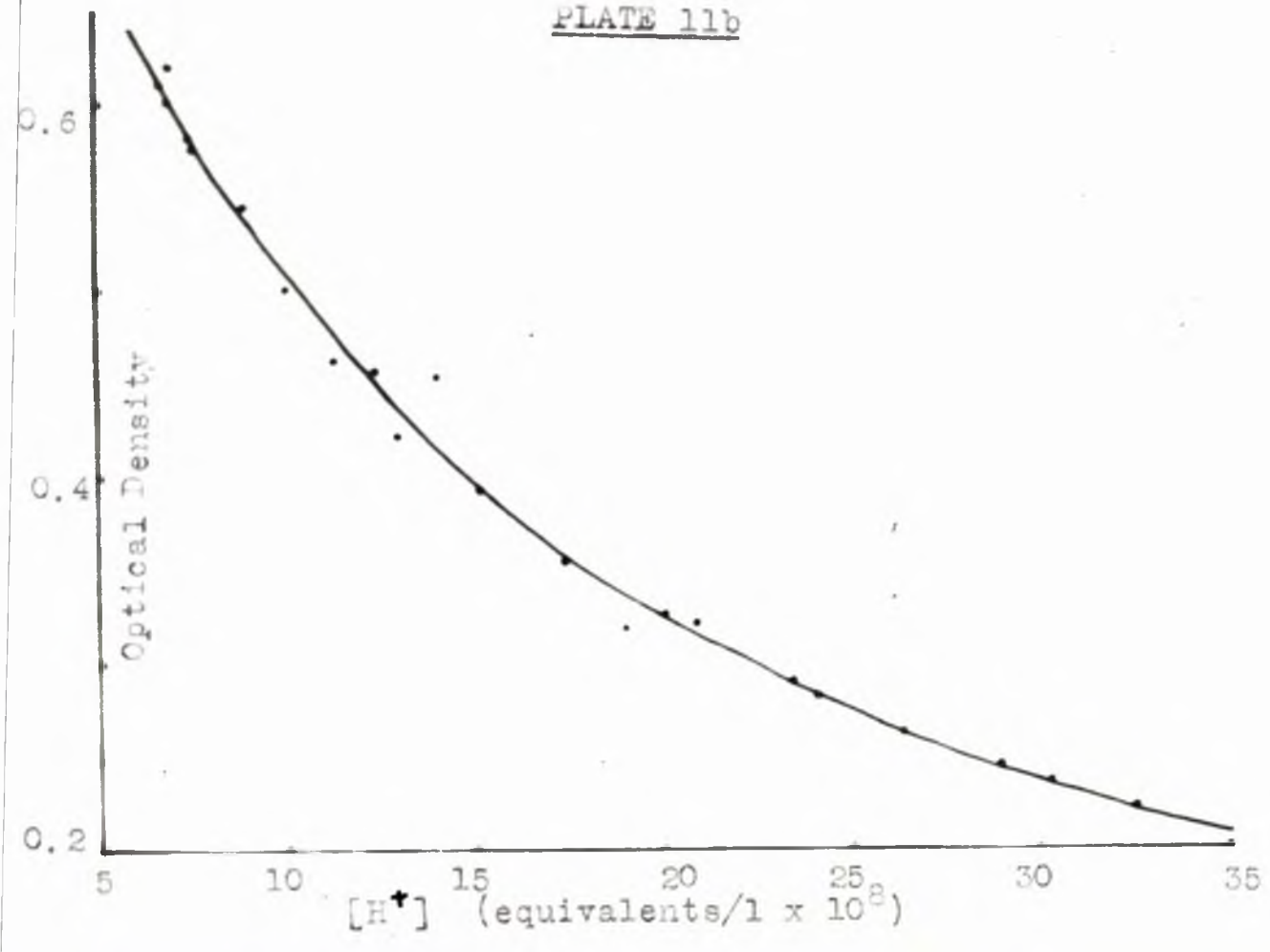


PLATE 11a

Variation of optical density at 615 mμ of bromothymol blue with pH.

Successive additions of a M/15 Na_2HPO_4 solution containing 0.0163 g/l bromothymol blue were made to a M/15 KH_2PO_4 solution containing the same concentration of indicator, and the pH and optical density of the mixture were measured after each addition.

PLATE 11b

Variation of optical density at 615 mμ of bromothymol blue with hydrogen ion concentration.

The hydrogen ion concentration in the mixture after each addition was calculated from the corresponding pH values.

can be seen that the peak at 615 mμ in the basic solution is completely absent at the lower pH, and, consequently, if the indicator is used for following a change in pH, measurements of optical density at 615 mu will not contain any contribution from the yellow component shown in curve B at 430 mμ. It is to be expected that bromothymol blue would be an ideal indicator for this purpose.

(c) pH Dependence of the optical absorption of bromothymol blue.

The change in optical density of bromothymol blue at 615 mμ with pH was determined and the results are shown in Plate 11a.

The change in $[H^+]$ with optical density was calculated from the above figures and the results plotted (Plate 11b). This graph shows that $[H^+]$ is not proportional to optical density, and that therefore, when using this indicator to follow phosphorylations, readings of the optical density of the system would not give a direct measure of the extent of the reaction. However, this curve can be used for correction purposes.

From the dissociation of 'a' moles of the indicator in solution according to the equation

$$\underset{(a-x)}{HIn} \rightleftharpoons H + \underset{(x)}{In},$$

where HIn is the species of the indicator present in

PLATE 12

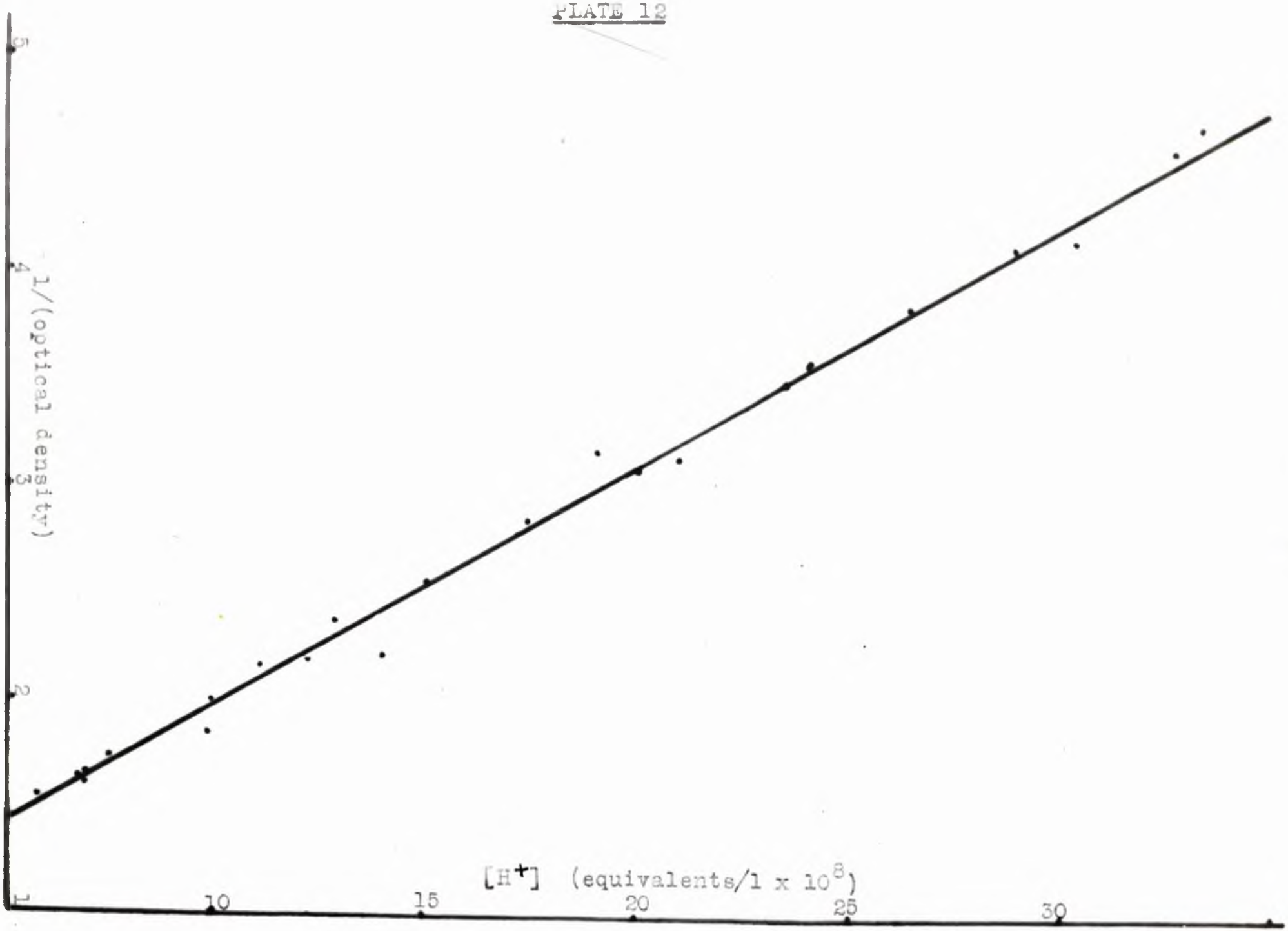


PLATE 12.

Graph of hydrogen ion concentration against reciprocal of optical density at 615 mμ of bromothymol blue.

Points calculated from data of Plate 11b.

acid solution and In^- that in alkaline solution, the following relationship can be derived.

$$K = \frac{[H^+]\,[In^-]}{[HIn]}$$

$$[H^+] = K\,\frac{[HIn]}{[In^-]}$$

$$[H^+] = \frac{K(a-x)}{x} \quad \ldots\ldots (i)$$

Assuming Beer's Law for light absorption,

$$(O.D) \propto [In^-]$$

$$\text{or, } k(O.D.) = [In^-] \quad \ldots\ldots\ldots (ii)$$

Substituting equation (ii) in equation (i),

$$[H^+] = K\,\frac{a-k(O.D.)}{k(O.D.)}$$

$$= \frac{K.a}{k(O.D.)} - K$$

Since 'a', 'k' and 'K' are all constants, it would be expected that a graph of $[H^+]$ against $1/(O.D.)$ would be linear. Plate 12 shows that this is so, thus

proving, incidentally, that the assumption of the validity of Beer's Law in the above calculation was justified.

(d) K_m measurements using the spectrophotometric indicator method.

In order to test this method of following hexokinase phosphorylations, the Michaelis Constants for the complexes of the enzyme with fructose and mannose were determined.

The test-system contained, in addition to the substrate being investigated and hexokinase, magnesium chloride, ATP and bromothymol blue. The amount of acid produced in the first five minutes after the addition of the enzyme was found from the corresponding change in the optical density of the indicator (Plate 11b).

It was found, however, that deviations occurred unless the starting point of each tube were approximately the same optical density. This was achieved, before the addition of hexokinase, by making a rough check with an EEL colorimeter. In order to have as small a change in total volume as possible, the acid and alkaline solutions used were relatively strong (2 N). Since the sensitivity of this assay method depends inversely on the buffering capacity of the solution, the fine adjustment of pH with

PLATE 13.

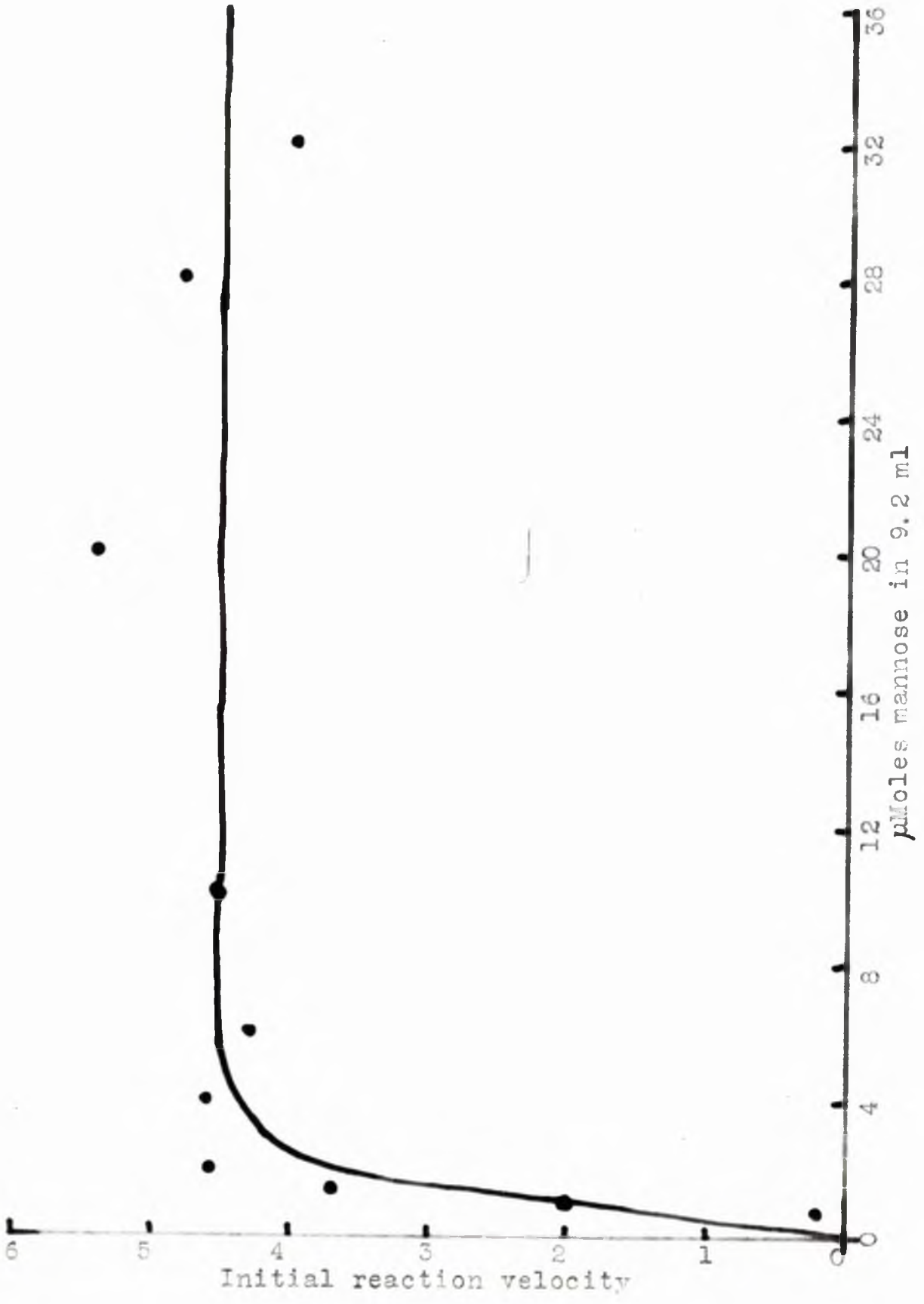

0
4
8
12
16
20
24
28
32
36
μMoles mannose in 9.2 ml
6
5
4
3
2
1
0
Initial reaction velocity

PLATE 13.

The variation of initial reaction velocity with substrate concentration for mannose in the hexokinase system.

The reaction velocity is expressed as the change in hydrogen ion concentration (equivalents/l x 10^8) over the first five minutes of reaction.

PLATE 14a.

The non-phosphorylation of fructosamine by hexokinase.

×—× glucose, 0.5 mMoles; hexokinase, 0.2 ml.

•—• fructosamine, 0.5 mMoles; Hexokinase, 0.2 ml.

Δ—Δ fructosamine; 0.5 mMoles; hexokinase, 1.0 ml.

PLATE 14b.

The non-phosphorylation of fructosamine by hexokinase.

•—• fructose, 0.5 mMoles.

×—× glucose, 0.5 mMoles.

▲—▲ fructosamine, 0.5 mMoles.

In each graph, the change in hydrogen ion concentration ($\Delta[H^+]$) is expressed in equivalents per litre x 10^8.

PLATE 14a.

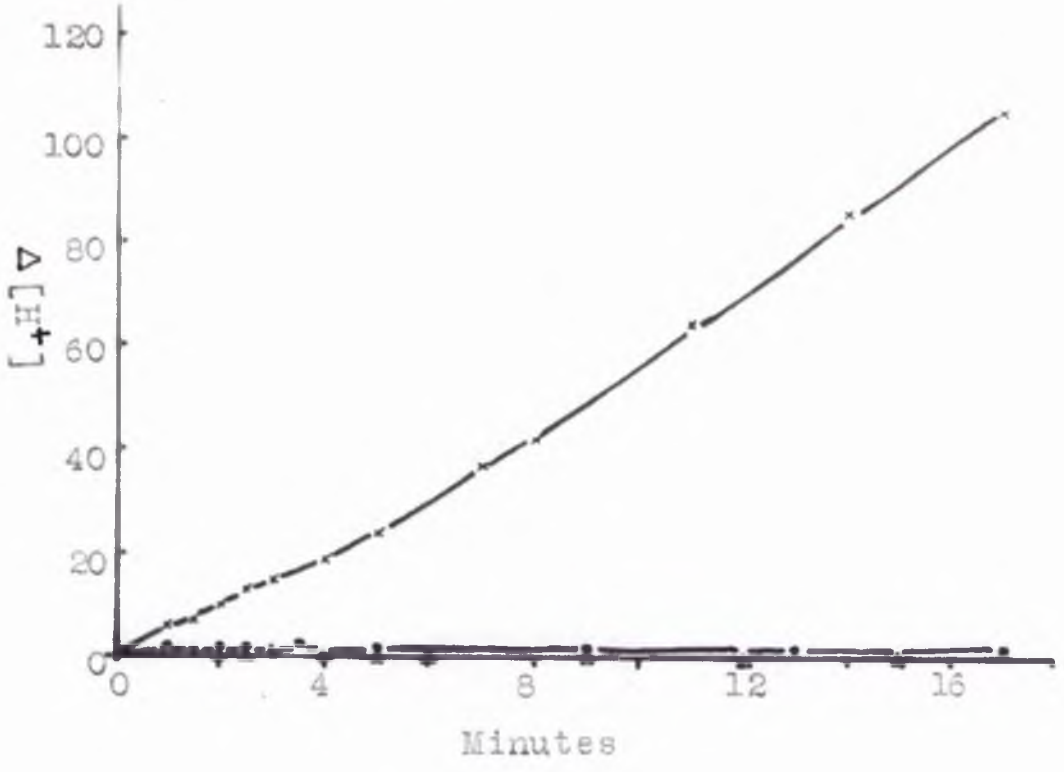
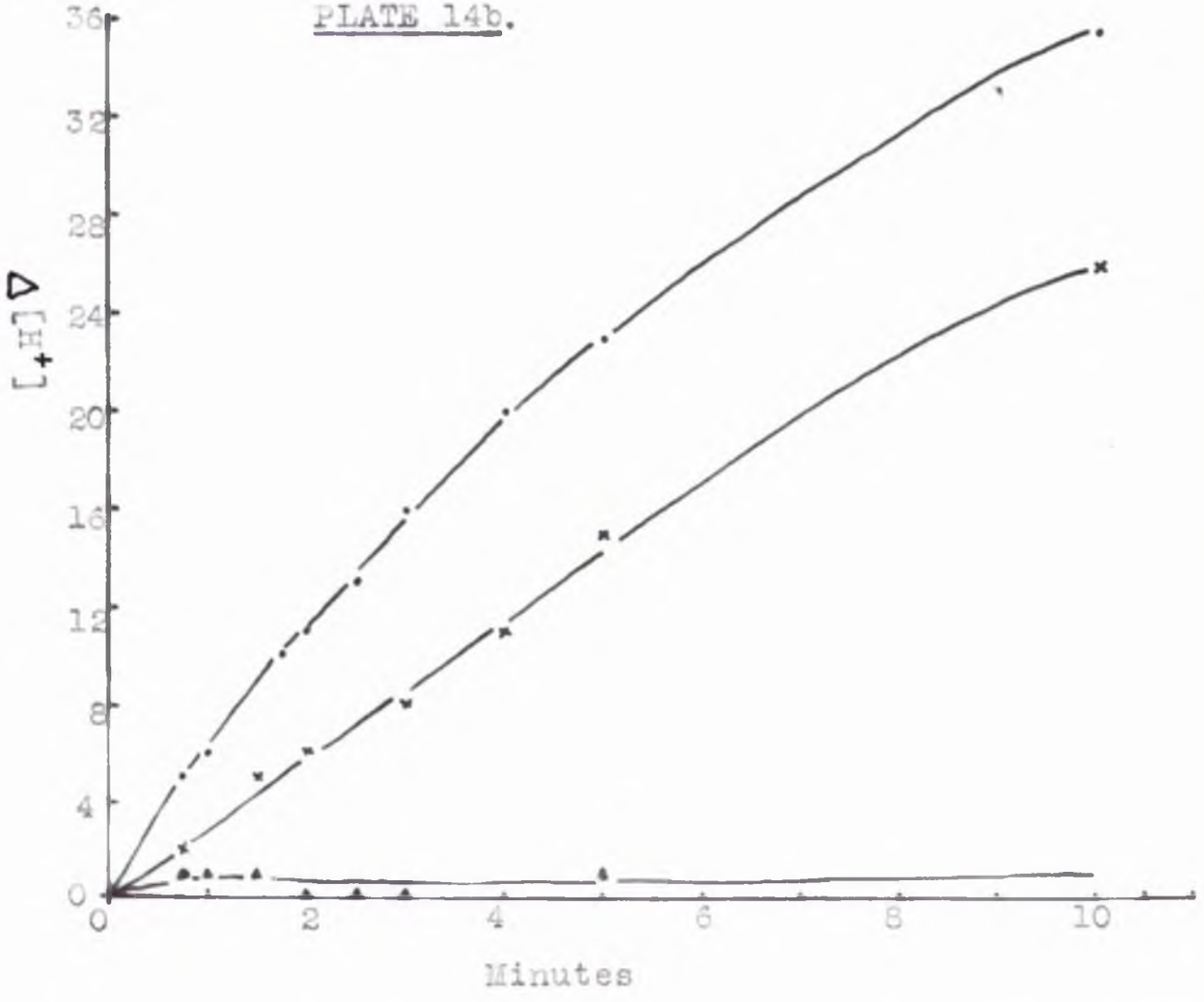


PLATE 14b.



strong acid or alkali is relatively difficult.

The deviations mentioned above can be ascribed to the buffering action of the three phosphates (ATP, ADP and substrate 6-phosphate) present in the solution, differing at different pH values. It was found that if the optical density at the start of the reaction in each tube lay within 0.02 units of that of any other, good reproducibility resulted, and if they were within 0.04, the results were just acceptable.

Plate 13 illustrates the change in reaction velocity with substrate concentration for the hexokinase-mannose system. From it, the K_m for mannose (Mannose concentration at half maximal velocity) is found to be 1.09×10^{-4} M. Similarly, the K_m for fructose was found to be 0.59×10^{-3} M. These values agree well with those obtained by other workers. (Table 7).

2 <u>The effect of fructosamine in the hexokinase reaction.</u>

Fructosamine was tested for substrate activity in the hexokinase system using the spectrophotometric method for following the reaction. The aminosugar was not found to give any signs of being phosphorylated. Plate 14a shows this, and for comparison, the phosphorylation of the same concentration of glucose. The concentration of hexokinase

PLATE 15.

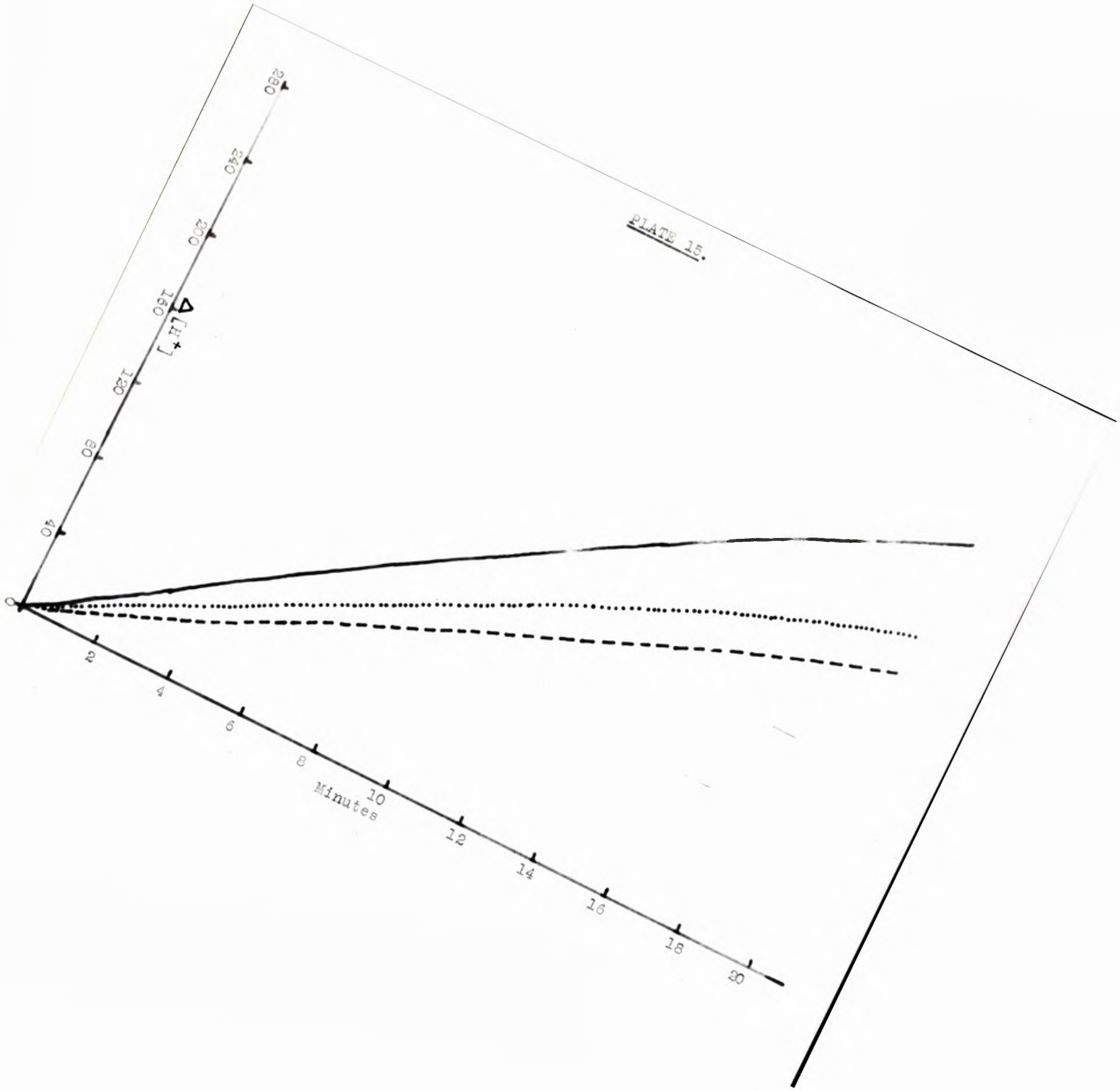


PLATE 15.

Inhibition of the phosphorylation of glucose by hexokinase in the presence of fructosamine.

—— glucose 0.05 M;

.... glucose 0.05 M; fructosamine 0.05 M

– – – glucose 0.05 M; fructosamine 0.10 M.

The phosphorylations were followed using the spectrophotometric assay method.

$\Delta[H^+]$ is expressed as (equivalents/l x 10^8).

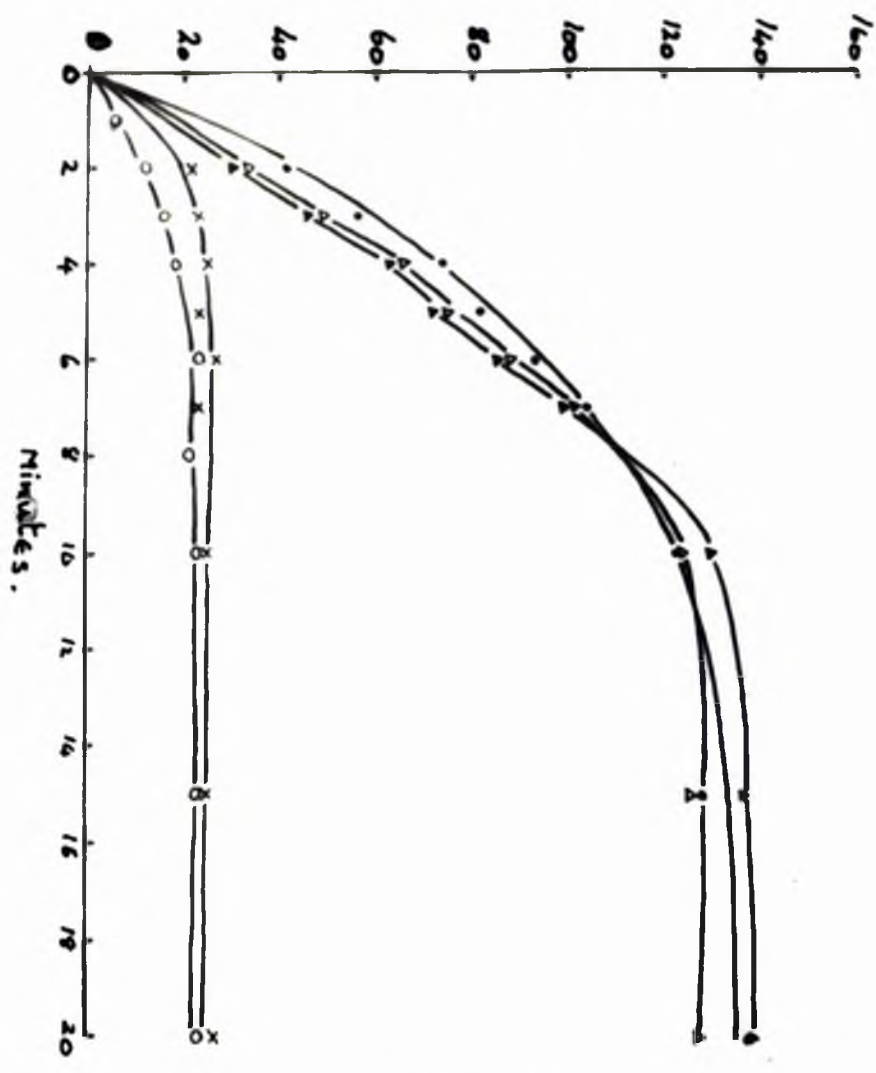


PLATE 16b.

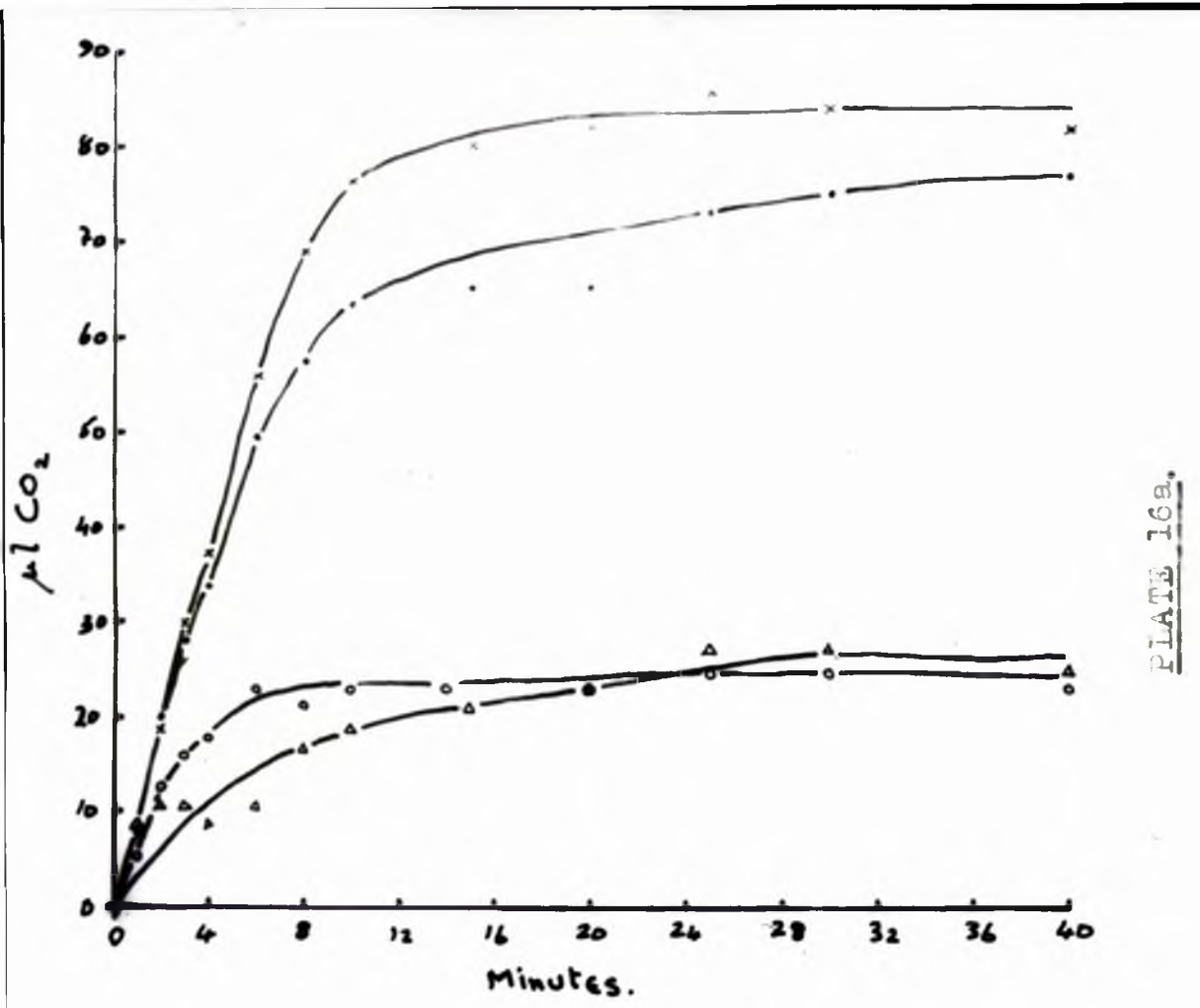

90
80
70
60
50
40
30
20
10
0
µl CO2
0
4
8
12
16
20
24
28
32
36
40
Minutes.

PLATE 16a.

PLATE 16a

The non-phosphorylation of fructosamine by hexokinase.

×—× fructose, 0.03 M

•—• glucose, 0.03 M

○—○ fructosamine, 0.03 M

△—△ blank.

PLATE 16b.

The effect of fructosamine on the phosphorylation of fructose by hexokinase.

▲—▲ fructose, 0.06 M

•—• fructose, 0.03 M; fructosamine, 0.03 M

△—△ fructose, 0.03 M; fructosamine, 0.03 M

×—× fructosamine, 0.06 M

○—○ blank.

The above phosphorylations were followed by the manometric assay method.

was increased to five times the usual test value, and still a reaction of fructosamine was not evident.

Graph 14b gives the results of another experiment substantiating the above result. This time a less active sample of the enzyme was used, and the phosphorylations of both glucose and fructose are shown.

The effect of fructosamine on the phosphorylation of glucose by hexokinase is shown in Plate 15. The inhibitory effect of fructosamine on this reaction is evident for equimolar amounts of glucose and fructosamine, while an increased inhibition was obtained when the fructosamine concentration was doubled.

In case the non-phosphorylation of fructosamine was only apparent, due to the buffering effect of the amino-sugar damping the sensitivity of the assay method, further investigations were carried out.

The titration curve of fructosamine acetate was found (Plate 3), and over the pH region of the test, the amino-sugar was found to have virtually no buffering action at all.

As a further check, the activity of fructosamine in the hexokinase reaction was tested using the manometric method of Colowick and Kalckar (1943), since this method involves a fully buffered system. Plate 16a shows that even here phosphorylation is not evident.

PLATE 17a.

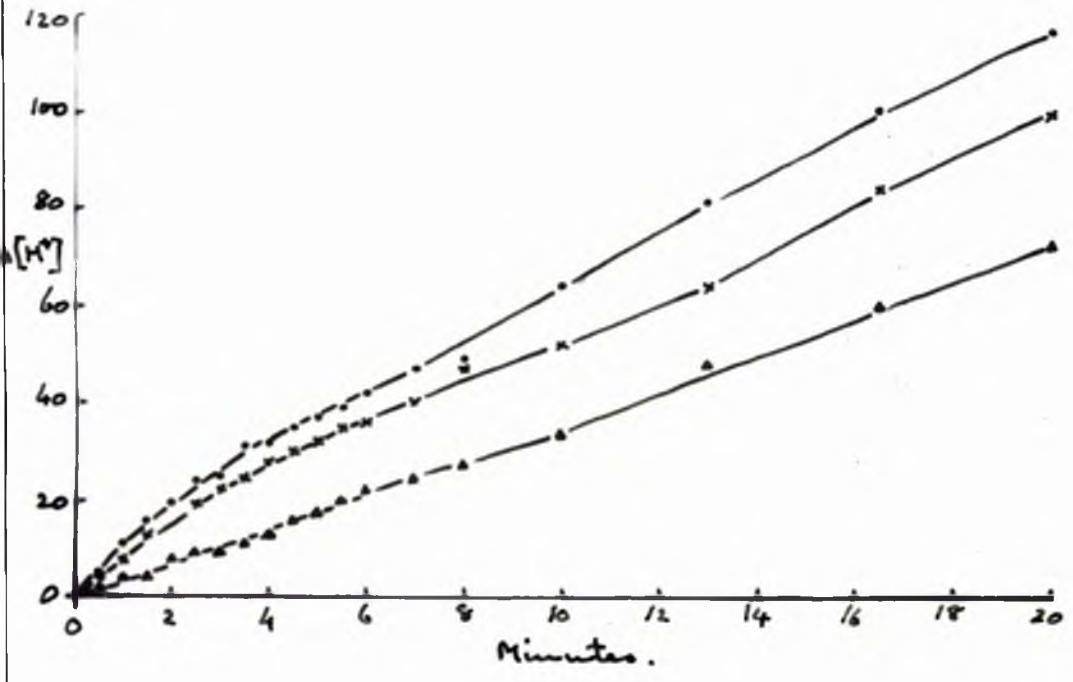


PLATE 17b.

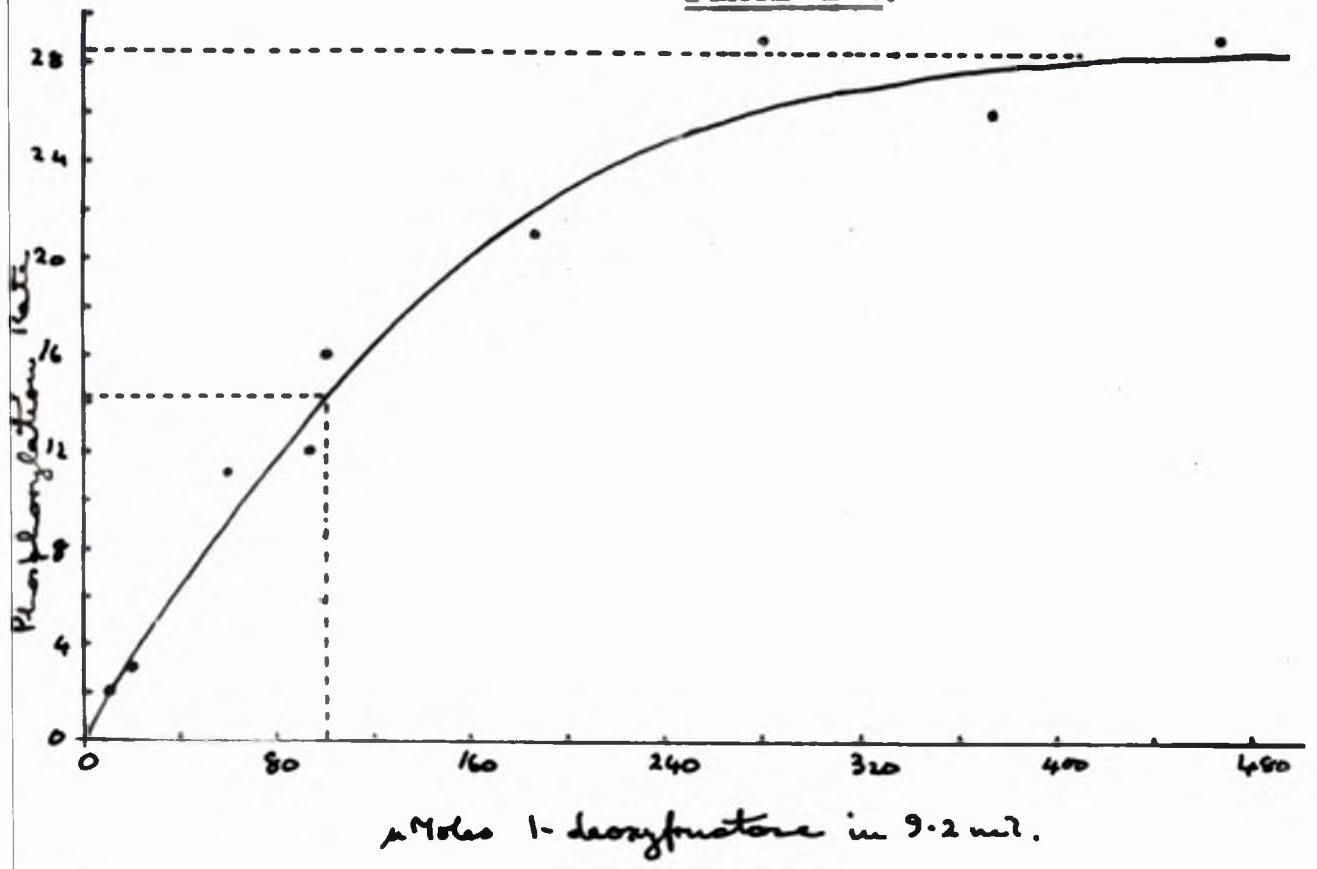


PLATE 17a.

The phosphorylation of 1-deoxyfructose by hexokinase; and the inhibition of the phosphorylation by hexokinase of fructose (by 1-deoxyfructose).

•—• fructose, 0.022 M

×—× fructose, 0.022 M,; 1-deoxyfructose, 0.022 M

▲—▲ 1-deoxyfructose, 0.022 M.

The phosphorylation was followed by the spectrophotometric indicator assay method.

Δ $[H^+]$ is expressed in (equivalents/l x 10^8).

PLATE 17b.

The variation of initial reaction velocity with substrate concentration for 1-deoxyfructose in the hexokinase system.

The reaction velocities are expressed as the change in hydrogen ion concentration (equivalents/l x 10^8) over the first five minutes of reaction.

PLATE 18a.

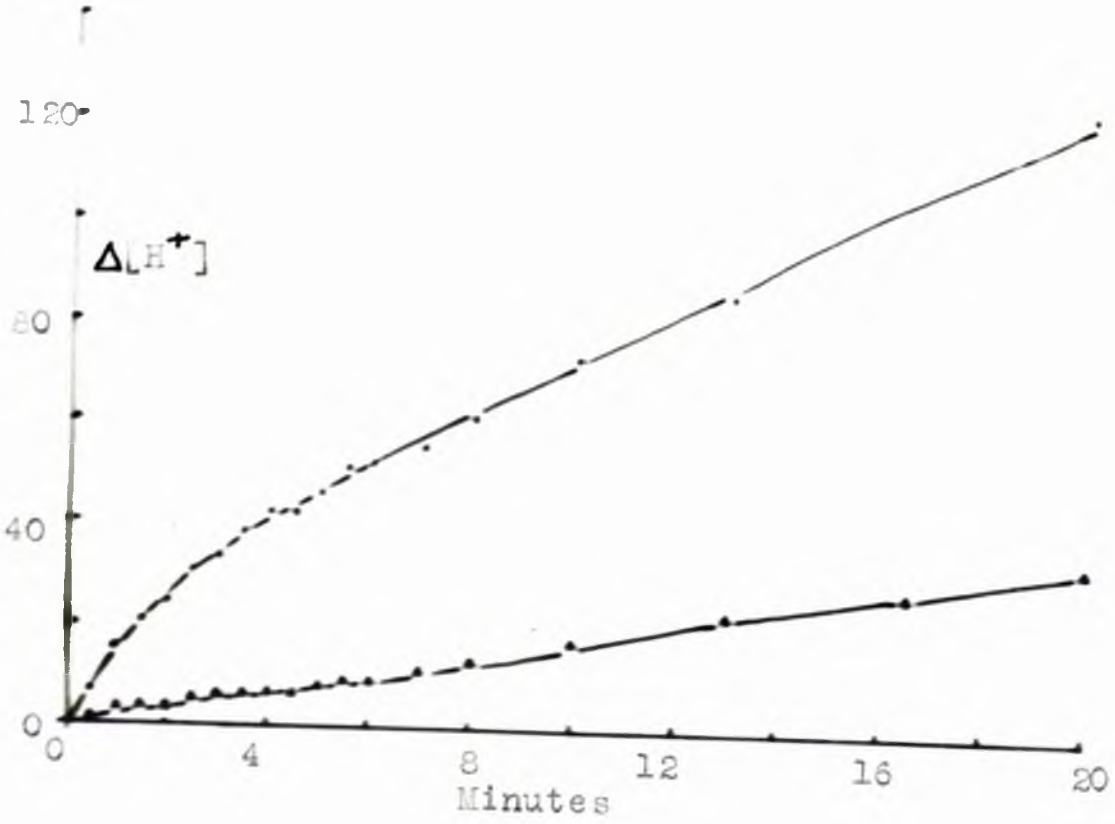


PLATE 18b.

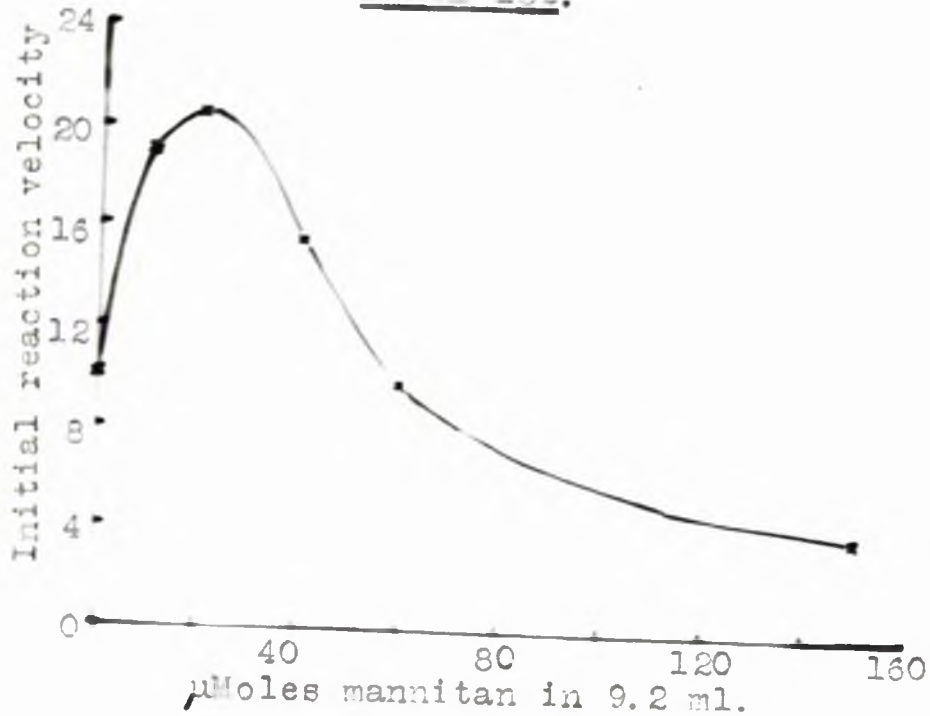


PLATE 18a.

The phosphorylation of 2,5-mannitan by hexokinase.

•—• fructose, 0.0022 M

▲—▲ 2,5-mannitan, 0.0022 M.

The phosphorylations were followed by the spectrophotometric indicator assay method.

$\triangle[H^+]$ is expressed in (equivalents/l x 10^8).

PLATE 18b

The variation of initial reaction velocity with substrate concentration for 2,5-mannitan in the hexokinase system.

The reaction velocities are expressed as the change in hydrogen ion concentration (equivalents/l x10^8) over the first ten minutes of reaction.

The inhibitory effect of fructosamine was investigated in this system, this time on fructose, but no inhibition was observed. This may be due to the lesser sensitivity of this assay method. (Plate 16b).

3 The phosphorylation of 1-deoxyfructose.

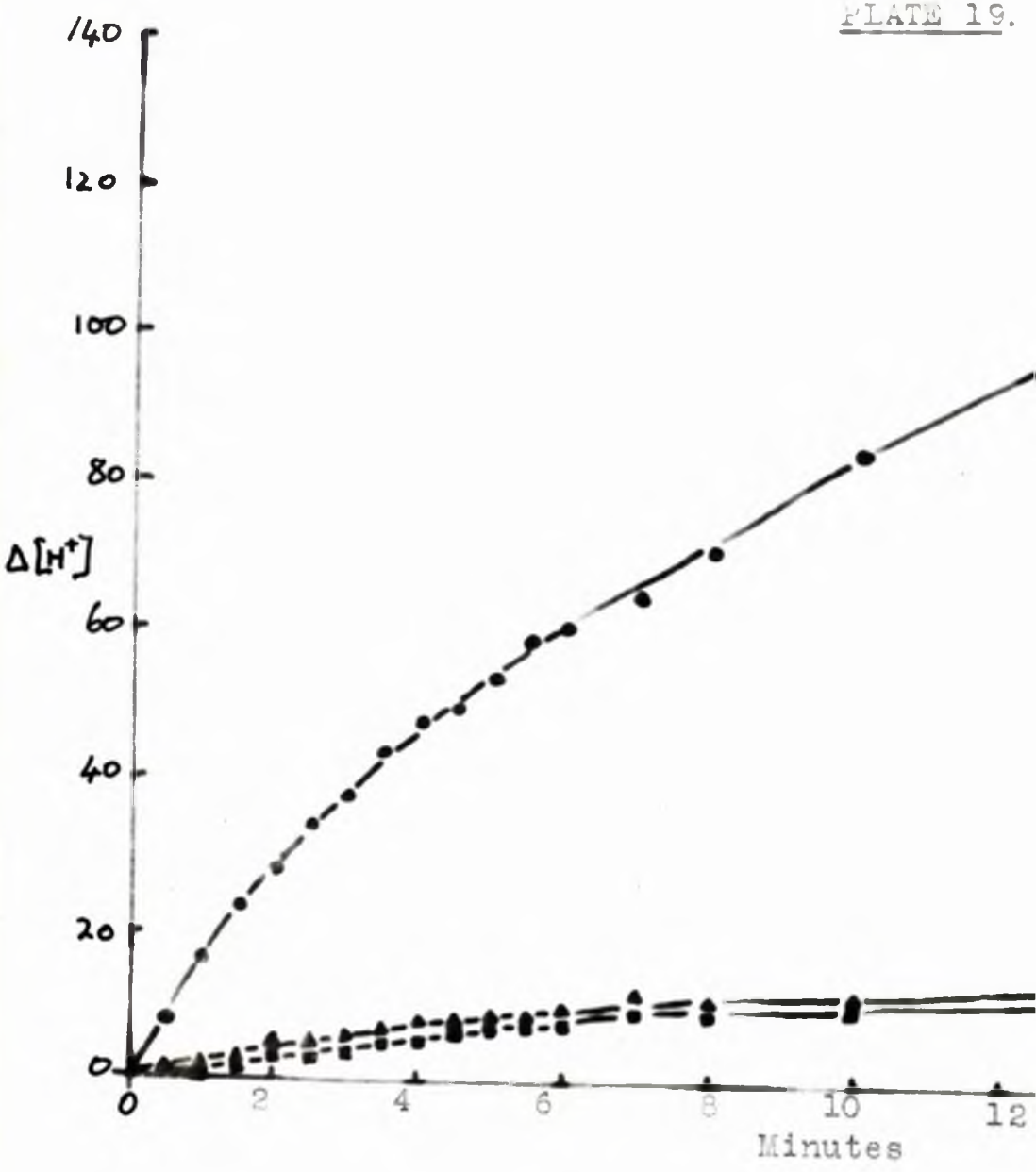
1-Deoxyfructose was found to act as a phosphate acceptor in the hexokinase reaction. Plate 17a illustrates this phosphorylation and compares it with that of fructose. It also shows that the deoxysugar inhibits fructose phosphorylation. 1-Deoxyfructose is phosphorylated at 0.41 times the rate of fructose.

The change of initial reaction velocity with the concentration of 1-deoxyfructose is shown in Plate 17b. From it, the substrate concentration at half maximal velocity is found to be 1.09×10^{-2} M.

4 The action of hexokinase on 2,5-mannitan.

2,5-Anhydromannitol is phosphorylated at 0.2 times the rate of fructose by hexokinase. Plate 18a illustrates this. The effect on reaction velocity of altering the concentration of 2,5-mannitan, in an attempt to evaluate the Michaelis Constant of the hexokinase mannitan complex (Plate 18b), revealed a case of substrate inhibition. A plot of initial rate against substrate concentration shows

PLATE 19.




14
16
18
20

PLATE 19.

The non-phosphorylation of 2,5-sorbitan by hexokinase.

●—● fructose, 0.0022 M

▲—▲ 2,5-sorbitan, 0.0022 M

■—■ blank.

The reaction was followed by the spectrophotometric indicator assay method.

Δ $[H^+]$ is expressed in(equivalents/l x 10^8).

that the Michaelis-Menten Law is not obeyed, but that as the substrate concentration increases, the rate rises, passes through a maximum and finally falls.

The fact that the curve passes below the zero level can be ascribed to the residual glucose, present in the hexokinase preparation, phosphorylation of which is inhibited by excess of the mannitan.

5 The action of hexokinase on 2,5-sorbitan.

2,5-Anhydrosorbitol is not phosphorylated by hexokinase (Plate 19) under the conditions of testing.

3. DISCUSSION OF THE SUBSTRATE SPECIFICITY OF YEAST HEXOKINASE.

'It is probably true that there is no basic difference between enzymic and non-enzymic catalysis in the nature of the reaction catalyzed, or even the method of catalysis, but that the difference lies in the extreme chemical and steric specificity found in enzymic reactions'. This statement of Gibson's (1957) will have to be modified somewhat in the light of present-day knowledge of enzyme specificity, since enzymes are continually being shown to be far less exacting in their requirements as regards potential substrates.

It has long been understood that enzymes catalyze reactions by first forming a complex with their substrates, and it is on this understanding that the whole approach to the problem of the substrate specificity of hexokinase has been based. Although the exact nature of these enzyme-substrate complexes has not been determined, they are known to be formed by a stoichiometric combination of substrate molecules with a certain area, or areas, (active sites) on the surface of the enzyme. Hydrogen bonds and van der Waals forces are probably involved in the formation of these complexes.

Inhibition of the utilization of one substrate by

another is believed to arise either from a competition of the two types of substrate molecule for the same active site (with account being taken of the relative ease with which the products of the enzyme action are dissociated from the active site), or from the attachment of the second substrate at an inhibitor site where its presence interferes with the reaction of the first (Crane and Sols, 1954).

Inhibition of the utilization of one substrate by a structural analogue which is itself not a substrate, is thought to be due to the inhibitor combining (reversibly or irreversibly) with the active site or with the inhibitor site mentioned above. In order to do this, the inhibitor must have some structural affinity with a normal substrate, but it apparently lacks certain structural features which would enable it to be acted on further by the enzyme.

Bayne (1958) has summarized the possible reasons for a certain sugar not taking part in an enzyme-catalyzed reaction, although it has formed a complex with the enzyme. These are:-

(1) The potentially active group is absent or not suitably situated.

(2) There has been a loss of a binding group.

(3) A group, or groups, in the sugar interferes sterically with a binding group.

From a study of the molecular structures of various carbohydrates that have been tested in the hexokinase system,

it is easily seen that there are definite structural requirements that have been fulfilled by those that exhibit any substrate or inhibitory activity.

The following sugars are phosphorylated by ATP in the presence of magnesium ions and hexokinase. References are given in Table 7.

glucose, mannose, glucosamine, 2-deoxyglucose, glucosone, allose, galactose, 1,5-sorbitan, fructose, 2,5-mannitan, 1-deoxyfructose, arabinose.

Mutual competitive inhibition in a large number of pairs of these substrates (see review) has been demonstrated, thereby proving that the enzyme contains only one type of active site and that this is catalytically active for all the substrates.

The following sugars inhibit the phosphorylation by hexokinase of one or more of the above-listed substrates, and are not themselves phosphorylated. References are given in Table 4.

Nacetylglucosamine, 6-deoxy-6-fluoroglucose, manno-heptulose, 2-C-hydroxymethylglucose, xylose, fructos-amine, 3-O-methylglucose, 3-O-methylfructose.

The following sugars have no substrate or inhibitory activity in the hexokinase system. References are given in Table 5.

L-sorbose 1-phosphate, glucose 1-phosphate, glucose 6-phosphate, galactose 1-phosphate, mannose 6-phosphate, glucose 6-phosphite, fructose 1-phosphate, fructose 6-phosphate, glucosone phosphate, galactosone, galactosamine, L-glucosone, methyl α-glucoside, methyl β-glucoside, 1-O-methylfructose, 3-O-methylglucose, 3-O-methylfructose, 4-O-methylfructose, 6-O-methylglucose, L-sorbose, L-arabinose, sorbitol, 1,4-sorbitan, 2,5-sorbitan, 1,5-mannitan, gluconic acid, L-rhamnose, lactose, maltose, sucrose, trehalose, raffinose.

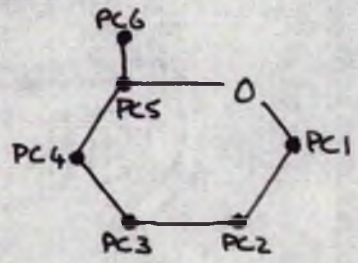
The actual spatial configurations of the molecules when they combine with hexokinase to form an enzyme-substrate or an enzyme-inhibitor complex, of only a few of these substrates or inhibitors are known. Whether glucose and mannose are present in the α- or β-configuration appears to make no difference to their respective rates of phosphorylation, although both of these compounds have to take up the pyranose form before they can be utilized by hexokinase (Gottschalk, 1943, 1947).

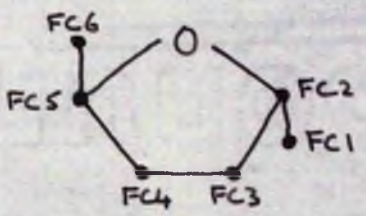
On the other hand, fructose has been shown to be in the β-furanose form when it is acted on by hexokinase (Gottschalk, 1943, 1945; Slein *et al.*, 1950). Results given in this thesis indirectly support the conclusions about fructose. Also among the substrates, 1,5-sorbitan and 2,5-mannitan have fixed structures, the former

containing a pyranose-type ring and the latter a furanose-type one.

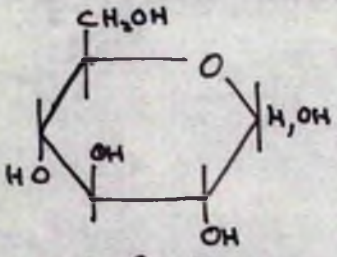
Due to this lack of specificity of hexokinase with regard to the ring structure of its substrates, analogues of the substrates and inhibitors will have to be considered under the two possibilities, pyranose or furanose. In some cases it will appear obvious in which form a sugar will inhibit the hexokinase reaction or act as a substrate in it, in others it will not be so easy to make such a prediction. For various reasons, however, quite a strong case can be made out for the probability of one form being prefered to the others that are possible.

For the purposes of the discussion, the carbon atoms in these rings will be numbered according to the following plans:-

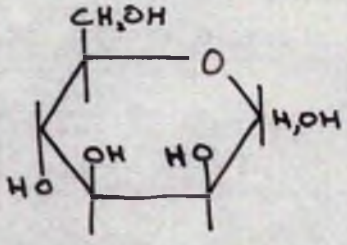




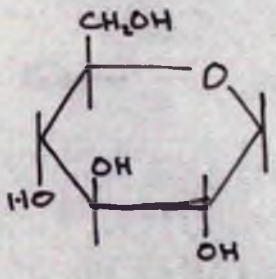
The structures of those substrates whose active forms are known are;-



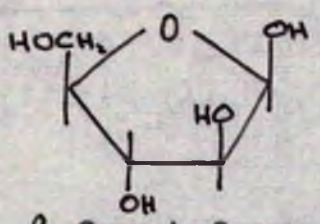
α-& β-gluco-pyranose



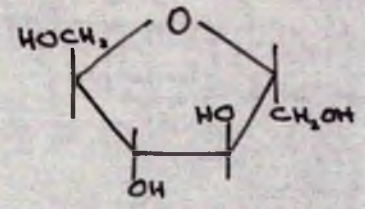
α- & β-manno pyranose



1,5-sorbitan



β-fructofuranose



2,5-mannitan

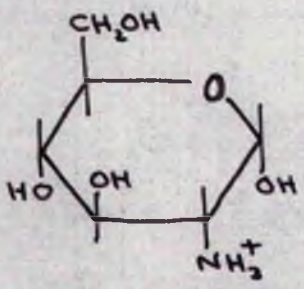
Considering these compounds alone, certain conclusions can be drawn concerning the substrate specificity of hexokinase. A certain amount of variation on PC1 does not interfere with hexokinase activity, and, in fact, a reducing group need not be present at all at either PC1 or FC2. In all these cases, PC5 and FC5 have a hydroxymethyl group above the ring (PC6 and FC6 respectively), and in the cases of glucose, mannose and fructose, it is here that the point of chemical change, catalyzed by the enzyme, is situated. This is known since the sugar 6-phosphate is the product of the reaction for each of these sugars. By analogy, it is to be expected that the mannitan and the sorbitan will be phosphorylated at FC6 and PC6 respectively too.

Not only should the configuration of each substrate molecule be taken into account for purposes of relating enzyme specificity with substrate structure, but also it's actual conformation.

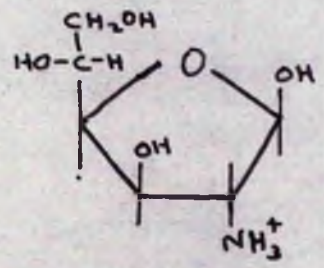
In α-glucopyranose, the hydroxyl group on PC1 is in an axial position, all the other hydroxyl groups and the

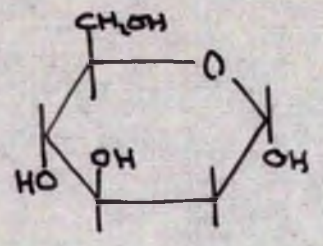
primary alcohol group are equatorial. In β-glucose, all the hydroxyl groups and the primary alcohol group are equatorial. A conformational analysis indicates that the hydroxyl group on PC1 of α-glucose is in a sterically hindered position, and that the reactions directly implicating this hydroxyl group will take place more readily with β-glucose (Bently, 1955). However, although a sugar in solution in the free state may be known to assume a certain conformation, there is no proof that it retains that conformation when it is combined with an enzyme. Consequently, although the importance of the conformation of a sugar must not be ignored, only a little work has as yet been done with regard to the part played by substrate conformation in biological systems, (Reeves, 1951; Bentley, 1955; Le Fevre and Marshall, 1958; Barker et al., 1959), and such a conformational analysis of substrate structure is not possible at this stage as regards the hexokinase reaction.

Glucosamine, 2-deoxyglucose, glucosone, allose and galactose are each fairly certain to be phosphorylated in the pyranose form.

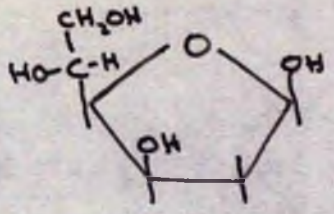


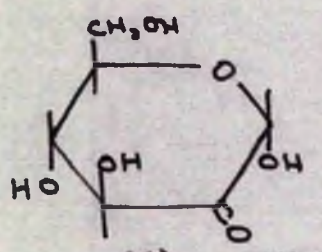
glucosamine



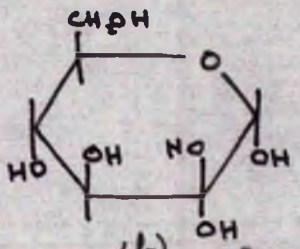


2-deoxyglucose



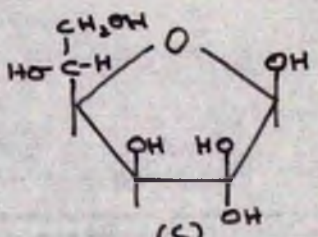


(a)

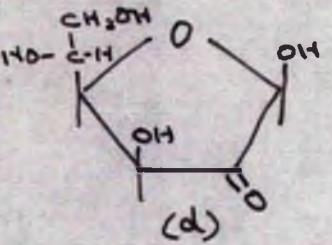


(b)

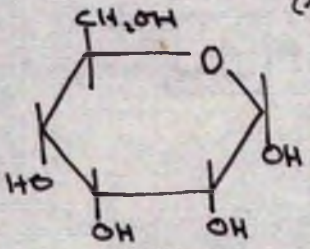
glucosone



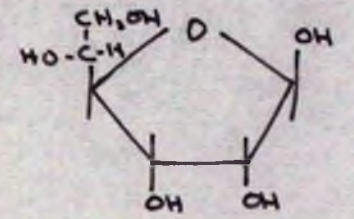
(c)

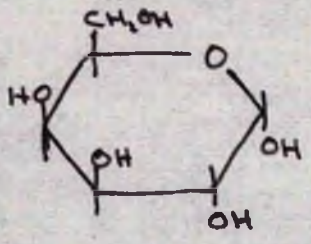


(d)

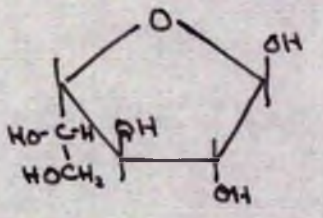


allose





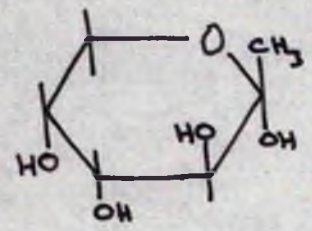
galactose



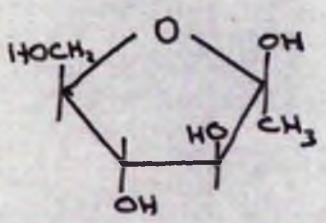
Each of the above compounds in the pyranose form differs in only one respect from glucopyranose and in four from fructofuranose when in the furanose form. Structure (b) of glucosone (a hydrate) has been suggested by Bayne and Fewster (1956) as being present in a solution of this compound, and structurally, or in the the free keto form (a), would be the most likely to be phosphorylated by hexokinase.

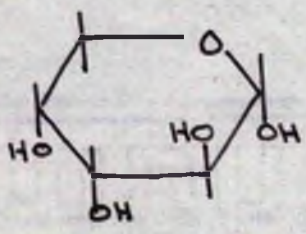
1-Deoxyfructose and arabinose are the remaining substrates of hexokinase not discussed so far. Consideration of their structures shows that they both differ

from fructofuranose at PC1 only while in the furanose form, but that in the pyranose form PC6 is absent and there are also variations from glucopyranose at other carbon atoms. Infra-red spectroscopic evidence indicates that 1-deoxy-fructose is predominately furanose in the solid amorphous state.

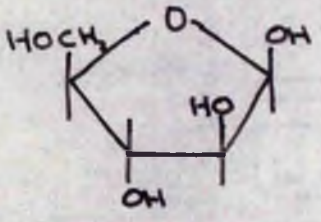


1-deoxyfructose

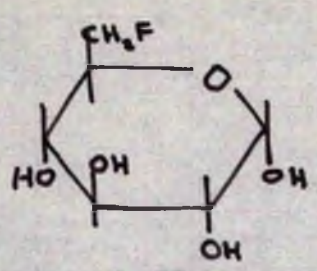




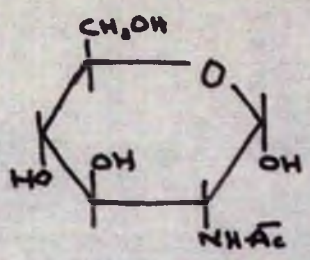
arabinose



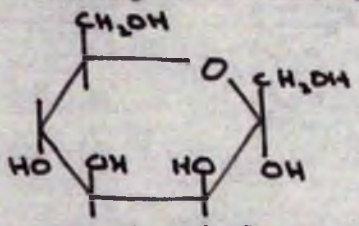
Assuming that for a carbohydrate analogue to act as an inhibitor of a substrate, it must attach itself to a site on the enzyme surface, it is reasonable to assume that similar forces must be employed in binding both compounds to the enzyme, and hence that some degree of similarity is to be expected between their respective molecular structures. Many of the above substrates are known to inhibit the utilization of other substrates, and the structures of substrate analogues that are known to inhibit the hexokinase reaction, but are not themselves phosphorylated, are given below:-



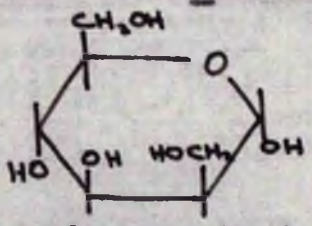
6-deoxy-6-fluoroglucose



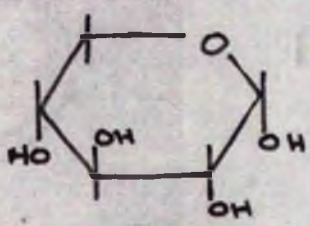
N-acetylglucosamine



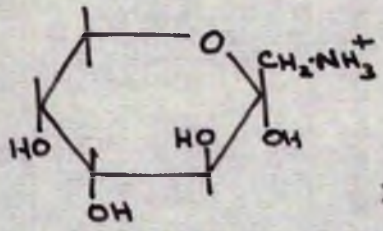
mannoheptulose



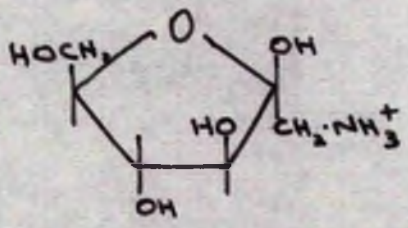
2-C-hydroxymethylglucose



xylose



fructosamine



In the case of these compounds the main problem to be solved is why, if they are attached to the enzyme, are they not phosphorylated.

Crane and Sols (1954) reported the non-competitive inhibition of brain hexokinase by glucose 6-phosphate and analogues of it, and showed that these inhibitors exhibited a pattern of specificity different from that of the substrates of this enzyme. This indicated that brain hexokinase possesses, in addition to binding sites for substrates and ATP, a third specific binding site.

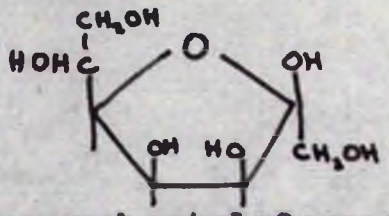
Despite the essential similarity between hexokinase and brain hexokinase (Sols *et al.*, 1958) as regards their substrate specificities, no evidence of a comparable third site has yet been obtained for hexokinase. This

enzyme is not inhibited by glucose 6-phosphate or sorbose-1-phosphate, both of which are strong non-competitive inhibitors of brain hexokinase; and even allowing for the higher degree of activity of the latter enzyme, were such an inhibition to exist, it would have been detected by the experimental procedures now available.

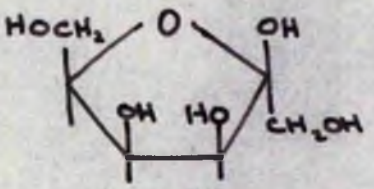
The fact that hexokinase has some affinity for glucose 6-phosphate has been proved by the demonstration that the enzyme can catalyze the reverse of its normal reaction (Gamble and Najjar, 1954; 1955; Kaufmann, 1955; Ågren and Engström, 1956), but conflicting reports of the inhibitory activity of glucose 6-phosphate have been published. Weil-Malherbe and Bone (1951), Sols and Crane (1953) and Mitchell (1954) failed to show any inhibition by glucose 6-phosphate in the hexokinase reaction, while Wajzer's (1953) results did show it. However, using the spectrophotometric indicator assay method, Wajzer does not seem to take into account the buffering action of the phosphate group in this compound, and this can account for the apparent depression of enzyme activity. Najjar and McCoy's (1958) statement that glucose 6-phosphate strongly inhibits yeast hexokinase is not supported by any experimental evidence, and can therefore be discounted.

Mannoheptulose differs from mannopyranose in having

a hydroxymethyl group substituted on PC1, when it is in the pyranose form. Mannoheptulofuranose differs similarly from tagatofuranose. In the latter case the hydroxymethyl group is on PC6.



mannoheptulofuranose



tagatose

There has been no evidence reported on the effect of tagatose in the hexokinase reaction. Armstron and Armstrong (1934) did show that tagatose is not fermented by whole yeast, but as there is probably a stage prior to the hexokinase-catalyzed one in the utilization of carbohydrate by whole yeast (Sols, 1956; Burger et al., 1959), no conclusion can be drawn from this fact concerning the fate of tagatose in the isolated hexokinase system. Mannose on the other hand, is a well known substrate of hexokinase, and if the mannoheptulose molecule is attached to the enzyme in the -pyranose form, it is easy to see how the hydroxymethyl group on PC1 can sterically hinder the access of ATP to PC6 where phosphorylation would take place.

In 2-C-hydroxymethylglucose, the hydroxymethyl group probably has a steric effect similar to that postulated for PC1 in the case of mannoheptulose.

Xylose and 6-deoxy-6-fluoroglucose in the pyranose form have no potentially active group. This is the only difference between these compounds and glucopyranose, and is understandably sufficient to render them inactive as substrates, while the relatively slight changes in them do not prevent them from being attached to the enzyme and thereby inhibiting the utilization of other sugars.

The affect of N-acetylation in glucosamine is evidently insufficient to prevent complex formation with the enzyme, but in that complex, the bulky acetyl group lying between the enzyme and carbohydrate moieties must induce some strain into the complex and thereby prevent phosphorylation.

The effect of fructosamine is harder to explain since insufficient evidence of its properties has been collected. The non-phosphorylation of this compound was demonstrated using both the spectrophotometric indicator and manometric assay methods. The former is normally the more sensitive method of the two, but in the presence of a strongly buffering compound its sensitivity is decreased. A titration curve of this amino-sugar showed that, in the region of pH where the spectrophotometric assay was operating, the buffering action was minimal. In the fully buffered manometric method, which is relatively insensitive, no substrate or inhibitory activity was demonstrable, but

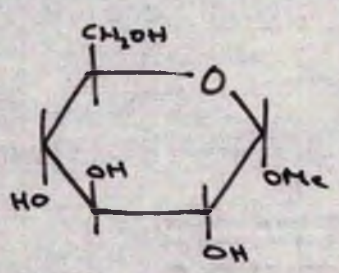
results presented in this thesis show, with the spectrophotometric method, that fructosamine inhibits the hexokinase reaction.

Kuhn *et al.* (1958) have shown that some N-substituted fructosamines exist in the pyranose form in solution, and infra-red spectral evidence shows that the pyranose form is more likely for the crystalline state of fructosamine acetate as well. In the pyranose form, however, the fructosamine molecule is very different from the glucopyranose molecule; configurations of, or substituents on, PC1, PC2, PC3, and PC5 being altered. In the furanose form, for which there is as yet no experimental evidence, fructosamine differs from fructofuranose at PC1 only, and it is in the latter form that it would be expected to exert any effect on the hexokinase reaction. The only recorded evidence of any biological effect of fructosamine is the fact that it is more inhibitory towards fructose fermentation than towards glucose fermentation by whole yeast (Woodward, Cramer and Hudson, 1953). For the reason given before, no analogy can be drawn between fermentation by the whole cell and phosphorylation by hexokinase.

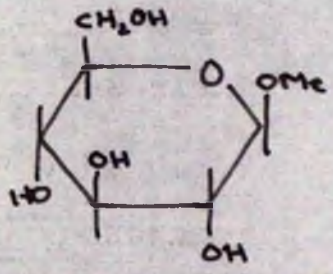
All the other carbohydrates that have been tested in the hexokinase reaction appear to have neither substrate nor inhibitory activity. These compounds will be discussed next in the light of how their structures might

be considered as modifications of those of known substrates and inhibitors.

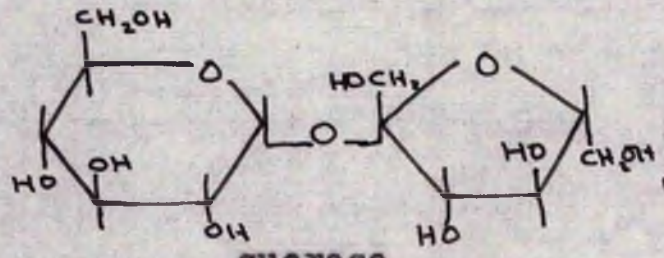
It has been shown that a certain amount of toleration exists in hexokinase towards modification at PC1 in its substrates. The enzyme will phosphorylate α- and β-glucose and 1,5-sorbitan. A slightly greater variation at PC1, as in mannoheptulose, destroys the substrate activity of the basic molecule, although the latter still retains its ability to form a complex with the enzyme. Other compounds, which differ to a greater or less degree from known substrates at PC1 only, and which have no substrate or inhibitory activity, are:-



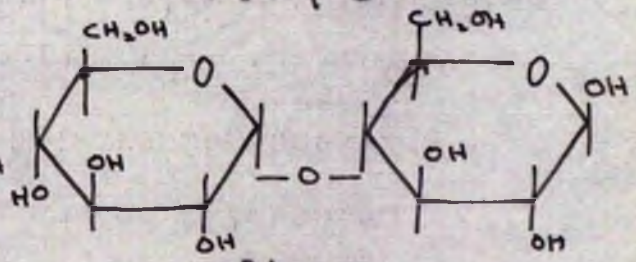
methyl α-glucoside



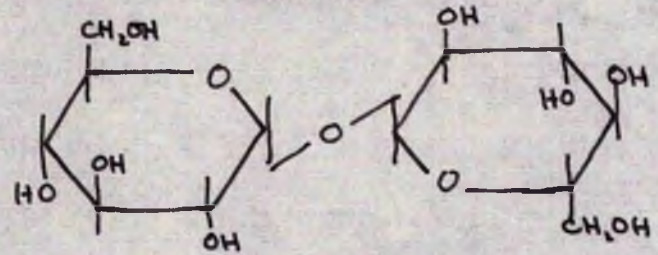
methyl β-glucoside



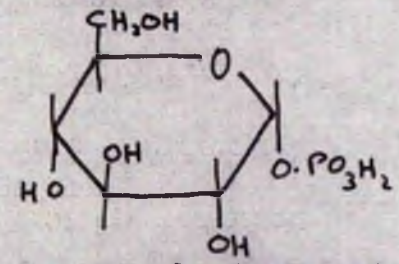
sucrose



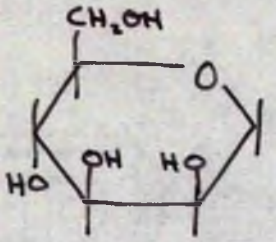
maltose



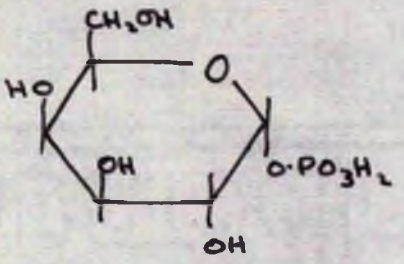
trehalose



glucose 1-phosphate



1,5-mannitan

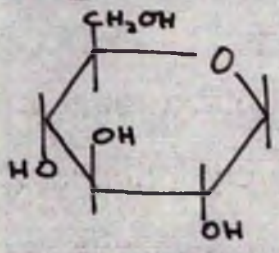


galactose 1-phosphate

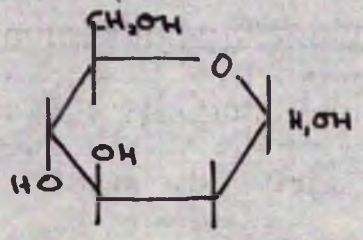
It can be deduced that the hydroxyl group on PC1 in glucopyranose is probably not essential for binding purposes, and that the modifications at this point leading to inhibition by, or inertness of, the sugars, bring about these effects through steric hindrance. there is a slight anomaly in that the methyl glucosides are inert, while the substitution of a bulkier group at PC1, as in mannoheptulose, does not destroy the sugar's binding properties. The answer to this may lie in the relatively non-polar nature of the methyl groups in the glucosides compared with the hydroxyl or even the hydroxymethyl group on PC1 in mannoheptulose.

The tolerance of hexokinase towards variations on PC2 appears to be much greater than it is towards variations on PC1. In fact, no compound, which differs from glucopyranose at PC2 only, has been found to be inert in the hexokinase reaction. Mannose, glucosone, glucosamine, and 2-deoxyglucose are all substrates, while N-acetylglucosamine and 2-C-hydroxymethylglucose are inhibitors.

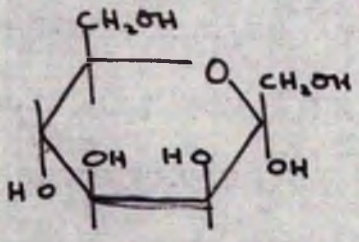
As in the case of PC1, PC2 does not appear to be involved in binding the sugar to the enzyme. It would be interesting to learn the effect of 2-O-methylglucose in a purified hexokinase system, but it might be predicted to be inert. This prediction can be made after drawing an analogy from the effects that the different substituents at PC1 have made in the hexokinase reaction. 1-Deoxyglucose (1,5-sorbitan) and 2-deoxyglucose are both substrates: 1-C-hydroxymethylglucose (mannoheptulose) and 2-C-hydroxymethylglucose are both inhibitors: 1-O-methylglucose (α- and β-glucosides) is inert.



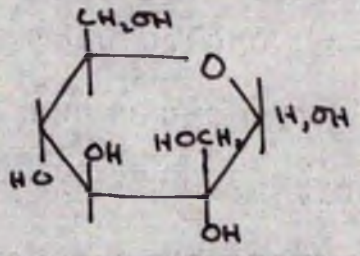
1,5-sorbitan



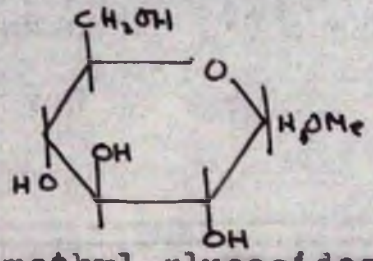
2-deoxyglucose ← substrates



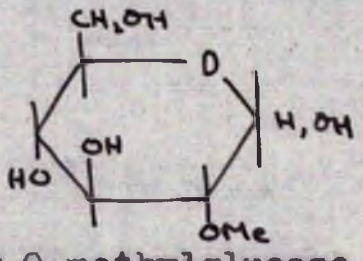
mannoheptulose



2-C-hydroxymethylglucose ← inhibitors



methyl glucosides

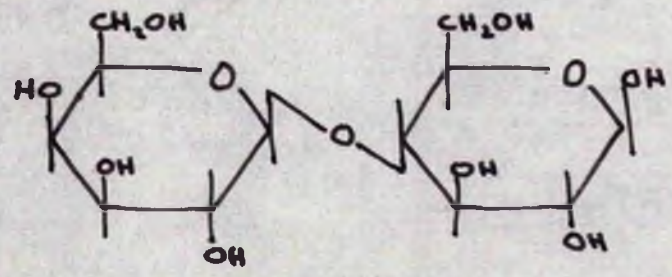


2-O-methylglucose ← inert (?)

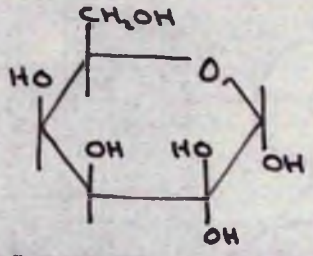
Mitchell (1954) failed to find any substrate activity in either 1,5-mannitan or 1,5-sorbitan, but the latter was shown later (Sols *et al.*, 1958) to be phosphorylated at a rather low rate. Had Sols and his co-workers used their more sensitive experimental procedures to investigate the effect of hexokinase on 1,5-mannitan it is probable that they would have found that it too is phosphorylated, since the mannitan bears the same relationship to mannose that the sorbitan does to glucose.

Allose and 3-O-methylglucose are the only PC3 analogues of glucopyranose that have been tested for hexokinase activity. The former is a substrate and the latter is inert. Here again is evidence that a polar group is necessary for bonding purposes.

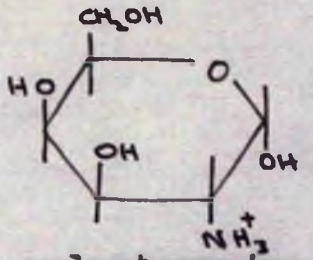
Galactose exhibits a low degree of substrate activity with hexokinase; so low that early workers in the field were not able to demonstrate it. Maltose and lactose, which can be considered as PC4-substituted glucopyranoses, are inert; as also are galactosone and galactosamine respectively.



lactose



galactosone hydrate

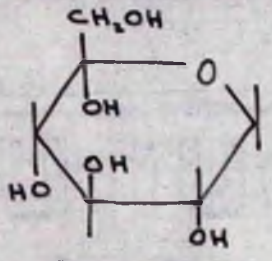


galactosamine

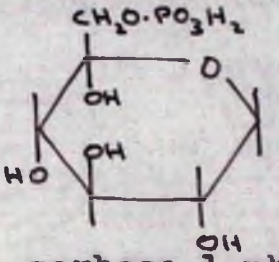
Taking PC5 and PC6 together, any change at these two points removes substrate activity completely. Xylose and 6-deoxy-6-fluoroglucose have been described as showing inhibitory activity, and the latter has also been reported as inert in the hexokinase reaction.

Glucosone phosphate (which is probably a 6-phosphate), 6-O-methylglucose, glucose 6-phosphite, and mannose 6-phosphate have all been described as inert with regard to hexokinase. Glucose 6-phosphate has already been discussed.

In the pyranose form, L-sorbose can be considered as an analogue of glucopyranose (modifications at PC1 and PC5), and both it and L-sorbose 1-phosphate are inert.



L-sorbose

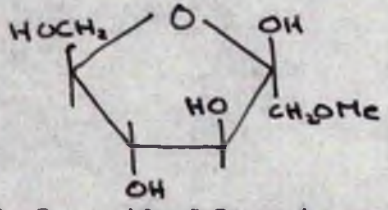


L-sorbose 1-phosphate

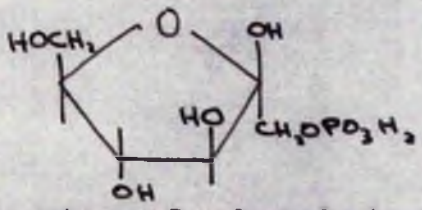
Hexokinase appears to have a much higher degree of specificity with regard to possible furanose substrates and inhibitors than it does to pyranose ones. This may

be due to the fact that not quite so many furanose as pyranose compounds have been tested in the reaction it catalyzes.

Alteration of FCl in β-fructofuranose leaves, in certain cases, complexing ability with hexokinase and in other cases, removes it. 1-Deoxyfructose and arabinose are substrates (the latter has also been claimed to be inert), fructosamine is an inhibitor, and 1-O-methyl-fructose, fructose 1-phosphate and sucrose are inert.

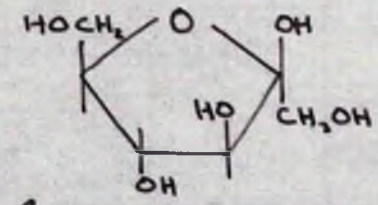


1-O-methylfructose

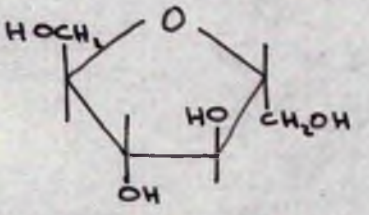


fructose 1-phosphate

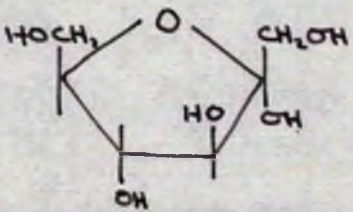
Structurally, 2,5-mannitan can be considered as 2-deoxy-β-fructofuranose and 2,5-sorbitan as 2-deoxy-α-fructofuranose.



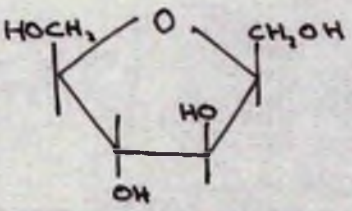
β-fructofuranose



2,5-mannitan



α-fructofuranose

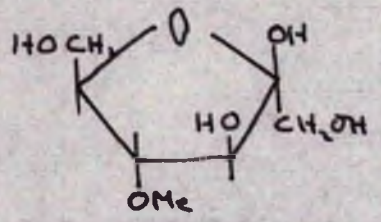


2,5-sorbitan

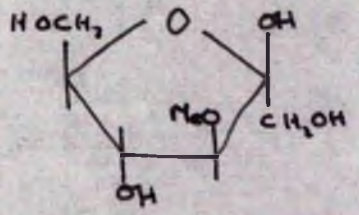
2,5-Mannitan is a substrate of hexokinase and appears to inhibit phosphorylation of itself. 2,5-Sorbitan, in contrast with its activity as a substrate of brain hexokinase, is inert. This result affords some indirect confirmation of the supposition that fructose is phosphorylated in the β-furanose form. Inspection of their respective structural formulae shows this.

The inhibition by substrate in single-substrate reactions has been observed in other enzyme systems, e.g. cholinesterase (Augustinsson, 1949), acetylcholinesterase (Wilson and Bergmann, 1950), urease (Laidler and Hoare, 1949), fumarase (Alberty et al., 1954) and carboxypeptidase (Lumry et al., 1951). An explanation of this phenomenon, as far as hydrolyitic enzymes go, is that reaction normally occurs between a substrate molecule and a neighbouring bound water molecule; this water molecule may be displaced by substrate at higher concentrations, but reaction may still occur, at a diminished rate, between bound substrate and unbound water. To adapt this explanation to the case of hexokinase and 2,5-mannitan, it will have to be assumed that it is ATP and not water molecules that are displaced from the enzyme surface.

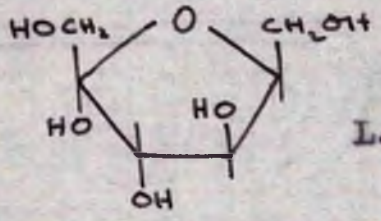
The following derivatives of fructose have no action on the hexokinase reaction:-



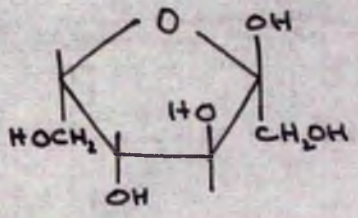
4-O-methylfructose

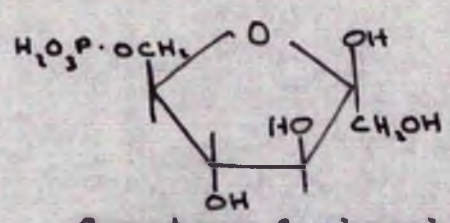


3-O-methylfructose



L-sorbose





fructose 6-phosphate

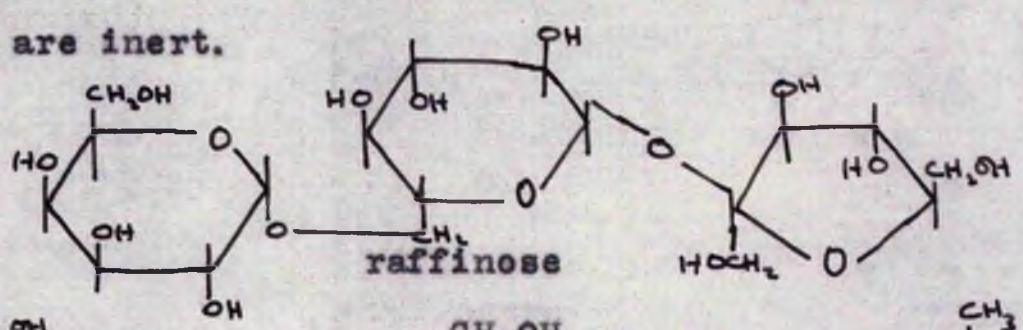
The slight degree of inhibition found by Wildy (1953) for 3-O-methylfructose was not confirmed by Sols et al. (1958). However, as in the case of the O-methyl derivatives of glucose, the inertness of the O-methylfructose does not necessarily mean that the particular hydroxyl groups that are blocked by alkyl substitution are essential for bonding purposes.

In the furanose form, L-sorbose can be considered as an analogue of fructofuranose, differing from it either at FC5 or ('upside-down') at FC2 and FC5. L-sorbose 1-phosphate which is also inert, can be considered similarly as an analogue either of fructose 1-phosphate

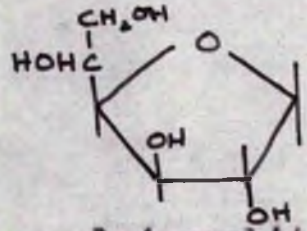
or of fructose 6-phosphate.

As with the products of the reaction of hexokinase with its pyranose substrates, fructose 6-phosphate does not inhibit phosphorylations by hexokinase.

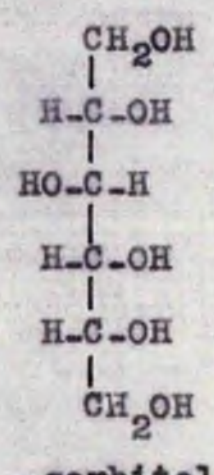
The following miscellaneous compounds have also been tested for activity in the hexokinase reaction and all of them are inert.



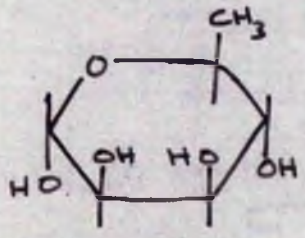
raffinose



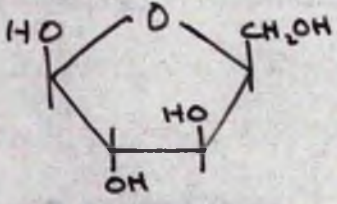
1,4-sorbitan



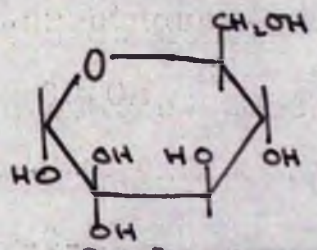
sorbitol



L-rhamnose



L-arabinose



L-glucosone

The inertness of raffinose, a PCl-substituted galactose or else a sucrose derivative, is easily understood, since galactose itself shows a very low level of activity in this reaction and sucrose is inert. 1,4-Sorbitan is so very different from fructofuranose that it is not surprising that hexokinase does not react

with it.

Sorbitol, a straight chain derivative of fructose and glucose, emphasizes the importance of a ring structure in the substrates and inhibitors of hexokinase.

Hexokinase, in common with most other enzymes, exhibits stereospecificity. L-Glucosone and L-arabinose are both inert, whereas their enantiomorphs are both substrates. L-Rhamnose (6-deoxy-L-mannose) is also inert.

Not enough evidence has been accumulated on the effects of fructofuranose analogues in the hexokinase reaction to make any wide generalizations concerning the specificity of the enzyme towards this type of compound. An investigation into the effects of hexokinase on 3-deoxy-, 4-deoxy- and 6-deoxyfructofuranose would help to throw some light on this aspect of the problem.

The present investigation, to sum up, has added to the knowledge of the substrate specificity of hexokinase with regard to its substrates which are fructose analogues. Two new substrates have been synthesized chemically and their substrate activity demonstrated with a purified preparation of the enzyme. One of these substrates exhibits the so far unique (for hexokinase) characteristic of substrate self-inhibition. A third analogue has been tested in the hexokinase reaction and inhibition of the

latter demonstrated, while a fourth compound is inert in it. These results have been shown to fit in with other information on hexokinase specificity.

4. EXPERIMENTAL DETAILS.

1. Investigation of the assay method.

(a) Absorption spectra of bromothymol blue.

The absorption spectra of bromothymol blue in acidic and basic solution was obtained from mixtures of 0.015 % (1 ml) aqueous bromothymol blue with 0.1 N sulphuric acid (10 ml) and with 0.1 N sodium hydroxide (10ml) respectively, using a Unicam SP 500 spectrophotometer.

(b) pH Dependence of the optical absorption of bromothymol blue.

Successive additions of a M/15 Na_2HPO_4 solution containing 0.0163 g/l bromothymol blue were made to a M/15 KH_2PO_4 solution containing the same concentration of indicator. The pH and optical density at 615 mu of the mixture were measured after each addition.

(c) K_m measurements.

The test system contained the following constituents (the concentration values in brackets refer to the final concentration of each constituent after dilution to 9.2 ml).

$MgCl_2$ . . . . . 1 ml, 0.046 M (0.005 M)

ATP . . . . . 1 ml, 0.025 M (0.0027 M)

bromothymol blue . . . . . 1 ml, 0.015 % (0.00163 %)

3. Preparation of purified hexokinase.

The enzyme was prepared from bakers' yeast by DCL. The procedure employed was that of Ber (1946), as modified by Gamble and Najjar (1955). enzyme solution obtained was divided into 2 ml sealed in glass ampoules and stored at -30°. Be the solution was diluted 50 times.

The concentrated solution retained virtuall its activity after being kept for 1 week at 0-5° fresh diluted sample was made up each day. The tion was free of any adenosine triphosphatase a

water and substrate solution . . . . . 6 ml.

hexokinase solution . . . . . 0.2 ml.

All the constituents, excluding the enzyme, were mixed together in a colorimeter tube. The initial optical densities of all tubes in a series were adjusted to the same value in an EEL colorimeter using 2 N sulphuric acid and sodium hydroxide solutions.

The enzyme solution was then added to the mixture, the tube inverted to ensure thorough mixing and a sample transferred to a spectrophotometer cell. Readings of optical density were taken in a Unicam SP 500 spectrophotometer at intervals for as long as was required. The optical density readings were plotted against time, an extrapolation made to zero time and the change in optical density found for the first five minutes. The corresponding change in hydrogen ion concentration was found from the calibration graph (Plate 11b).

2. <u>The effect of the fructose analogues on the hexokinase reaction</u>.

The procedure used in these experiments is that described in 4.1(c) above.

SUMMARY

1. The effect of hexokinase on four analogues of fructose is reported, and the substrate specificity of the enzyme is discussed. Other properties of these analogues are investigated.

2. 1-Amino-1-deoxyfructose is shown to inhibit the hexokinase reaction. The amino-sugar is shown to react with ninhydrin to form glucosone.

3. The synthesis of 1-deoxyfrucose is described. It is shown to be phosphorylated by hexokinase ($K_m = 1.09 \times 10^{-2}$) and to inhibit the phosphorylation of fructose by this enzyme.

4. 2,5-Anhydrosorbitol is shown to be inert in the hexokinase reaction.

5. The synthesis of 2,5-anhydromannitol is described. It is shown to be phosphorylated by hexokinase, and to inhibit phosphorylation of itself.

6. A systematic investigation of the spectrophotometric indicator assay method for following hexokinase phosphorylations is described.

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APPENDIX

TABLES I to XIV

TABLE I. Data for Plate 3.

A 0.01 M solution of fructosamine (25 ml) titrated successively with hydrochloric acid and sodium hydroxide. Additions expressed in terms of µl 1.009 N acid or base.

µl HCl	pH	µl HCl	pH	µl NaOH	pH	µl NaOH	pH
0	6.59	164	3.98	150	4.83	370	8.86
5.5	6.18	175	3.80	160	4.94	390	9.06
8.2	6.04	186	3.58	170	5.04	410	9.30
10.9	5.86	196	3.29	180	5.17	420	9.44
13.6	5.82	207	3.12	190	5.29	430	9.61
16.4	5.75	µl NaOH		200	5.45	440	9.84
21.8	5.60	0	2.81	210	5.66	450	10.19
27.3	5.49	20	3.20	220	5.99	460	10.61
32.7	5.40	30	3.46	230	6.62	470	10.87
38.4	5.31	40	3.68	240	7.24	480	11.03
43.6	5.24	50	3.86	250	7.58	490	11.15
54.5	5.11	60	4.00	260	7.77	500	11.24
65.5	4.99	70	4.12	270	7.94	510	11.31
76.3	4.88	80	4.22	280	8.06	530	11.42
87.2	4.77	90	4.32	290	8.17	550	11.51
109	4.57	100	4.41	300	8.26	580	11.60
120	4.48	110	4.50	310	8.36	630	11.72
131	4.36	120	4.59	320	8.44	700	11.82
142	4.24	130	4.67	330	8.54		
153	4.12	140	4.76	350	8.70		

TABLE II. Data for Plate 10.

Absorption spectra of bromothymol blue in (A) alkaline and (B) acid solutions. (A) 0.015 % in 0.1 N NaOH. (B) 0.015 % in 0.1 N H_2SO_4.

mμ	optical density A	B	mμ	Optical density A	B	mμ	Optical density A	B
320	.255	.174	480	.088	.232	610	.910	.001
330	.160	.188	490	.121	.180	615	.930	-
340	.120	.188	500	.150	.142	620	.920	.002
350	.132	.186	510	.193	.104	625	.890	.-
360	.153	.187	520	.232	.070	630	.890	.002
370	.175	.205	530	.285	.050	640	.660	.001
380	.203	.244	540	.346	.038	650	.460	.001
390	.213	.274	550	.410	.023	660	.289	.001
400	.208	.313	560	.488	.013	670	.167	.001
410	.276	.354	570	.573	.008	680	.092	.001
420	.131	.380	580	.680	.009	690	.058	.002
430	.090	.398	585	.699	-	700	.023	.001
440	.063	.394	590	.750	.004	720	.010	-
450	.052	.368	595	.800	-	750	.004	-
460	.057	.335	600	.850	.004	800	.003	-
470	.070	.284	605	.890	-	-	-	-

TABLE III. Data for Plates 11a, 11b & 12.

M/15 KH_2PO_4 containing 0.0163 g/l bromothymol blue and M/15 Na_2HPO_4 containing 0.0163 g/l bromothymol blue mixed in varying proportions.

O.D.	1/O.D.	pH	$[H^+]x10^{+8}$	O.D.	1/O.D.	pH	$[H^+]x10^{+8}$
.000	-	4.42	-	.327	3.06	6.70	20.0
.003	-	4.80	-	.319	3.14	6.72	19.1
.013	-	5.32	-	.354	2.83	6.76	17.4
.034	-	5.74	-	.392	2.55	6.82	15.1
.048	-	5.80	-	.453	2.21	6.85	14.1
.070	-	5.92	-	.422	2.37	6.89	12.9
.079	-	6.03	-	.457	2.19	6.91	12.3
.100	-	6.12	-	.462	2.17	6.95	11.2
.116	-	6.28	-	.500	2.00	7.00	10.0
.160	-	6.33	-	.545	1.84	7.05	8 .91
.186	-	6.40	-	.575	1.74	7.12	7.59
.200	5.00	6.44	-	.620	1.61	7.16	6.92
.215	4.65	6.48	-	.600	1.67	7.16	6.92
.221	4.53	6.49	32.4	.610	1.64	7.17	6.76
.236	4.24	6.52	30.2	.645	1.55	7.24	5.75
.245	4.08	6.54	28.8	.705	1.42	7.32	-
.263	3.80	6.58	26.3	.735	1.36	7.40	-
.282	3.55	6.62	24.0	.760	1.32	7.42	-
.290	3.45	6.63	23.4	.793	1.26	7.51	-
.322	3.11	6.68	20.9	.880	1.14	7.74	-

TABLE IV. Data for Plate 13.

Enzyme solution	0.2 ml
Bromothymol blue	0.00163 %
$MgCl_2$	0.005 M
ATP	0.0027 M
Water	To 9.2 ml

Mannose μMoles	Initial $[H^+]$ $\times 10^8$	$[H^+]$ after 5 mins $\times 10^8$	$\Delta[H^+]$ $\times 10^8$	$\Delta[H^+]$-blank $\times 10^8$
0	14.4	18.2	3.8	0
0.8	13 5	17.5	4.0	0.2
1.0	13.8	19.6	5.8	2.0
1.4	15.9	23.4	7.5	3.7
2.0	14.2	22.6	8.4	4.6
10	13.8	22.1	8.3	4.5
20	13.9	23.1	9.2	5.4
0	14.2	15.9	1.7	0
4.0	13.7	20.0	6.3	4.6
6.0	14.0	20.0	6.0	4.3
14	12.9	19.1	6.2	4.5
28	15.0	21.5	6.5	4.8
32	13.4	19.1	5.7	4.0

TABLE V. Data for Plate 14a.

Bromothymol blue 0.00163 %

$MgCl_2$ 0.005 M

ATP 0.0027 M

Enzyme solution ml	0.2					1.0		
Glucose µMoles	0	0		500		0	0	
Fructosamine µMoles	0	500		0		0	500	
Time (mins)	$\Delta[H^+]$	$\Delta[H^+]$	a	$\Delta[H^+]$	a	$\Delta[H^+]$	$\Delta[H^+]$	a
0	0	0	0	0	0	0	0	0
1	0	2	2	7	7	5	7	2
1.5	2	3	1	9	7	17	17	0
2	3	5	2	13	10	22	20	0
2.5	3	6	3	16	13	27	27	0
3	4	7	3	19	15	33	32	0
4	5	8	3	24	19	37	38	1
5	7	9	2	31	24	39	39	0
7	9	-	-	44	37	-	38	0
9	10	12	2	-	-	-	-	-
11	-	-	-	74	64	-	-	-
12	-	-	-	-	-	40	-	-
13	11	13	2	-	-	-	40	0
14	-	-	-	97	86	-	-	-
17	12	14	2	118	106	40	40	-

$\Delta[H^+]$ expressed as equivalents/l x 10^8.

Columns headed with 'a' are the blank-corrected values.

TABLE VI. Data for Plate 14b.

Bromothymol blue 0.00163 %

$MgCl_2$ 0.005 M

ATP 0.0027 M

Enzyme solution 0.2 ml

Glucose (μMoles)	500	0	0
Fructose (μMoles)	0	500	0
Fructosamine (μMoles)	0	0	500
Time (mins)	$\Delta[H^+]x10^8$	$\Delta[H^+]x10^8$	$\Delta[H^+]x10^8$
0	0	0	0
0.75	2	5	1
1	-	6	1
1.25	5	-	-
1.75	-	10	1
2.	6	11	0
2.5	-	13	0
3	8	16	0
4	11	20	-
5	15	23	1
10	26	35	2

TABLE VII. Data for Plate 15.

Bromethymol blue 0.00163 %

$MgCl_2$ 0.005 M

ATP 0.0027 M

Enzyme solution 0.2 ml

Glucose µMoles	0	50	50	50	50	50	50
Fructosamine µMoles	0	0	0	50	50	100	100
Time (mins)	$\Delta[H^+]$	$\Delta[H^+]$	$\Delta[H^+]$	$\Delta[H^+]$	$\Delta[H^+]$	$\Delta[H^+]$	$\Delta[H^+]$
0	0	0	0	0	0	0	0
0.5	1	8	7	7	4	5	6
1	0	14	13	11	11	10	8
2	3	24	21	13	-	13	15
2.5	3	-	28	20	25	-	21
3	4	42	35	30	29	19	27
4	5	52	51	38	41	32	31
5	7	64	59	50	50	45	-
6	8	-	-	-	59	-	-
7	9	-	-	70	-	-	65
8	9	-	-	-	80	-	-
9	-	123	118	-	-	79	-
10	10	-	-	104	-	-	86
11	-	-	-	-	108	-	-
13	11	-	-	-	-	-	110
15	-	-	-	136	-	121	-
17	12	199	205	-	-	-	-
20	13	229	231	179	180	148	161

TABLE VIII. Data for Plate 16a

Enzyme solution	0.75 ml.
$NaHCO_3$	0.025 M
$MgCl_2$	0.010 M
ATP	0.005 M

Glucose µMoles	50	-	-	-
Fructose µMoles	-	50	-	-
Fructosamine µMoles	-	-	50	-
Time (mins)		µl CO_2		
1	8	7	8	5
2	20	19	10	12
3	28	30	11	16
4	34	37	8	18
6	49	56	10	23
8	57	69	17	21
10	63	76	19	23
15	65	80	29	23
20	65	82	23	23
25	73	86	27	25
30	75	84	27	25
40	77	82	25	23

TABLE IX. Data for Plate 16b.

Enzyme solution 0.75 ml

$NaHCO_3$ 0.025 M

$MgCl_2$ 0.010 M

ATP 0.005 M

Fructose µMoles	100	50	50	-	-
Fructosamine µMoles	-	50	50	100	-
Time (mins)			µl CO_2		
2	41	33	30	21	12
3	56	49	46	23	15
4	74	66	63	25	18
5	82	75	72	23	22
6	93	88	85	27	21
7	104	102	99	23	22
10	123	124	130	25	23
15	128	126	137	25	22
20	138	128	139	27	23

TABLE X. Data for Plate 17a.

Bromothymol blue 0.00163 %

$MgCl_2$ 0.005 M

ATP 0.0027 M

Enzyme solution 0.2 ml

Fructose µMoles	200	200	-	-
1-Deoxyfructose µMoles	-	200	200	-
Time (mins)	$\Delta[H^+]$ (equivalents/l x 10^8)			
0.5	7	6	4	2
1	15	12	8	4
1.5	22	19	10	6
2	27	-	15	7
2.5	32	27	17	8
3	35	32	19	10
3.5	42	35	22	11
4	44	40	25	12
4.5	48	43	29	13
5	51	46	31	14
5.5	54	50	35	15
6	58	52	38	-
8	69	67	47	20
10	85	73	54	21
13	103	86	70	22
16.5	122	106	82	22
20	140	123	95	23

TABLE XI. Data for Plate17b.

Enzyme solution	0.2 ml
Bromothymol blue	0.00163 %
$Mg\ Cl_2$	0.005 M
ATP	0.0027 M
Water	To 9.2 ml

1-Deoxyfructose µMoles	$\Delta[H^+]$ for 5 mins $\times 10^8$	$\Delta[H^+]$-Blank $\times 10^8$
0	9	0
10	11	2
20	12	3
60	20	11
93	21	12
100	25	16
186	30	21
279	38	29
372	35	26
465	38	29

TABLE XII. Data for Plate 18a.

Bromothymol blue 0.00163 %

$MgCl_2$ 0.005 M

ATP 0.0027 M

Enzyme solution 0.2 ml

Fructose (µMoles)	20	-	-
2,5-Mannitan (µMoles)	-	20	-
Time (mins)	$\Delta[H^+]$ (equivalents/l x 10^8)		
0.5	8	2	1
1	16	4	0
1.5	23	6	2
2	28	7	3
2.5	34	9	3
3	38	11	4
3.5	44	12	5
4	48	13	5
4.5	50	14	7
5	54	16	7
5.5	59	17	7
6	61	18	8
7	65	21	9
8	71	23	9
10	84	28	10
13	98	35	11
16.5	118	40	12
20	137	47	13

PLATE XIII. Data for Plate 18b.

Enzyme solution	0.2 ml
Bromothymol blue	0.00163 %
$MgCl_2$	0.005 M
ATP	0.0027 M
Water	To 9.2 ml

2,5-Mannitan µMoles	$\Delta[H^+]$ for 10mins equivalents/l $x10^8$
0	10
10	19
20	21
40	16
60	10
150	5

TABLE XIV. Data for Plate 19.

Bromothymol blue 0.00163 %

$MgCl_2$ 0.005 M

ATP 0.0027 M

Enzyme solution 0.2 ml

Fructose (µMoles)	20	0	0	0
2,5-Sorbitan (µMoles)	0	20	20	0
Time (mins)	$\Delta[H^+]$ equivalents/l x 10^8			
0.5	8	1	1	1
1	16	2	2	0
1.5	23	3	3	2
2	28	3	5	3
2.5	34	5	5	3
3	38	5	6	4
3.5	44	6	7	5
4	48	7	8	5
4.5	50	7	8	7
5	54	9	9	7
5.5	59	9	9	7
6	61	9	10	8
7	65	10	12	9
8	71	11	11	9
10	84	12	13	10
13	98	14	13	11
16.5	118	15	14	12
20	137	15	14	13