SELF-DECOMPOSITION OF [^{14}C]-CARBOHYDRATES

REACTIONS OF CARBOHYDRATES WITH INORGANIC OXY-ACIDS

A Thesis submitted by

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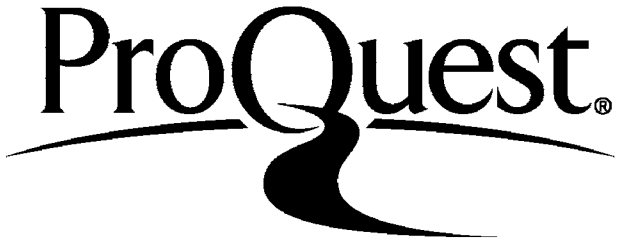

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ABSTRACT

PART I

A commercial sample of D-[^{14}C] glucose, from the Radiochemical Centre, Amersham, was found to have decomposed under the influence of its own β-radiation. The products of the decomposition have been examined by chromatography, ionophoresis and carrier dilution analysis, and found to be mainly oxidation products. The fact that the actual rate of self-decomposition was *ca.* ten times that of the theoretical rate suggested the participation of a secondary effect in the decomposition. A similarity between the products of self-decomposition and the products of hydroxyl radical attack on D-glucose was noted. It was inferred that water in the D-[^{14}C] glucose sample enhanced self-decomposition by absorbing some of the β-radiation and yielding hydroxyl radicals *in situ*.

PART II

The products of the oxidation of D-glucitol with a deficiency of periodate were identified and measured.

The results were explained on a stereochemical basis assuming the formation of a 5-membered cyclic intermediate in the periodate reaction. Periodate oxidation of the glucitol-borate complex in solution at pH 10 was attempted and compared with a similar oxidation of glucitol in phosphate buffer. The increased yield of L-xylose in the borate buffered oxidation was explained by the complexing of borate with glucitol. Certain anomalous results in both borate and phosphate were noted and are discussed.

The complexing of molybdate with carbohydrates has been examined with a view to elucidating the structure of complexes and utilising the complexes. The results of (mainly) ionophoresis experiments have shown that the pyranose ring will complex with molybdate only if it possesses three hydroxyl groups in a cis-cis-1:2:3-triol arrangement. Ionophoresis in molybdate solution has been found to be a valuable analytical method for carbohydrates, complementary with the borate method.

CONTENTS

PART I

THE SELF-DECOMPOSITION OF [^{14}C]-CARBOHYDRATES

THEORETICAL

INTRODUCTION

The increased production of radioactive compounds for use in chemistry and biochemistry has created its own problems. One of these is the decomposition of such compounds due to self-irradiation by the radioactive "labelling" atom. This work is concerned with the instability of sugar molecules which contain the radioactive ^{14}C isotope of carbon as well as the stable isotopes which are present in natural carbon, ^{12}C (abundance 98.9%) and ^{13}C (abundance 1.1%).

Carbon-14 is a long-lived isotope (half-life ca. 5000 years) which emits weak β-rays (max. energy 0.154 Mev.). It was thought originally that there was little probability of serious decomposition because the average energy of these β-particles is very low (50 Kev.). Experience, however, has shown otherwise.

Self-decomposition was first reported in 1953 from three American laboratories. Tolbert et al.[1], after storing methyl-labelled choline chloride (specific activity 13 μC/mg.) in evacuated tubes in the dark for one year, found that 63% of the material had decomposed.

Their data suggested that decomposition may proceed through some type of chain mechanism and that destruction of labelled compounds might be more severe than hitherto expected. This startling rate of decomposition is not general and it has subsequently been shown[2] that choline chloride is particularly susceptible to radiation damage. The same workers[1] have shown that calcium glycollate samples (C_1 and C_2 labelled - Sp. Activities 5.8 and 4.0 μC/mg. respectively) decompose to a lesser extent. Skraba et al.[3] examined the products of self-decomposition of [^{14}C] methanol by carrier dilution analysis. The specific activity of the methanol was very high (8-10 C/m.mole) and the storage time was 21 months. Considerable quantities of methane and hydrogen were evolved and the non-volatile products were ethylene glycol, glycerol and erythritol in the ratio 1360:15:1. They showed that all of these products could be formed by chain reactions, initiated by the free radicals formed in the reaction:

$$^{14}CH_3OH \rightsquigarrow H + {}^{14}\dot{C}H_2OH$$

Following these reports, Wagner and Guinn[4] calculated the expected amount of decomposition for [^{14}C] methyl iodide (0.12 mC/m.mole) in 3 years. The value was found to coincide with the experimental value, found from spectroscopic measurements of the wine-red iodine colour that developed in the liquid. A chain reaction was not apparent in this system. Lemmon[5] has reviewed the above American work. At the commencement of this work the only reference to the self-decomposition of carbohydrates was by Bacon and Allen[6] in 1956. These workers found that it was necessary to purify D-[^{14}C] glucose because 2% of its radioactivity migrated, during paper chromatography, in the disaccharide region, obscuring the disaccharides they were hoping to produce. The effect was not attributed to self-decomposition but this seems a most likely explanation.

The self-irradiation effect so far, then, can be divided into two parts:-

(a) The primary effect, that of direct destruction by β-rays.

(b) The secondary effect (which may or may

not be operative), that of a chain reaction initiated by the free radicals, or other entities, produced by the primary effect.

There is another reaction involved, part of (a), which is of theoretical importance. The carbon decay reaction to nitrogen causes destruction of the molecule in which it is situated. Wolfgang[7] found that in 50% of the decay reactions the nitrogen atom remained attached to an adjacent carbon atom. The extent of decomposition due to this effect may be readily calculated from the emissions per unit time and Avagadro's Number and amounts to

1.937×10^{-4} %/year/mC/m.mole

Attempts have been made to calculate the extent of the whole of the primary radiation effect. Wagner and Guinn's calculation[4] assumed that all the destruction is accomplished solely by ionization induced by electron impact; that ionization is always accompanied by permanent molecular destruction and that all the energy is absorbed by the labelled compound. A further assumption was that an ion pair of average energy 32.5 eV. (calculated from the

β particle energy of 51 KeV.) is formed. This ion pair would then destroy 1570 molecules in addition to the one containing the disintegrating atom. On this basis, a preparation containing 1mC/m.mole would destroy itself at the rate of 0.304% per year. As these workers state, decomposition could be enhanced by chain mechanisms and reduced by radical recombination. The calculation is really based on an ideal gas system and holds very well for their liquid methyl iodide system. Carbohydrates, however, are usually stored in the form of freeze-dried syrups. Under these conditions, most of the above assumptions are invalid. At the time of writing, Bayly and Weigel[8] are preparing for publication a more realistic approach (with respect to carbohydrates). Their results will be discussed in the theoretical section. They have also coined expressions for the various radiation effects. The ^{14}C decay effect is termed the "internal primary effect"; and the direct destructive action of β-rays, the "external primary effect".

The decomposition during storage of high specific activity preparations presents a serious problem. The value of samples increases with specific activity and hence the most valuable stocks possess the highest rate of decomposition. It is, therefore, in the interests of both the manufacturers and the buyer, that self-decomposition should be reduced in some way. Lemmon[5] has suggested the following methods:-

(a) The distribution of materials of low specific activities. This method is acceptable for many purposes especially now that low-level scintillation counting has been developed. The method cannot be generally satisfactory as high level activities are often necessary, for example, in biological experiments.

(b) Storage of materials in dilute solution. Solvents would have to be chosen with great care.

(c) Storage as an intimate admixture with an insoluble, inert substance.

The preservation methods envisaged by the Radiochemical Centre[9] include storage as a mixture with barium carbonate; storage in frozen solution; storage at -80°C and on dry paper. These methods are mostly concerned with trapping most of the radiation in material other than the sugar. At present, data supplied with each compound gives the date of preparation and analysis, and the expected level of self-decomposition.

The importance of radiation self-decomposition to the scientist is two-fold. Materials obtained from the Radiochemical Centre at great cost are known to be rigorously purified by ion-exchange resins and chromatography. The scientist, particularly the biologist, will reasonably assume that these tracer chemicals are pure when he comes to use them at a later date. This work has shown that in sugars (commonly used in biochemistry, etc.) the impurities in an old sample may be of the same order of magnitude as the amount of product which might be expected from the experiment in which the sample is used, for example, bacterial action on the sugar.

As previously inferred, the practical importance

of self-decomposition lies mainly in its nuisance value and the phenomenon has no special application at present. A study of the products of such a decomposition is of theoretical interest, however, because the effect of self-irradiation is quite likely to be similar, if not identical, to the effect of external irradiation with β-rays, cathode rays or even γ-rays. This possibility leads one into the field of radiation chemistry but excludes the need for a radiation source. It will be of value then to review the literature on the radiation chemistry of carbohydrates and other relevant compounds.

The field of radiation chemistry has flourished in recent years. The immediate reason for this is the construction of atomic piles, wherein substances may be irradiated. Reactors, while in action producing either "peaceful" energy or plutonium, also produce large quantities of highly radioactive isotopes as by-products. These by-products are useless as far as the working of the reactor is concerned, and have to be removed at intervals. Disposal of the waste presents a problem. It was soon realised, however, that these products formed a vast reserve of portable

energy. Subsequent to many departments acquiring sources from the U.K.A.E.R.E., a great effort has been made to harness the waste energy by making it promote chemical reactions. Reviews by Dainton[10], Collinson and Swallow[11] and Bourne, Stacey and Vaughan[12] cover the general aspects and trends of this work.

The examination of the radiation susceptibility of natural products is essential for other reasons. High energy radiation induces sterility and death in all forms of life and is therefore useful in the sterilization and preservations of food stuffs. This application is still in its infancy but encouraging results have been obtained on the irradiation of cheese, butter, meat and wheat, to mention but a few examples. The method introduces the problem of possible damage to foodstuffs as well as to the bacteria in them. For example, milk can be sterilized in this way but the accompanying changes in colour and taste render it quite unacceptable to the palate. This is an instance where knowledge of the radiation chemistry of lactose and protein is required. Another reason

for the interest in the damage of natural products is the susceptibility of the genetic processes in humans. Much work is being carried out on solutions of nucleic acids[13].

Most of the radiation chemistry of carbohydrates has been carried out in dilute aqueous solution. In this state, chemical change is invariably due to a secondary reaction of hydroxyl radicals with sugar, the hydroxyl radicals being produced in the initial reaction:

$$H_2O \xrightarrow{\sim\sim} H + {}^{\bullet}OH$$

The same type of decomposition would then be expected for any form of radiation used under these conditions. Work has been conducted using ultra-violet light, X-rays, cathode rays, accelerated electrons, pile radiation (a mixture) and γ-radiation. The latter is of great use now because one of the waste products mentioned above, cobalt-60, is a very powerful emitter of γ-rays. Some water was present in the samples investigated in this work, so that a review of the radiation chemistry of aqueous solutions

of carbohydrates is not out of place.

The radiation chemistry of water is well reported in the literature[14-18] because clearly it is fundamental to most other studies. Kelly[19] has used mixtures of water and heavy water to elucidate the mechanism of α breakdown. The initial reaction is the dissociation of water to a hydrogen atom and a hydroxyl radical. This is followed by numerous reactions of these radicals with water producing H_2O_2, HO_2 and oxygen. There appears to be no agreement on the precise sequence of secondary reactions. These are, however, probably of minor importance in the presence of a labile solute which will tend to react with OH radicals as they are formed.

The first reference to the irradiation of sugar solutions was by Kailan[20] who reported that glucose, unlike sucrose, was not inverted on irradiation. Holtz and Becker[21] irradiated solutions with X-rays. D-Glucose, in phosphate buffer (pH 7.6), produced a solution which reduced methylene blue and possessed an absorption maximum at 275-290mμ. Irradiation of glucose, fructose, arabinose, glycerol and

glucosamine solutions with U.V. light[22] also produced reducing solutions with absorption maxima at 265-290 mμ. This suggested that a readily enolised carbonyl group was formed (Haworth et al.[23]). These workers did not characterise the products of such irradiation. Peat and Whelan[24] examined the breakdown of amylose and allied carbohydrates during irradiation with ultra-violet light. The products obtained from oxygenated solution were found to differ from those obtained in an atmosphere of pure nitrogen. Phillips[25] irradiated D-glucose, D-galactose and D-mannose in dilute aqueous solution ($\lesssim 5 \times 10^{-2}$ M) with 1 MeV. electrons. Contrary to expectations, highly specific changes apparently resulted. The only detectable products were the hexuronic acids, attack by radicals being confined to C(6). Within the range of the concentrations studied (5×10^{-2} to 5×10^{-5} M) the yields of acids were independent of the concentrations, suggesting the indirect nature of the radiation effect. During the irradiation, a mixture of hydrogen (87%) and

carbon dioxide (16%), was evolved with a trace of carbon monoxide. (The evolution of CO_2 indicates a decarboxylation reaction hence a yield of 5-carbon product would be expected). He has recently repeated the above work and finds that the breakdown pattern suggested originally was quite erroneous (see Theoretical Section). The inversion of sucrose to glucose and fructose has been brought about by the β-irradiation of a 50% aqueous solution[26].

Reactions similar to this but outside of the carbohydrate field are of direct interest. The products obtained by the irradiation of aqueous solutions of α-hydroxy acids, such as lactic acid, depend to a large extent on the conditions under which the reactions are performed. Thus, radiation of oxygenated lactic acid solutions gave pyruvic acid as the major product[27], whereas large doses of γ-radiation in the absence of oxygen afforded mainly acetaldehyde[28]. Ethyl alcohol has been converted to acetaldehyde[13(i)].

$$CH_3.CH_2OH \longrightarrow CH_3.CHO$$

In the aldose irradiation, evidence for this stage is

not easily gained as the hexodialdose intermediate is probably unstable. In an attempt to prove the presence of this stage, Phillips[25] irradiated a mannitol solution and found that mannose was produced and that its conversion to mannuronic acid did not start until a fair concentration of aldose had been built up. Irradiation of solutions of nucleic acids[29] causes the chain to break at the ribofuranose residues which appear to be the most susceptible part of the molecule. A small amount of radiation damage in these and other polymeric compounds profoundly alters their physical properties. The literature on polysaccharide irradiation[30-34] deals mainly with physical changes due to polymer degradation. Bourne, Stacey and Vaughan[34], however, have studied acid production during the irradiation of amylose solutions and find two independent reactions:

(a) polymer degradation and

(b) acid production from the small units produced in (a).

At the commencement of this work, the only published information on the irradiation of solid

carbohydrates was by Coolidge[35] in 1925. The results may be summarised in his own words:- "Crystals of cane sugar turn white and, upon subsequent gentle heating, evolve considerable quantities of gas. An aqueous solution of cane sugar becomes acid to litmus upon being rayed". Colour changes on the γ-irradiation of solid sugars have been noted[28] but melting points and optical rotations indicated that extensive decomposition had not occurred with aldoses.

Towards the end of the present work, two papers appeared, one on the irradiation of solid sugars[36] and one on the aqueous solution irradiation of carbohydrates[37]. Very recently two more departments have published work on the irradiation of aldoses[38,39]; the results confirm those of the present investigation. They will be discussed later. The deterioration of [^{14}C]-labelled glucose on storage has been attributed recently to the presence of a foreign ion introduced during the preparation[40]. This idea will be discussed in the light of findings in the

theoretical section.

The present work stems from the synthesis of [^{14}C] dextran sulphate of high specific activity; this was required for tracer work in rats[41]. An inactive test preparation yielded stable material; [^{14}C] dextran sulphate (Sp. Activity 22.4 mC/g. atom of carbon), however, decomposed almost completely in ca. 3 weeks. The high decomposition rate was attributed to a secondary radiation effect arising from the prior liberation of sulphuric acid. At that time Dr. R. J. Bayly, of the Radiochemical Centre, Amersham, was investigating the problem from the manufacturer's point of view and supplied material for further research. This material was the residue from the paper chromatographic re-purification of [^{14}C] sucrose and [^{14}C] fructose. At this stage, the author would like to acknowledge the help of Dr. Bayly who made available his initial results on the decomposition of [^{14}C] sucrose and discussed developments freely. A memorandum of the [^{14}C] sucrose results is displayed (Fig. 1).

Figure 1

Radiation decomposition of [^{14}C] sucrose

This memorandum records some preliminary observations on the decomposition on storage of sucrose-C14.

Six samples of sucrose-C14 with various histories have been examined by paper chromatography in butanol-ethanol-water; the percentages of the total activity in the various spots are recorded below. In this connection the terms "sucrose", "glucose", and "fructose" refer to these sugars or substances with the same R_F values.

Histories

Tube	Batch	Method of sealing	Storage		Specific Activity (μc/mg)
			Temperature	Time(months)	
A	3	Normal	Normal	13	104
B	3	Vacuum	Normal	13	104
C	3	Vacuum	- 80°C	13	104
D	1	Normal	Normal	10	51
E	1	Normal	Normal	29	51
F	2	Normal	Normal	29 ≠	51

≠ This sample was a small one (10μc) resulting from a sub-division of Batch 1 after 9 months normal storage. It was therefore much more dispersed than the original batch.

Analyses

Spot	Tube						Original batch	
	A	B	C	D	E	F	3	1
Sucrose	81.9	84.1	89.7	95.4	86.8	93.2	98.9	99.8
Glucose	5.9	5.7	4.2	3.0	5.1	2.7	0.4	0.1
Fructose	3.1	2.6	1.6		2.4	1.3		
Backward trail	6.1	4.3	2.6	1.3	2.7	2.2	0.6	0.2
Forward streak	3.1	3.3	1.9	0.3	3.0	0.5	-	-

Note:- "Backward trail" is area from origin to sucrose spot.
"Forward streak" refers to activity which moves ahead of fructose.

Figure 1 (continued)

Conclusions

1. There is no evidence of a "specific activity barrier" in the range studied, though the production of slow-moving substances may be more than proportionately greater with the higher specific activity.

2. Ordinary vacuum sealing reduces decomposition slightly - particularly the production of slow-moving substances. (Note:- More recently, methods of vacuum sealing have been used which reduce the tendency to syrup formation).

3. Storage at -80°C (with vacuum sealing) significantly reduces decomposition - thereby demonstrating that all the decomposition observed is not primary radiation decomposition - but it does not eliminate it.

4. Dispersion without dilution reduces decomposition.

5. Glucose/Fructose ratio is approximately 2:1 in all cases.

27th February, 1956.

The work now described was undertaken with the above information available. A commercial sample of D-[^{14}C] glucose which had been stored in these laboratories for more than one year was also examined. Later some [^{14}C] sorbitol, on purification for other work, yielded a small quantity of decomposition product which was examined. An attempt has been made to establish the products and to form some idea of the mechanisms of degradations. Comparison with conventional chemical degradations with known mechanisms was also used to support theories.

EXAMINATION OF THE PRODUCTS OF SELF-DECOMPOSITION OF [^{14}C] SUCROSE AND [^{14}C] FRUCTOSE

Purified [^{14}C] sucrose and [^{14}C] fructose (specific activities, 3.0 and 4.3 mC/g.-atom of carbon, respectively) were found to undergo considerable self-decomposition during storage at the Radiochemical Centre, Amersham. The materials were stored as freeze-dried samples in vacuum sealed ampoules, in the dark at room temperature. Both compounds had to be repurified before distribution to customers as the level of impurity rendered them quite unacceptable for tracer purposes. The purification was effected by chromatography on thick paper. The sections of the chromatograms containing the impurities were sent to these laboratories for further examination. Of the fructose impurities, only those compounds which migrated slower than fructose during chromatography were available. On receipt, the degradation products were eluted from the paper with cold water, freeze-dried and stored at 0°C.

Radiochromatograms (Expts. 1 and 3) of the [^{14}C] sucrose products revealed twelve components (Expt. 8). Three of the components had R_G values identical with those of sucrose, glucose and fructose. The latter two were present in the ratio of ca. 1:3. This differed considerably from the ratio (2:1) found by Bayly (Fig. 1). A large amount of radioactivity remained on the origin line during chromatography and was thought to be polymeric.

Radiochromatograms of the fructose products revealed eight components (Expt. 9), including fructose, and a streak moving faster than fructose. Paper ionophoresis in phosphate buffer (in which only acidic compounds migrated) gave very similar results for both sets of products (Expts. 8 and 9).

The polymeric component of the [^{14}C] sucrose products was isolated by chromatography and subjected to an acidic hydrolysis (Expt. 10). A chromatographic analysis of the hydrolysate revealed some radioactivity on the origin line and a spot moving at the same rate as D-glucose. On this evidence, and the discrepancy between the values for the glucose: fructose ratio,

it is proposed that, since Bayly's analysis, much of the glucose had become incorporated in a glycosidic polymer. It seemed very unlikely that radiation could have accomplished the whole of this change and a more reasonable explanation was that bacterial contamination had occurred. Discussion revealed that, prior to being despatched, the papers containing the impurities had been exposed to the air at room temperature for ca. six weeks. The major premise in this work is that the compounds had decomposed under the influence of self-irradiation. There must obviously be no cause for doubt that radiation caused the decomposition. In view of the probability of bacterial contamination, neither the sucrose nor fructose degradation products were examined further.

PRELIMINARY EXAMINATION OF THE PRODUCTS OF SELF-DECOMPOSITION OF D-[^{14}C] GLUCOSE

A Description of the Sample Analysed

A commercial sample of generally labelled D-[^{14}C] glucose (481μC at a specific activity of 14.44 mC/m.mole) was stored in these laboratories for ca. 1 year. It was an ideal sample for research, being a representative "old" sample bought from the Radiochemical Centre. The precise history of the sample was obtained.[42] The material had been prepared by photosynthesis in a tobacco leaf fed with $^{14}CO_2$, extracted, purified by ion-exchange resins and chromatography and crystallised. After assay for radiochemical purity, it was dispensed in aqueous solution, into a glass ampoule, freeze-dried and sealed under vacuum. The ampoule had previously been treated with steam under pressure to remove any alkalinity from the glass. The preparation and analysis of this batch were completed in January 1956. The ampoule was stored in these laboratories, at room temperature, in the dark, until it was first opened in March, 1957.

A Discussion of Analytical Methods

Since the first part of the thesis is concerned mainly with the analysis of a very small quantity of a mixture of radioactive carbohydrate derivatives, the possible analytical methods will be reviewed.

The small quantity of material (ca. 6 mg.) was the factor which limited the number of methods available. If, for example, the D-[^{14}C]-glucose had decomposed to the extent of 17% in one year the total weight of decomposition products would have been 1 mg. The relatively high specific radioactivity of the mixture, however, made possible the application of a very powerful and sensitive tool - Isotope Dilution Analysis. This method, as originally applied,[43] consisted of the addition of a labelled compound of known specific activity to an aliquot part of a mixture of unknown composition. The compound was then isolated by normal chemical methods. If the compound added was also present in the mixture, the isolated material would be a representative sample of

the added compound and that present in the mixture. The per cent recovery was thus unimportant. The amount of compound originally in the mixture could then be calculated from the fall in specific activity. The degree of accuracy of the method was independent of the method of isolation and the yield of the isolated product. The degradation products of a sample of generally labelled D-[^{14}C] glucose necessarily possess the same specific activity (per g.atom of carbon) as one another and the parent compound. Hence, though the quantity of possible product was quite unknown, its specific activity was identical with the known value of the D-[^{14}C] glucose.

The analysis was carried out by the addition of an inactive carrier to the radioactive mixture, i.e. the reverse procedure to that of the original method. The correct terminology for the technique is "Reverse isotope dilution analysis", though the term "Carrier dilution analysis" is acceptable and will be used here. The term "Dilution analysis" will also refer to this technique. The essential

requirements for the full exploitation of the method are listed below.

(a) Some knowledge of the types of compounds present in the mixture.

(b) Pure carrier materials for dilution.

(c) Homogenous mixing of the carriers and radioactive components in all forms.

(d) A method of purification of the carriers after dilution.

Paper chromatography (Expt. 1) and paper ionophoresis (Expt. 2) proved to be the ideal methods for the tentative characterisation of compounds prior to the selection of carrier materials. The use of selective detecting agents was not possible owing to the very small quantities involved. Every carbon containing compound was radioactive, however, and could be detected by virtue of the fact that β-radiation darkens a photographic plate (Expt. 3). A very small quantity of radioactivity ($10^{-3}\mu$C) could be detected by a 24 hour exposure of a chromatogram against X-ray film.

The isolation of carrier compounds was effected by repeated recrystallisation when possible. Large scale chromatographic and ion-exchange resin separations were also used in conjunction with recrystallisation. The fact that the accuracy was independent of the yield of carrier was very valuable, as the yield often dropped to one tenth of its original value during several recrystallisations to constant specific activity.

Homogeneous mixing of carrier and component presented difficulty when the component existed in two or more forms in equilibrium. The relatively rapidly attained equilibrium between α- and β-D-glucose was found to cause no error. That between free acids and lactones, however, caused appreciable error when insufficient time was allowed for the equilibrium to establish itself. Dilution analyses for acids using the potassium salts as carriers gave rise to low values. The analyses were repeated successfully using the free acids as carriers.

Preliminary Qualitative and Quantitative Work and Results

In an analysis of a small amount of carbohydrate mixture it is essential to minimise the risk of bacterial action. For this reason, it was decided to sub-divide the sample of D-[^{14}C] glucose into smaller quantities which could conveniently be utilized as soon as each ampoule was opened. The bulk of the material, in this way, remained stored in a sterile state until required for use. This sub-division introduced complications with regard to the rate of self-decomposition. The geometry of the freeze-dried material which was then spread over a larger surface area was altered by sub-division. The amount of radiation absorbed by the material, and hence the rate of self-decomposition, would probably be reduced. This change, however, was preferable to having bacterial action on the sugar.

The D-[^{14}C] glucose was sub-divided into ten approximately equal portions (50μC), eight of which were stored in the freeze-dried state, vacuum-sealed, at room temperature (Expt. 11). A bacterial count on one portion (Expt. 12) revealed the presence of two

types of colony. A colony of glucose-fermenters (3900/mg.) appeared with a colony of non glucose-fermenters (2000/mg.). This count was rather high but in view of the similar values for both colonies it was considered that bacterial action was not extensive. Non glucose-fermenters could have multiplied by utilizing the products of glucose-fermenters or of radiation decomposition. These products have been shown to be distributed in approximately twenty 1% fractions and, therefore, such utilization is unlikely.[44] The physical state of the material was such that it did not favour the growth of most bacteria, as no salts, air or carbon dioxide were present in the syrup during storage. It is concluded that there was a possibility of some slight bacterial action but only during the time between radioassay and freeze-drying prior to despatch from the Radiochemical Centre.

The tenth portion of the sub-divided sample was used for chromatographic and ionophoretic analyses. The sample, when not in use, was stored in frozen

aqueous solution, at -20°C. A radiochromatogram prepared using an n-butanol solvent (Expt. 13) revealed twelve components (Fig. 2). A comparison with reference compounds indicated the possible presence of polymeric material, sugar acids (e.g. gluconic acid), arabinose, acid lactones, erythrose, 1.6 anhydro-β-D-glucopyranose and glyceraldehyde. Glucuronic acid did not appear to be present. Borate ionophoresis (Expt. 14) revealed nine components but a clear resolution was not possible due to the similarity of the M_G values (Fig. 3). The experiment, however, ruled out the possibility of 1.6 anhydro-β-D-glucopyranose being present as a reference sample migrated only by endosmotic flow and had no counterpart in the radioactive mixture. Phosphate ionophoresis (Expt. 14) indicated that most of the acidic impurity was similar in character to gluconic acid (Fig. 4). These analyses were repeated 24 months after the preparation of the D-[^{14}C] glucose and the distribution of radioactivity on the papers measured (Expt. 4). The results revealed that 14.5% of the

glucose had decomposed and 8.2% of the total activity (i.e. 56.5% of the impurity) was acidic. Hence the rate of decomposition of this sample was 7.2% per year.

This preliminary analysis showed that the sample was well worth further study. Carrier dilution analyses were carried out to make (a) an accurate estimation of the rate of decomposition and (b) a characterisation of some of the products and hence to formulate a breakdown mechanism.

Figure 2.

Radiochromatogram of the Products of Self-decomposition of D-[^{14}C] Glucose.

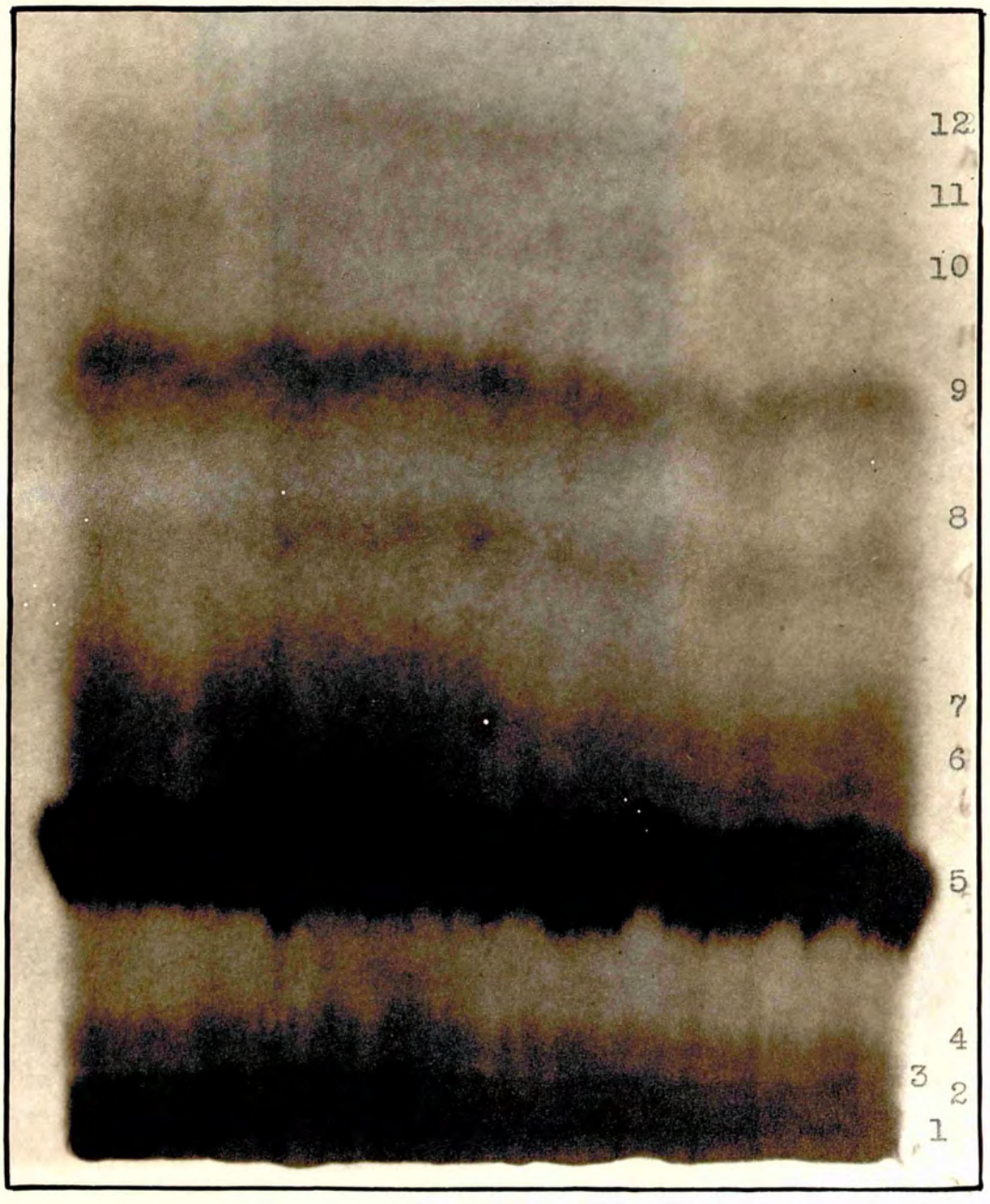


Figure 3.

Borate Ionophoresis of the Products of Self-decomposition of D-[^{14}C] Glucose.

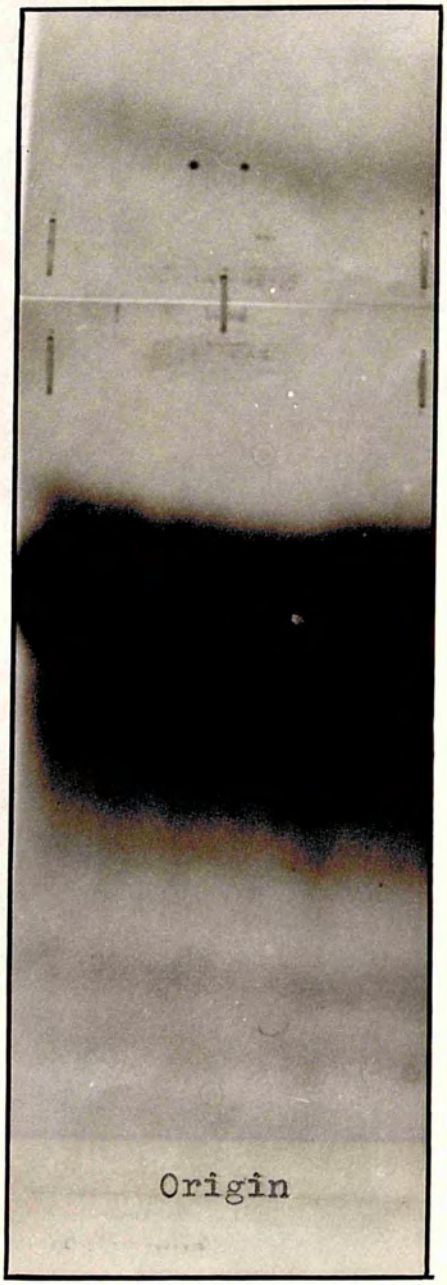
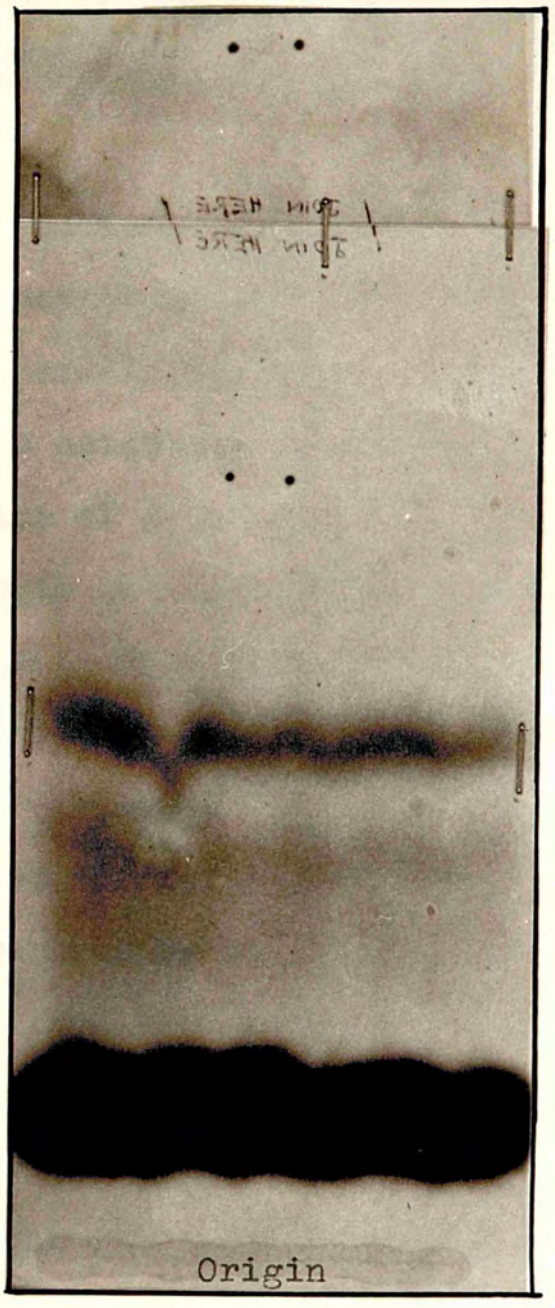


Figure 4.

Phosphate Ionophoresis of the Products of Self-decomposition of D-[^{14}C] Glucose.



CARRIER DILUTION ANALYSIS OF THE PRODUCTS OF SELF-DECOMPOSITION OF D-[^{14}C] GLUCOSE

Preliminary analysis had indicated the possible presence in the self-decomposition mixture of glucose, arabinose, erythrose, glyceraldehyde, aldo-hexonic acids and probably arabonic acid. A consideration of general radiation chemistry suggested that keto-aldoses and keto-acids may have been present. From a consideration of the D-glucose molecule, most of these products would be probably "D"-isomers. Some of these compounds were available for carrier dilution analysis. D-Arabonic acid was prepared by the bromine oxidation of D-arabinose. Analyses were made by dissolving a carrier compound in an aliquot part of the decomposed D-[^{14}C] glucose. The mixture was allowed to equilibrate for sufficient time and then the carrier isolated and purified to constant specific activity. The details of these dilutions are given in Expts. 15-23. Table 1 gives the results of the carrier dilution analysis and also compounds whose presence have been fairly firmly established by chromatography. The analysis for undegraded D-[^{14}C] glucose was made 26

months after preparation. Other compounds were estimated near this time. The low yields made a variation of 2 months of little consequence when calculating the rate of formation per year.

Table 1. The products of the self-decomposition of D-[^{14}C] glucose

Carrier Compound	Yield of [^{14}C] product from D-[^{14}C] glucose after 26 months (Weight per cent)	Rate of formation (% per year)
D-Glucose	79.95	9.25*
D-Arabinose	0.43	0.20
D-Erythrose	indicated by chromatography	-
D-Glyceraldehyde		-
D-Gluconic acid	0.62	0.29
2-keto-D-Gluconic acid	0.38	0.18
D-Glucuronic acid	< 0.10	< 0.05
D-Arabonic acid	0.07	0.03
Oxalic acid	< 0.0005	< 0.0002
D-Glucosone	negligible (by chromatography)	-
Polymeric material	ca. 1.0	ca. 0.5

* Rate of decomposition

VERIFICATION OF CARRIER DILUTION RESULTS AND FURTHER CHARACTERISATION OF COMPOUNDS IN THE SELF-DECOMPOSITION PRODUCT

The bands of a chromatogram of the type displayed in Fig. 2 (prepared using ca. 50μC of radioactivity) were cut out and eluted. Various experiments were carried out on the separate components, which were labelled from 1 to 12 (Fig. 2).

The "Polymeric" Component (No. 1)

The material which remained on the origin line of the chromatogram was purified from slow moving materials and subjected to sulphuric acid hydrolysis (Expt. 24). After neutralization of the hydrolysate with barium carbonate, no radioactivity could be detected in the filtrate (either by the film method or with a Geiger-Müller counter). A reasonable interpretation of this result is that either the polymer or the hydrolysate was acidic and precipitated by barium carbonate. Two dimensional chromatographic-phosphate ionophoretic analysis showed that component 1 was acidic. The long streak produced

indicated that the material varied in molecular size. An attempted hydrolysis using Amberlite IR-120 [H^+] acidic resin[45] failed completely.

The facts available suggested that the material is a polymer, probably non-glycosidic, with some acidic repeating units. It was of interest, therefore, when Barker et al.[46] recently reported that γ-irradiation of aqueous solutions of α-hydroxy and α-amino acids in vacuo produced acidic polymers, the structures of which are under investigation.

Further Chromatographic Analyses

The following chromatographic work is reported fully in Expt. 25. Components 2, 3 and 4 (Fig. 2) produced two spots each, on re-chromatogaphy. The faster running spots possessed the R_G values of D-gluconolactone and D-arabonolactone (i.e. those of components 8 and 9). Analysis of components 2 and 3 by phosphate ionophoresis gave spots with M_{GA} values of 1.0 and component 4 gave a spot migrating with D-arabonic acid (M_{GA}, 1.1). Fractions of components 8, 9 and 10 also migrated during phosphate ionophoresis, component 9 having the M_{GA} value of arabonic acid.

Chromatography of these components (8, 9 and 10) produced the original spots and also spots migrating with the R_G values of the acids. The evidence suggests that components 8, 9 and 10 are the lactones of the slow moving acids.

The glucose band (No. 5), on elution and chromatography in a phenol solvent, was found to be homogeneous. Thus the self-decomposition of D-[^{14}C] glucose yielded no detectable D-glucosone.[62]

Component 6 migrated with D-arabinose in several solvents including phenol, in which the usual order of arabinose and xylose is reversed. Component 9 represented a relatively large amount of radioactivity so an effort was made to resolve it further. Borate ionophoresis resolved it into 3 spots, two of which migrated with D-arabonic acid and D-erythrose.

Component 12 was shown to be neutral and non-migrating in borate buffer. It is, therefore, probably a fairly low molecular weight, stable ring compound, formed perhaps by intramolecular condensation.

A factor which complicated all these analyses was the adsorption of glucose during paper chromatography.

D-[^{14}C] glucose, being present in a large excess, badly contaminated all compounds with R_G values less than unity. This effect has been noticed and reported by Kowkabany and Hordis.[46A]

An attempt was made to find ene-diol containing compounds by ionophoresis of their molybdate complexes (Expt. 40).[47] It was only partially successful. D-Glucosone migrated with an M_S value [Expt. 2(iii)] of 0.85. Acidic compounds, however (L-ascorbic acid and 2-keto-D-gluconic acid) migrated at the same rate as D-gluconic acid. The method was thus not applicable to the self-decomposition product.

The rapid reaction of phenylhydrazine with di-keto groups could be used to characterise such compounds on chromatograms. D-Glucose reacted under the conditions employed, however, and complicated the matter when applied to the self-decomposition product (Expt. 41). The quinoxaline derivative formed from O-phenyl~~amine~~ ene diamine and a di-keto group[48] also afforded a possible method of characterizing such compounds. The R_G values of the resulting compounds (Expt. 41) were very similar and the method was abandoned.

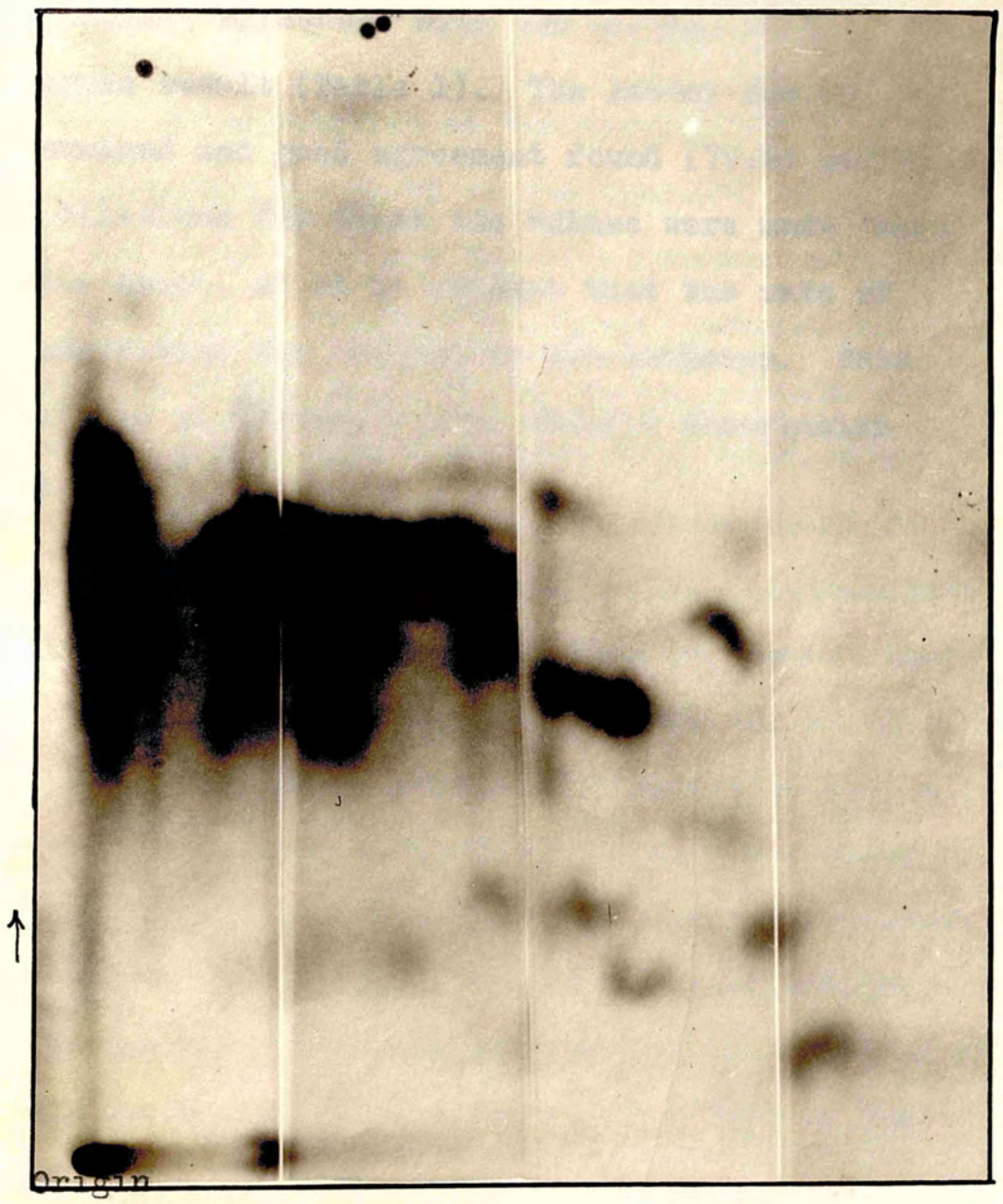
Here again the small amount of material available limited the methods to chromatographic ones. Grant and Ward[49] have applied this reaction to the colorimetric estimation of D-glucosone.

The amount of radioactivity accounted for by carrier dilution analysis was only 81.15% of the total. The picture displayed in Fig. 2 was, perhaps, a simplified one. Further resolution of the mixture was obtained by chromatography followed by borate ionophoresis in a direction at right angles (Expt. 26). The radiogram produced on exposure to X-ray film revealed the presence of at least 37 components (Fig. 5). The distribution of radioactivity was measured and the results given in Table 10. The material used in this experiment was from a freshly opened tube and there was no possibility of bacterial contamination.

The quantity of radioactivity in the glucose region was 86.4% of the total [Expt. 4(i)] indicating a decomposition of 13.6%(6.3% per year). The method of estimation cannot be regarded as accurate when one component is present in a very large excess. There is a variation in the amounts of decomposition given in Tables 4 and 10; both of these values are only in

Figure 5.

Chromatography/Borate Ionophoresis of the Products of Self-decomposition of D-[^{14}C] Glucose.



approximate agreement with the carrier dilution analysis result (Table 1). The latter was re-determined and good agreement found (79.95 and 80.0%). The dilutions for these two values were made three months apart, so it is evident that the rate of decomposition was reduced by sub-division. This result is in agreement with Bayly's observation (Fig. 1).

The Possible Contribution of a Secondary Radiation Effect

A calculation at the appropriate level of specific activity, based on the approach of Bayly and Weigel[8], shows that the maximum rate of decomposition of the D-[^{14}C] glucose sample, due to absorption of all of the β-ray energy, should be 4.27% per year. Their approach also takes into account the thickness and density of the layer of material when these factors are known. In this case the factors were unknown but it can be assumed that only a fraction (ca. one tenth) of the energy is absorbed. The actual rate of decomposition should be, therefore, only a fraction of the above value (i.e. ca. 0.5% per year). There is markedly a large discrepancy between the theoretical value and the experimental value (9.25% per year) which points clearly to the participation of a secondary effect in the degradation.

Secondary effects have already been discussed in the introduction and can be often caused by chain mechanisms. Another effect is envisaged in this case. D-Glucose was shown to freeze-dry only as far as the

monohydrate (Expt. 42); the presence of an absorption band (1640 cm.$^{-1}$) in the infra-red spectrum of the freeze-dried D-glucose also indicated water. This amount of water constituted ca. 10% of the total weight. It seemed reasonable, therefore, to assume that the freeze-dried D-[^{14}C] glucose sample also contained water. This water, whether it were bound as the sugar hydrate, or merely mixed as a syrup, would tend to absorb β-radiation and break down. The action of ionizing radiation on water has been discussed and it was seen that the primary reaction is the scission of the water molecule to a hydrogen atom and a hydroxyl radical. Thus the interaction of the β-particles from ^{14}C with the water could produce hydroxyl radicals. These radicals are very reactive and would react with glucose on formation and enhance the overall degradation. It was decided to study the effect of chemically generated hydroxyl radicals on D-glucose to test this hypothesis.

THE EFFECT OF A RADIOMIMETRIC AGENT ON D-GLUCOSE

An obvious choice here was Fenton's reagent[50] (a mixture of hydrogen peroxide and ferrous ions), particularly since some knowledge of its effect on carbohydrates was already available.

A Survey of the Literature on Fenton's Reagent

The free radicals produced on adding ferrous ions to hydrogen peroxide have been studied in great detail by Abel.[51] They are remarkably similar to those produced on the irradiation of water. The reader is referred to a good general review by Waters;[52] a more recent discussion is that of Dainton and Hardwick.[53] The initial reaction is that of ferrous ion with hydrogen peroxide according to the equation

$$Fe^{++} + HO\text{-}OH \longrightarrow Fe^{+++} + {}^{\bullet}OH + OH^{-}.$$

Ferrous ion may then be regenerated and though the initial rate of reaction will depend on the ferrous ion concentration, the overall chemical change will depend only on the amount of hydrogen peroxide present. The similarity of the mechanism of the radiation decomposition of a compound in aqueous solution and

of the Fenton oxidation of the compound has been previously noted. Feinstein and Nejelski[54], using alginic acid as a model for testing radiomimetric reagents, have found an exact correlation between ionizing radiation and Fenton's reagent.

The action of Fenton's reagent on carbohydrates has been studied intermittently since 1894. The progress up to 1956 has been well reviewed by Pigman.[55] The production of mannose from mannitol was reported by Fenton and Jackson[56] in early work (cf. Phillips[25]). Characterisation of a number of compounds produced by the oxidation of D-glucose was accomplished by Kuchlin.[57] The following products were characterised: D-glucosone, 2-keto-D-gluconic acid and 2-3 di-keto-D-gluconic acid. D-Glucuronic acid has so far only been detected[58] in small yield by the oxidation of glucose with hydrogen peroxide at 37°C (no catalyst). Recent work has been connected with the depolymerizing effect of Fenton's reagent on polysaccharides and a comparison with the radiation chemistry in this field.[54,59]

It is therefore apparent that Fenton's reagent can cause the following reactions:

(i) $-CH_2OH \longrightarrow -CHO$

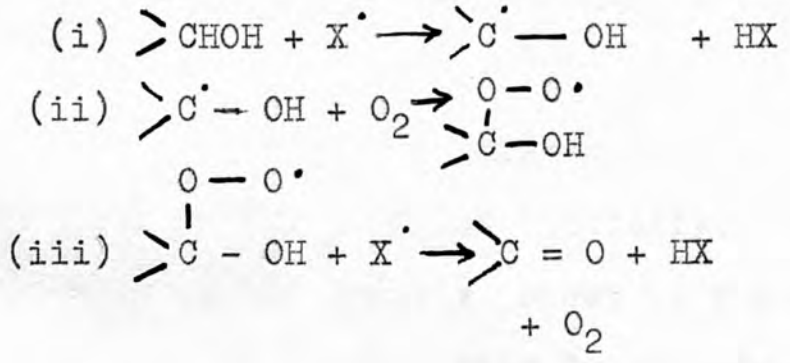
(ii) $\rangle CHOH \longrightarrow \rangle C = O$

There exist some very important physical differences when oxidation by Fenton's reagent is compared with the present radiation decomposition. If these differences are not realised the comparison is a dangerous one. The mobilities of the reacting species differ markedly in the two systems. Much of the energy available for self-decomposition is probably lost by recombination of reacting species near the sites of their formation. The other difference lies in the part played by oxygen. Self-decomposition, under the normal storage conditions, takes place in the absence of oxygen. It is impossible to study the Fenton oxidation under oxygen-free conditions even by de-gassing the solutions. Hydrogen peroxide undergoes a slow, continuous decomposition to oxygen and water during storage and consequently, an unknown amount of oxygen will always be present in Fenton's reagent. It is thought[60] that oxygen enhances oxidation in two ways. The reaction $H + O_2 \longrightarrow HO_2^{\cdot}$ increases the concentration of OH radicals by inhibiting

the back reaction:

$$H + \dot{O}H \longrightarrow H_2O$$

The second effect is a combination of oxygen with an intermediate free radical which cannot then recombine. For example, in the conversion of an alcohol group to a keto-group, a two-stage reaction, oxygen takes part as follows:-



where X is any oxidising radical.

As long as these differences are borne in mind, the comparison was felt to be a useful one.

D-Glucose, D-fructose and sucrose were oxidised (Expts. 27 and 29). The products from D-glucose were compared with those from the self-decomposition of D-[^{14}C] glucose. Pure D-[^{14}C] glucose was also oxidised with Fenton's reagent and some of the

products characterised by carrier dilution analysis.

Chromatographic Analyses of the Products of the Oxidation of D-Glucose, D-Fructose and Sucrose with Fenton's Reagent

D-Glucose, D-fructose and sucrose were each oxidised with one molecular proportion of Fenton's reagent (Expts. 27 and 29) according to the method of Morrell and Crofts.[61] Hydrogen peroxide was added in ten portions and time allowed, after each addition, for the ferric ion formed to be reduced back to ferrous ion. This change was evident by the slow fading of the brown colour produced initially. The rates of oxidation varied greatly (shown by the rate of fading of the colour) in the order fructose > sucrose > glucose.

Chromatography of oxidised glucose resulted in a very similar picture to that obtained with the self-decomposition products of D-[^{14}C] glucose. A direct comparison of the two reactions was made by superimposing the mixtures on the same paper and irrigating with an acetone solvent (Expt. 28). Exposure to X-ray film revealed radioactive products and the silver nitrate spray reagent revealed the non-radioactive

Fenton oxidation products. Six of the eight coloured spots had R_G values similar to those of six of the radioactive components.

A comparison was made between the oxidation products of glucose, fructose and sucrose (Expt. 29). Oxidised sucrose contained glucose and fructose. The use of a p-anisidine spray revealed a small amount of pentose in the glucose products. All three oxidations gave bright pink spots (p-anisidine) with R_G values ca. 0.2. This value was too low for glucuronic acid. 2-Keto-D-gluconic acid was shown to possess the same R_G value and to give the same bright pink colour with p-anisidine. The same component gave an orange colour on spraying with molybdate solution at pH 5.0. This colour reaction is characteristic of an ene-diol grouping.[47] A reference spot of L-ascorbic acid behaved identically in R_G value and towards the spray.

Chromatography of the components with R_G, 1.0 in a phenol solvent,[62] in all three cases indicated the presence of much D-glucosone (Expt. 30). Kuchlin showed, by carrying out the reaction in the presence of phenylhydrazine acetate, that D-glucosone was probably the first product of oxidation of D-glucose

and that keto-acids were produced from this. The similarity of the products from D-glucose, D-fructose and sucrose supports this. A more detailed analysis of the glucose oxidation products was undertaken.

Acid Production from D-Glucose by Fenton's Reagent

Ionophoresis of the oxidation products in phosphate buffer indicated that ca. 50% of the material was acidic. Slow titration of an aliquot with standard alkali (Expt. 31) showed that 56.7% of the glucose had been oxidised to acid. This figure was obtained by assuming that one molecule of glucose was oxidised to give one molecule of monocarboxylic sugar acid. This assumption probably gave rise to a rather high value.

The production of an ascorbic acid type of compound was supported by a very strong absorption band in the ultra-violet when the solution was made alkaline[23] (Expt. 32). The shift in the absorption maximum on making the solution alkaline (21mμ) was very similar to that of L-ascorbic acid (20mμ) and mannosaccharolactone[23] (34mμ). A second incompletely resolved peak in the

absorption curve coincided with an experimental absorption maximum obtained for 2-keto-D-gluconic acid. An acidic solution of the oxidation product decolorized a solution of iodine at room temperature (Expt. 33) and also showed the presence of 3.7% of a six-carbon ascorbic acid type of compound.* Part of this value could have been caused by the slow acid-catalysed enolization and lactonization of a keto-acid.[63]

* assumed for the purposes of calculation.

Further Results on Oxidised D-Glucose using Non-radioactive Techniques

D-Glucosone was determined by reaction in the cold with phenylhydrazine acetate[64] after the removal of acidic compounds by precipitation as their barium salts (Expt. 34). The phenylhydrazine derivative possessed, after recrystallisation, a m.p. and mixed m.p. and $[\alpha]_D$ coinciding with those of D-glucosazone. The weight, before recrystallisation (250 mg.), represented 12.4% of the original glucose. The value was calculated on the assumption that all the osone present was a six-carbon product (e.g. D-glucosazone). This is only approximate as other osones were probably present, e.g. D-arabinosone.

Carbon dioxide was removed from the oxidised glucose reaction mixture in a stream of nitrogen and determined gravimetrically as barium carbonate (Expt. 35). The most likely source of CO_2 was from decarboxylation of a sugar acid produced during the oxidation. The proportion of the original glucose that appeared as CO_2 (0.49%) indicated that 3.53% of the acid decarboxylated to give CO_2.

Formaldehyde was determined by the chromotropic

acid method[65] but a negligible quantity (0.02%) was found (Expt. 36).

Carrier Dilution Analysis

Pure D-[^{14}C] glucose was oxidised with Fenton's reagent and the products analysed by dilution analysis (Expts. 37 and 38). The yields of all the products found by all methods are given in Table 2.

Table 2. The products of the Fenton oxidation of D-Glucose

Product	Yield (weight per cent)	Analysis reference
D-Glucose	40.12	Expt. 38
D-Arabinose	0.49	Expt. 38
D-Erythrose D-Glyceraldehyde	(Traces found by chromatography	(Expt. 27
Sugar Acid	56.70	Expt. 31
D-Gluconic acid	11.68	Expt. 38
2-Keto-D-Gluconic acid	2.32	Expt. 38
D-Arabonic acid	1.11	Expt. 38
Ascorbic (type) acid	3.70	Expt. 33
Oxalic acid	1.02	Expt. 38
Formaldehyde	0.03	Expt. 36
Carbon dioxide	0.49	Expt. 35
D-Glucosone	12.40*	Expt. 34

* total osone, calculated as D-glucosone.

The "Polymeric" Component of Oxidised D-Glucose

The formation of a polymer in the radiation self-decomposition of D-glucose was an interesting reaction. The presence of a similar component (R_G,0) in the glucose oxidation products suggested the possibility of a similar polymer-forming reaction in the Fenton degradation.

The "polymer" could be precipitated from aqueous solution with acetone. It dialysed through cellophane. Analysis gave 17.9% iron. It decomposed on standing (2 months) producing a precipitate of an iron compound, and sugar products. The bulk of the compound is thought to be either a ferrous salt of a sugar acid or possibly a ferrous complex of the type reported by Küchlin[57] and quite unrelated to the radiation-produced polymer. The irradiation of α-hydroxy acids in aerated solution did not produce polymers[46] (cf. irradiation in a de-gassed solution under vacuum p. 46). In the light of this knowledge, the formation of polymers by Fenton's reagent would not be expected, because of the presence of oxygen in the latter.

A COMPARISON OF THE RESULTS OF THE TWO DEGRADATIONS OF D-GLUCOSE AND THE RECENT WORK ON THE IRRADIATION OF D-GLUCOSE

The similarity between the products of the self-decomposition of D-[^{14}C] glucose and the Fenton oxidation of D-glucose is apparent from Tables 1 and 2. The differences in the physical conditions of the two degradations has already been discussed (p.56). Some differences between the two sets of products have been found experimentally. The production of polymeric material was apparently confined to the self-decomposition. As has already been seen, this was probably a consequence of the absence of oxygen in the case of the D-[^{14}C] glucose sample (p.65).

The absence of D-glucosone from the self-decomposition products may be due to the fact that it is a very reactive compound. It would be subject to the same radiation flux and free radical attack as the glucose and thus may have possessed a higher decomposition rate (including chemical decomposition) than the very slow rate of formation from glucose. The irradiation of degassed[49] and non-degassed[66] D-glucose

solutions yielded D-glucosone as did the Fenton oxidation. In these cases, however, the rate of formation of the compound was very much greater than that of any compound in the self-decomposition reaction.

The Fenton oxidation, under the conditions used, caused more extensive oxidation, evidenced by the greater overall destruction of glucose and the presence in the products of a small quantity of oxalic acid. The oxidation was relatively specific, however, compared with the self-irradiation effect which was found to produce 37 compounds (Fig. 5). The wide variety of products from the radiation degradation was probably due to the primary external radiation effect giving rise to random decomposition of glucose and its degradation products. The course of the hydroxyl radical decomposition (secondary radiation effect) would also be expected to vary somewhat from the Fenton oxidation, in which all the free radicals are completely mobile.

The choice of Fenton's reagent for a comparative reaction was justified further when Streuhli[67] showed that electron irradiation of carbohydrates in solution, and in the solid state, produced acids, aldoses and

reductones. The ultra-violet absorption curves of the irradiated solution in acid and in alkali were almost identical with those obtained with the Fenton oxidation product of Expt. 27 (Fig. 7). Streuhli also found that irradiated solid glucose, when dissolved in water, liberated a gas, some of which was carbon dioxide. Infra-red analysis indicated the presence of a carbonyl group in small quantity. Bothner-By and Balzas[68] have reported acid production by the X-ray and electron irradiation of D-glucose solutions. Under alkaline conditions the irradiated solutions possessed U.V. absorption maxima at 275mμ. No products were characterised but the absence of D-glucuronic acid in chromatographic examinations was noted (cf. Ref. 25). The authors did not mention the production of D-glucosone but an illustration of a phenol/water chromatogram possessed a spot with an R_G value of *ca.* 0.7 and suggested its presence.

A direct comparison between the self-decomposition products of D-[^{14}C] glucose and those of the irradiation of solid D-glucose was not possible. The small

quantity of the former available did not allow enough for either infra-red analysis or gas determination. The similarity of the products from the self-decomposition, from the peroxide oxidation of glucose and from irradiation of glucose (solid and in solution) suggests that the same type of mechanism is operative in all cases. The similarity is not surprising as internal irradiation would not be expected to produce results differing appreciably from external radiation. Choline chloride has been shown to be very sensitive to both types of radiation,[1,2] The products formed by the self-decomposition[3] and irradiation[69] of methanol are very similar.

One difference, that due to the decay reaction of ^{14}C, has been discussed (p.) and, in small molecules, is only of theoretical interest. The extent and importance of this effect on polymeric compounds will be discussed later.

The colour change produced by the electron irradiation of solid D-glucose[67] is of interest. D-Glucose changed to a light yellow colour during

irradiation. The colour slowly changed to pink on standing for 4 weeks at 0°C. Streuhli suggested that this change was due to the presence of free radicals and refers to work by O'Meara and Shaw.[70] Bourne et al.[71] noticed that γ-rays produced the same colour changes but neither the m.p's. nor the specific rotations of aldoses were changed. The inference here, then, is that the colour change was not due to extensive chemical decomposition of their samples. It was noted, at the start of the present investigations, that the D-[^{14}C] glucose sample was faintly yellow. O'Meara and Shaw[70] found by electron paramagnetic resonance spectra (EPRS) that anhydrous D-glucose produced a very high yield of free radicals on irradiation. After storage for eight days the free radical concentration was still 4 x 10^7 radicals per gram of D-glucose. The presence of a small amount of moisture in a glucose sample, however, greatly decreased the strength of the EPRS and the radical concentration fell very rapidly. It is probable, therefore, that in spite of the semi-solid nature of the D-[^{14}C] glucose medium, the water present (ca. 10%) profoundly affected both the rate of

decomposition and the final products.

It is concluded that water in the D-[^{14}C] glucose sample contributed to the overall degradation, by absorbing much of the β-radiation and yielding hydroxyl radicals *in situ*, thus causing extensive secondary degradation.

THE PRODUCTS OF SELF-DECOMPOSITION OF D-[^{14}C] GLUCITOL COMPARED WITH THOSE OF THE FENTON OXIDATION OF D-GLUCITOL

The mechanism of the self-decomposition of labelled compounds is a difficult problem to study because the system cannot easily be externally influenced in any way. To substantiate the D-[^{14}C] glucose break-down mechanism it would be ideal to examine a number of other [^{14}C]-labelled carbohydrates. These are not readily available. Fortunately, some D-[^{14}C] glucitol, required for work reported in Part II, was found to have decomposed to the extent of *ca.* 2% in six months.

The sample contained 100 μC of radioactivity in 2.45 mg. and thus possessed a specific activity of 7.43 mC/m.mole. The maximum amount of decomposition from primary effects expected on theoretical grounds[8] was 2.25% in one year.

The Rate and Products of Self-decomposition

Chromatographic and ionophoretic analyses (Expt. 43) showed that, while small amounts of acid

and lactone (0.38%), D-glucose (0.5%) and L-gulose (0.4%) and negligible amounts of L-sorbose and D-fructose were produced, the main impurity (2.3%) was of a different character. The larger part (80%) of this impurity moved as one spot in a butanol solvent, with an R_G value of 2.6. It was shown to be neutral, but not a lactone, pentose, tetrose or triose. Borate ionophoresis resolved it into 3 components. The nature of these compounds was not elucidated. 1.5-anhydro-D-glucitol and 1.4-3.6-dianhydro-D-glucitol did not migrate during borate ionophoresis and hence were shown to be absent from the mixture. The similar chromatographic behaviour of the three unknown compounds suggested that they were similar in size. The evidence, then, is for the production of approximately equal amounts of three compounds, similar in size but differing in the spatial arrangement of their hydroxyl groups (borate ionophoresis). These could have been produced by differing modes of intramolecular reaction.

The oxidation of D-glucitol with Fenton's reagent (Expt. 44) resulted in very little acid production and approximately equal quantities of D-glucose,

D-fructose, L-gulose and L-sorbose (Expts. 45 and 46). A small quantity of osone was also produced. The products were not characterised further as they were examined for comparative purposes only.

The lack of similarity between the self-decomposition and the Fenton oxidation of D-glucitol suggested that the hydroxyl radical secondary effect may not have been so marked in this case as in the D-[^{14}C] glucose decomposition. A second radiochromatogram of a one-year-old sample of D-[^{14}C] glucitol revealed 3.5% decomposition. This figure was higher than the theoretical figure (p.72) but not as high as the decomposition rate of D-[^{14}C] glucose, which, at the same specific activity, would have been *ca.* 4.50% per year (assuming a linear relationship between specific activity and decomposition effects).

It was shown (Expt. 42) that a 200 mg. freeze-dried sample of D-glucitol contained ca. 2% of water (*cf.* D-glucose, ca. 10%). The conversion of an alcohol group to a keto group is a two stage reaction requiring two hydroxyl radicals for completion.

$$>CHOH + \dot{O}H \longrightarrow >\dot{C} - OH + H_2O \qquad (1)$$

$$>\dot{C} - OH + \dot{O}H \rightarrow >C = O + H_2O \qquad (2)$$

Reaction (1) may occur readily enough, via OH radicals generated from the small amount of water, but the radical formed may then tend to react in some other way before a second OH radical becomes available to complete the reaction. Thus the products might be expected to differ from the Fenton oxidation products.

OTHER IDEAS ON SELF-DECOMPOSITION

Some time after the work on the self-decomposition of D-[^{14}C] glucose had been completed, a paper appeared which attributed the deterioration of D-[^{14}C] glucose during storage to an anionic impurity.[72] Two preparations were examined. "Batch I" was prepared from Canna leaf by photosynthesis and purified by chromatography. "Batch II" was identical but also had been further purified by Duolite A-4 anion exchange resin. The methods of examination of the decomposition were:-

(i) storage in frozen solution.
(ii) storage as a thin layer on an aluminium disc in air.
(iii) autoclaving in distilled water.
(iv) autoclaving in the presence of HCl.

Quantitative examinations were by chromatography and radio-assay of the chromatograms. No products were identified. Dry state storage (ii) of batch I caused 25% decomposition in 8 months; of batch II, 5% decomposition in the same time. Auto-

claving in distilled water produced decomposition, again batch I showed more decomposition than batch II. Autoclaving experiments (Batch I) with constant specific activity and varying glucose concentration showed that the extent of decomposition was proportional to the total glucose concentration rather than to the specific radioactivity. Autoclaving in the presence of HCl gave reduced decomposition and different products.

The conclusions were that an anionic substance was present in batch I which caused decomposition in frozen solution, the dry state and while autoclaving. The effect of HCl was to replace glucose in the reaction with the anion.

Sterile conditions were not used for the dry state storage and the conditions used in each experiment were, wittingly or unwittingly, such that self-decomposition would be very much reduced. The spreading of a small amount of a radioactive substance over a large area causes most of its β-radiation to be lost to the atmosphere. The deterioration observed under these conditions was probably due to the impurity, or possibly, the action of bacteria. Control

experiments were run with the autoclaving experiments and the results seem to point to the action of impurity, though the method of detection of decomposition in non-radioactive controls would be less sensitive.

The nature of the anionic impurity was not discussed. A small amount of amino-acid may have survived the purification procedure as the material was prepared from a natural source. A type of "Browning reaction" could then occur in the sample. An amino-acid would probably not enhance radiation decomposition. It would also possess very little radioactivity as the leaves would have been at a late stage of growth when fed with $^{14}CO_2$. Hence, the substance would not complicate radio-chromatograms badly.

The D-[^{14}C] glucose used for the present examination had been purified by ion-exchange resins and was stored under conditions where radiation decomposition would have been extensive. It is concluded that the material decomposed mainly under the influence of its own radiation. The de-composition may have been enhanced by an impurity

and also possibly by a small amount of bacterial action.

GENERAL CONCLUSIONS

It is difficult to make general predictions as to the stability of labelled carbohydrates. One prediction, however, may be made on theoretical grounds. A rate of self-decomposition expressed in the units - per cent/μC/m.mole/year, is constant for any labelled compound when only the primary radiation effects are considered. If, however, the rate is expressed on a weight basis and not on a molar basis (e.g. per cent/μC/mg./year) the figure will depend on the molecular weight of the compound. Thus, expressed on a weight basis, the rate of decomposition of maltose will be twice that of glucose if the compounds are made from carbon of the same ^{14}C level. Amylose of D.P. 1000 would, on this theory, decompose a thousand times faster than glucose. This rate would only hold initially and it would drop as more of the energy became absorbed by products. The rate would still be appreciably higher than glucose - the same applies to any polymer. The effect may not be serious for some chemical requirements, but in enzyme work, for example, where the molecule is required intact, the

effect could be catastrophic.

Damage caused by secondary reactions can only be approximately assessed by considering (i) the physical state of the material and (ii) the possibility of the liberation of an ion, compound or radical which could attack the material.

The results of this investigation emphasise the need for a general awareness of the phenomenon of radiation self-destruction and its consequences.

PART I

EXPERIMENTAL

GENERAL METHODS

Expt. 1. Paper Chromatographic Methods

Paper chromatographic analyses of mixtures were carried out on Whatman No. 1 or No. 3 paper. The solvent descending technique was used. Chromatograms were developed with the following solvents:-

Solvent (a) - the organic phase of a mixture (v/v) of n-butanol, ethanol and water (4:1:5).
Solvent (b) - acetone and water (4:1).
Solvent (c) - amyl alcohol, pyridine and water (5:5:4).
Solvent (d) - the organic phase of a mixture of n-butanol, benzene, pyridine and water (5:1:3:3).
Solvent (e) - ethyl acetate, acetic acid and water (9:2:2).
Solvent (f) - a mixture (w/w) of phenol and water (4:1).
Solvent (g) - n-butanol, pyridine, water and saturated aqueous boric acid solution (6:4:2:1). Mobilities of compounds were expressed relative to D-glucose (R_G value). Hence the R_G value of a compound =

$$\frac{\text{Distance migrated by the compound}}{\text{Distance migrated by D-glucose (on the same chromatogram)}}$$

Compounds were detected by autoradiography (Expt. 3) or by spraying with one of the following systems:-

(i) silver nitrate in acetone, followed by ethanolic sodium hydroxide solution[73].

(ii) a solution of p-anisidine hydrochloride (2%) in n-butanol, followed by heating at 100°C for 5 min. and viewing in U.V. light.[74]

(iii) an ethanolic solution of trichloroacetic acid (5%) and after heating at 100°C for 3 min., spraying with p-anisidine (ii). Ketoses appeared as yellow spots in the cold and aldoses, as brown spots, on heating at 100°C.[75]

(iv) a freshly prepared mixture of α-naphthol (1%) in ethanol (50 ml.) and phosphoric acid (5 ml.), and heating for 5 min. at 100°C. Ketoses developed a blue colour.[76]

(v) a solution of urea in phosphoric acid solution and heating for 5 min. at 100°C. Ketoses developed a blue colour.[77]

(vi) a 1% solution of sodium metaperiodate in water and after drying in the cold, with a 1% solution of

$KMnO_4$ in water. The paper was then washed with water after 5 min. and dried in air. It was then sprayed with benzidine solution. Compounds containing a-glycol groups appeared as blue spots.[78]

Expt. 2. Paper Ionophoresis

Paper ionophoresis was carried out in three electrolytes using the technique of Foster.[79] This apparatus gave a potential difference of 15 volts per cm. of paper.

Buffers and electrolytes:- (i) Borate buffer (0.2 M) at pH 10.0 in which polyhydroxy compounds migrated as negatively charged borate complexes.[80] (ii) Acids were separated from neutral material by using phosphate buffer (0.2 M) at pH 7.2. In this system acids moved rapidly, as their anions, towards the anode. Migration rates were expressed relative to D-gluconic acid, and the term M_{GA} infers this. (iii) A solution of sodium molybdate (0.1 M) and sulphuric acid at pH 5.0 was used for the analysis of the self-decomposition products of D-[^{14}C] glucitol.

Expt. 3. Autoradiography

After drying, chromatograms and ionophoretograms of [^{14}C]-labelled compounds were marked with radioactive ink spots and laid on sheets of Ilford Industrial (G) X-ray film in a light-proof cupboard. Exposure times varied from 2 days to 2 months according to the radioactivity applied. A spot of material containing $10^{-3}\mu$C marked a film in 2 days. After exposure, films were developed for 5 mins. in an Ilford high contrast developer, PQX-1. The resulting picture is termed a "radiochromatogram".

Expt. 4. Assay of the distribution of radioactivity on chromatograms and ionophoretograms

(i) By the use of a Geiger counter. Strips, 2 cm. wide, were cut from chromatograms and ionophoretograms and clamped between two aluminium plates. The top plate contained a rectangular hole (1 x 2 cm.). The size of this hole could be varied by partially blocking it with aluminium foil. The activity distribution was determined by taking 5 min. counts on successive 1 cm. sections of the strips.

(ii) By the use of an "Eel" scanner. A section of an autoradiograph film was cut out and the distribution of radioactivity measured by utilising the variation in the optical density of the film.

The results of (i) and (ii) above are also termed radiochromatograms.

Expt. 5. Carrier Dilution Analysis

(a) Application to a mixture of a very small amount (by weight) of radioactive materials of the same, but unknown, specific activity

$$\frac{\text{Wt. of component X}}{\text{Wt. of total material}} = \frac{\text{Amount of } {}^{14}C \text{ as X}}{\text{Total } {}^{14}C}$$

then percentage of X in mixture

$$= \frac{(\text{amount of } {}^{14}C \text{ as X}) \times 100}{\text{Total } {}^{14}C} \quad \ldots\ldots\ldots (1)$$

The amount of ^{14}C as X is given by the formula

$$\text{R.A.} = \frac{W \times N \times \text{Sp. acty.}}{\text{Mol. Wt.}} \quad \ldots\ldots\ldots (2)$$

where R.A. = amount of ^{14}C as X in μC.

W = weight of carrier (g.)

N = no. of carbon atoms per molecule.

Sp. acty.= specific activity of the isolated carrier compound in μC/g.-atom of carbon.

Mol. wt. = molecular weight of carrier (g.).

This method is applicable when the weight of the carrier is very much greater than that of the compound sought.

(b) Application to a mixture of larger amounts (by weight) of radioactive materials of known specific activity

The total radioactivity in a component of a mixture (given by formula 2) is unchanged by dilution with an inactive carrier. Then

$$\frac{x \times N \times So}{\text{Mol. wt.}} = \frac{(x + a) \times N \times Si}{\text{Mol. wt.}}$$

and hence $$x = \frac{a.Si}{So - Si} \quad \ldots\ldots\ldots\ldots (3)$$

where x = wt. of radioactive material sought.

a = wt. of carrier added.

S_o = specific activity of material sought.

Si = specific activity of isolated carrier.

(c) The purification of carriers after dilution

Methods of purification varied with each carried compound. In general, repeated crystallisation was used where possible. In some cases a chromatographic purification on Whatman No. 3 paper was used. It was found that 0.5 g. of material could be handled on 10 sheets of paper.

Expt. 6. Determination of Radioactivity

The sample (10-12 mg.) was combusted in a stream of CO_2-free oxygen in a silica glass tube. The gases were passed over platinum and silver gauzes, heated to 600°C, to ensure complete combustion. The resulting $^{14}CO_2$ was absorbed in CO_2-free ca. 0.15 N-NaOH (35 ml.). The [^{14}C] carbonate thus formed was precipitated as barium [^{14}C] carbonate with 1N-barium chloride (5 ml.) and 1N-ammonium chloride (5 ml.) and filtered through a demountable filter giving a disc of diameter 2 cm. The

amount of material combusted was sufficient to give a disc of "infinite thickness" (<20 mg./cm.^{2}). After drying on a vacuum hot-plate at 80°C for 20 min., the β-emission of the disc was measured for a time sufficient to give a standard error of better than ± 2%, except for samples of specific radioactivity lower than 1.6 μC per g.-atom of carbon. The counting system consisted of a G.E.C. Geiger-Müller end-window tube, surrounded by a 2" thickness of lead; an "EKCO" scaler/power pack (type N.529 A) and Probe unit (type N.558). All counts per min. given were corrected for the paralysis time of the system (2 μ sec.) and background count. The correction formula was

$$n = \frac{60 \times N}{60 - N \times p} - b \qquad \ldots\ldots\ldots(4)$$

where n = true c/min.
N = apparent c/min.
p = paralysis time
b = background c/min.

A sample of poly-([^{14}C] methyl methacrylate), supplied by the Radiochemical Centre, Amersham, was used as a

standard source of barium [^{14}C] carbonate.

The specific radioactivity of a compound is given by

$$S_c = \frac{S_s \times N_c}{N_s} \quad \ldots\ldots\ldots\ldots\ldots (5)$$

where S_c = specific radioactivity of compound

S_s = specific radioactivity represented by standard

n_c = true counts/min. of Ba $^{14}CO_3$ from compound

n_s = true counts/min. of standard

Expt. 7. Preparation of [^{14}C] Perspex Standard for specific activity determinations

Standard [^{14}C] perspex sheet (type CFP 1) was obtained from the Radiochemical Centre, Amersham. A circle of this material (2 cm. diameter) was cut out and fixed with chloroform on to a machined block of inactive perspex. The mount was machined such that, when placed in the counting system, the surface of the standard and that of a routine barium carbonate disc were the same distance from the window of the G.-M. tube. [^{14}C] perspex (10 mg.) was combusted (Expt. 6). The sample (of nominal specific activity 0.1 mC/g. of perspex)

gave a Ba $^{14}CO_3$ disc of activity 27.4 counts/min. The activity of the mounted perspex disc was 272 counts/min. Therefore the β-emission of the standard represented a specific radioactivity of 19.87 μC per g.-atom of carbon.

PRELIMINARY EXPERIMENTS ON THE SUCROSE AND FRUCTOSE SELF-DECOMPOSITION PRODUCTS

Expt. 8. Paper chromatography and ionophoresis of the [^{14}C] Sucrose self-decomposition products

(i) Radiochromatography (film method) using solvent (a) revealed 12 spots with R_G values of 0, 0.08, 0.2, 0.28, 0.44, 0.73, 1.0, 1.2, 1.8, 2.1, 2.3 and 2.5. Assay of radioactivity [Expt. 4(i)] in the glucose and fructose regions (R_G 1.0 and 1.2 respectively) showed that the ratio of glucose:fructose was ca. 1:3 (cf. Fig. 1).

(ii) Paper ionophoresis in phosphate buffer for 1½ hours [Expt. 2(ii)] revealed six components migrating 0, 4.2, 6.4, 8.4, 11.5 and 17.7 cm. respectively (detected by film method).

Expt. 9. Paper chromatography and ionophoresis of the [^{14}C] Fructose self-decomposition products

(i) Radiochromatography using solvent (a) revealed 8 components between the origin line and fructose and a streak of material travelling faster than fructose. The R_G values were 0, 0.05, 0.11, 0.18, 0.27, 0.37, 0.55 and

1.14 (fructose).

(ii) Ionophoresis under the conditions of Expt. 8(ii) revealed components which migrated 0, 4.0, 6.2, 8.2 and 11.5 cm. respectively.

Expt. 10. Examination of the component from the [^{14}C] Sucrose products which was immobile during chromatography

The sucrose products (ca. 20 μC) were applied to one sheet of Whatman No. 1 filter paper and irrigated with (i) solvent (a) for 2 days and (ii) solvent (e) overnight. The base-line strip was cut out. Cold water elution would not remove all radioactivity from the strip; therefore, it was suspended over water (10 ml.) which was refluxed for one hour. This method is recommended by Wylam[81] for the removal of oligosaccharides from paper. Complete removal of radioactivity was checked with a Geiger-Müller counter. The material was concentrated by partial freeze-drying and made up to 4 ml. Three hydrolyses were attempted[81].

(i) Complete hydrolysis. The concentrated eluate (1 ml.) and 0.5 $\underline{\underline{N}}$-$H_2SO_4$ (4 ml.) were refluxed for 2 hr.,

neutralised with $BaCO_3$ and freeze-dried.

(ii) Partial hydrolysis. The concentrated eluate (1 ml.) and 0.05 $\underline{N}$-H_2SO_4 (2 ml.) were refluxed for 1 hr., neutralised with $BaCO_3$ and freeze-dried.

(iii) Weak hydrolysis. The concentrated eluate (1 ml.) and 1% aqueous oxalic acid (2 ml.) were refluxed for 1 hr., neutralised with $CaCO_3$ and freeze-dried.

Chromatograms of the hydrolysates and the original material were run in solvent (a). The film method of detection was used. The complete hydrolysis resulted in part of the material remaining on the origin line and part moving with the same R_G as a glucose reference spot. The results of (ii) and (iii) were unsatisfactory, streaks being obtained (presumably due to partial hydrolysis).

PRELIMINARY EXPERIMENTS WITH D-[^{14}C] GLUCOSE 15 MONTHS AFTER PREPARATION

Expt. 11. Sub-division of D-[^{14}C] Glucose sample.

The vacuum in the sample tube was checked with a discharge leak tester and found satisfactory. The tube was then broken open and the contents dissolved in sterile water (10 ml.). Aliquots (1 ml.) were pipetted into 8 sterile test-tubes (3" x $^{3}/_{8}$") and freeze-dried immediately. One portion (1 ml.) was stored at -20°C for preliminary chromatographic analyses. The final portion was used for a bacterial count.

Expt. 12. Bacterial count of D-[^{14}C] Glucose

A standard text-book method was used.[82] A culture medium (600 ml.) was prepared containing:-

Agar	-	12 g.	(2%)
sodium chloride	-	4.8 g.	(0.8%)
peptone	-	3.0 g.	(0.5%)
D-glucose	-	6.0 g.	(1.0%)
phenol red	-	15 ml.	(B.D.H. solution).

The pH of the solution was adjusted to 7.6 and the medium sterilized at 15 lbs./sq. in. for 20 min. Ten agar plates were made from this medium. D-[^{14}C] glucose solution (1 ml. from Expt. 11) was diluted with water as shown in Table 3. The plates were innoculated with the solutions and incubated at 25°C for 4 days. The results are shown in Table 3. Two types of colony were apparent. A large colony with crenated edge was a non glucose-fermenter and a small colony was shown to be a glucose-fermenter (by the colour change in the indicator surrounding the colony). The number of glucose-fermenters in the original D-[^{14}C] glucose sample was 3917 per mg. and that of the non glucose-fermenter, 1917 per mg.

The sucrose and fructose residues were also assayed but negligible counts were obtained (ca. 100 per mg.)

Table 3

Plate No.	Dilution	Large colony	Small colony
1	1:10	12	26
1	1:10	11	21
2	$1:10^2$	2	0
2	$1:10^2$	1	1
3	$1:10^3$	0	0
3	$1:10^3$	0	0
4	$1:10^4$	0	0
4	$1:10^4$	0	0
5	$1:10^5$	0	0
5	$1:10^5$	0	0

Expt. 13. The chromatographic analysis of D-[^{14}C] Glucose self-decomposition products

Radiochromatograms (film method) of D-[^{14}C] glucose (solution from Expt. 11) in solvent (a) revealed 12 components (Fig. 2). The distribution of radioactivity on a similar chromatogram, run 24 months after the glucose had been prepared, was measured [Expt. 4(i)]. The percentage of radioactivity of the component with the R_G value of glucose was 85.5% (Table 4). The same solution was analysed chromatographically with the aid of reference compounds using solvents (a), (c) and (d). The results are shown in Tables, 5(i), 5(ii) and 5(iii).

Expt. 14. The ionophoresis of D-[^{14}C] Glucose self-decomposition products

A radioionophoretogram (film method) using borate buffer [Expt. 3(i)] revealed 9 components (Fig. 3). Clear resolution of the components was not possible because of the similarity of the M_G values [Table 6(i)]. A negligible amount of material remained on the origin line. A similar experiment using phosphate buffer [Expt. 3(ii)] revealed 5 components [Fig. 4, Table 6(ii)].

Repitition of this experiment using reference compounds showed that neutral material (unchanged glucose etc.) migrated only by endosmotic flow. A measurement of the distribution of radioactivity showed that 8.2% of the material moved towards the anode and was therefore acidic.

Table 4. Radiochromatogram of D-[^{14}C] Glucose (24 months after preparation)

Component No.	R_G value	Radioactivity (% of total)
1	0	2.9
2	0.1	2.6
3	0.2	1.6
4A	0.3	1.2
4B*	-	1.1
5	1.0	85.5
6	1.3	1.4
7	1.5	1.3
8	2.1	0.7
9	2.6	1.0
10	2.9	0.3
11	3.1	0.2
12	3.5	0.1
13 /	-	0.1

* trail between components 4A and 5

/ trail between component 12 and solvent front.

Table 5. Paper chromatograms of self-decomposed D-[^{14}C] Glucose and D-Glucose oxidized with Fenton's reagent

Table 5 (i) - Solvent (a)

Component No.	R_G values		Co-chromatographs with:-
	Self-decomposition	Fenton oxidation	
1	0	0	Polymeric material
2	0.1	0.1*	D-Gluconic and 2-Keto-D-Gluconic acids (R_G 0.12)
3	0.21	0.21	D-Arabonic acid (R_G 0.21)
4	0.32	0.34	
5	1.0	1.0	D-Glucose (R_G 1.0) D-Glucosone (R_G 1.0)
6	1.3	1.25*	D-Arabinose (R_G 1.3)
7	1.5	1.7	
8	2.1	-	D-Gluconolactone (R_G 2.2)
9	2.6	2.5	D-Erythrose (R_G 2.6)
10	2.9		D-Arabonolactone Levoglucosan (R_G 2.8)
11	3.1		
12	3.5		Glyceraldehyde (R_G 3.4)

* Pink after treatment with p-anisidine [Expt. 1(ii)].

Table 5 (ii) - Solvent (c)

Component no.	R_G values	
	Self-decomposition	Fenton oxidation
1	0	0
2	0.27	0.25
3	0.38	0.36
4	0.50	0.55
5	1.0	1.0
6	1.27	1.3
7	1.4	1.5
8	1.9	-

Table 5 (iii) - Solvent (d)

Component no.	R_G values		Co-chromatographs with:-
	Self-decomposition	Fenton oxidation	
1	0	0	Polymeric material
2	0.19	0.22	D-Gluconic and 2-Keto-)
3	0.34	0.35	D-Gluconic acids)
	-	0.44	D-Arabonic acid)*
4	0.62	0.70	
5	1.0	1.0	D-Glucose (R_G 1.0) D-Glucosone (R_G 1.0)
6	1.2	1.2	D-Arabinose (R_G 1.2)
7	1.45	-	
8	1.6	1.6	D-Gluconolactone (R_G 1.7)
9	1.9	2.0	D-Arabonolactone (R_G 1.9)
10	2.1	-	Glyceraldehyde and Erythrose (R_G's 2.0) Levoglucosan (R_G 2.1)
11	2.5	-	
12	2.6	2.7	

* R_G values indefinite (ca. 0.2)

Table 6. Ionophoresis of the products of self-decomposition of D-[^{14}C] Glucose

Table 6 (i) - Borate Buffer

Component no.	M_G value	Migrates with:-
1	0.12	
2	0.25	
3	0.37	
4	0.60	
5	0.70	Glyceraldehyde (M_G 0.69)
6	0.80	D-Arabinose (M_G 0.90) D-Erythrose (M_G 0.90)
7	1.0	D-Glucose (M_G 1.0)
8	1.15	Sugar acids (M_G 1.1)
9	1.87	Dicarboxylic acid

Table 6 (ii) - Phosphate Buffer

Component no.	M_{GA} value	Migrates with:-
1	0	D-glucose etc.
2	0.36→0.63	-
3	1.0	Aldohexonic acid
4	1.13	D-arabonic acid
5	2.43	Dicarboxylic acid

CARRIER DILUTION ANALYSIS OF THE PRODUCTS OF THE SELF-DECOMPOSITION OF D-[^{14}C] GLUCOSE

Expt. 15. Determination of Total Radioactivity

The tubes prepared in Expt. 11 were numbered 1 to 8. As each was broken open for use, the total radioactivity in the tube was determined. This was done by diluting an aliquot with a known, large quantity (ca. 4 g.) of an organic compound (e.g. D-glucose) and freeze-drying. The specific radioactivity of the freeze-dried material was determined (Expt. 6). The total amount of radioactivity in the tube was calculated from this. The results are shown in Table 7.

Table 7

Tube no.	Aliquot of tube taken	Glucose added (g.)	Total radioactivity in tube (μC)
1	0.04	0.099	45.66
2	0.10	1.003	46.61
3	0.10	1.000	46.30
4	0.20	4.000	49.20
5	0.10	1.950	46.92
6	Used for chromatography		

Expt. 16. Dilution analysis for D-[^{14}C] Glucose

Dry B.D.H. "ANALAR" α-D-glucose (3.998 g.) was dissolved in an aliquot part of a solution of the self-decomposition product (20.83 μC) in water (15 ml.). The pH of the solution was adjusted to 7.5 with ammonium hydroxide and the solution allowed to stand until the optical rotation reached the equilibrium value as shown in Table 8.

Table 8

Time (hr.)	0	0.33	2.0	5.0
Rotation	18.29°	13.66°	13.65°	13.65°
$[\alpha]_D^{20}$	73°	54.5°	54.4°	54.4°

The solution was then freeze-dried. The resulting solid was dissolved in 96% boiling methanol (15 ml.). Isopropanol (10 ml.) was added to a faint turbidity and the solution refrigerated. After filtering and drying in vacuo, some of the crystals (12 mg.) were combusted. Recrystallisation was continued until 3 consecutive samples possessed constant specific

radioactivity. The fall in activity with crystallisation was as follows:-

Recrystallisation	1	2	3	4	5	m.p.
Activity (counts/ min.)	-	541	402	382	385	147°C

The final value corresponded to a specific activity of 125.07 μC per g.-atom of carbon. The application of formulae (1) and (2) [Expt. 5] gave the percentage of D-[^{14}C] glucose remaining in the mixture of the self-decomposition products as 79.95%.

Expt. 17. Dilution analysis for D-[^{14}C] Gluconic acid

(i) Potassium D-Gluconate was recrystallised once from ethanol and water to give a m.p. 176°C. This material (1 g.) was added to a solution of the self-decomposition product (4.57 μC) in water (4 ml.). After 10 min., methanol (15 ml.) was added until incipient cloudiness was obtained and the material was crystallised at 0°C. The fall in activity with recrystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	m.p.
Activity(counts/min.)	-	78.5	30.0	12.0	11.7	10.8	176°C

The final specific activity (0.76 μC per g.-atom of carbon) indicated that 0.43% of the self-decomposition product of D-[^{14}C] glucose was D-[^{14}C] gluconic acid.

(ii) D-Glucono-δ-lactone (1.019g.) was dissolved in an aliquot part of the self-decomposition product (9.98 μC) in water (10 ml.). The solution was allowed to stand at room temperature until the optical rotation indicated that the system had passed its point of maximum concentration of free acid. Machell[83] gives the following figures for the hydrolysis of D-glucono-δ-lactone in aqueous solution:-

Time (hr.)	0	0.25	0.5	1.0	2.0	4.0	7.0	23.4	48.0
% lactone	100	70	56.4	42.8	24.7	12.2	5.4	8.1	15.0

These figures show that the maximum concentration of free acid occurred 7 hrs. after solution. The rotational

change during the lactone hydrolysis in this dilution analysis was as follows:-

Table 9

Time (hr.)	0.75	1.75	13.3	14.75
$[\alpha]_D^{20}$	23.0°	9.3°	6.6°	7.1°
% lactone	40.9	22.0	18.4	19.0

The percentage of lactone was calculated using the $[\alpha]_D^{20}$ values of Isbell and Frush[84] for δ-lactone and free acid (+ 66° and -6.7° respectively). The experimental values agreed reasonably with those of Machell and confirmed that the carrier and [^{14}C]-products had reached true equilibrium. The solution was neutralized, after 15 hr., to pH 7.0 with potassium hydroxide and evaporated under reduced pressure to ca. 5 ml. Ethanol (15 ml.) was added as in Expt. 17(i) and the potassium salt repeatedly recrystallised. The fall in activity with re-crystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	7	8	9	10	m.p.
Counts/min.	180	58	49	45	36	32	31	25	26	25	176°C

The final specific activity (1.81 μC per g.-atom of carbon) indicated that 0.62% of the self-decomposition product was D-[^{14}C] gluconic acid or lactone.

Expt. 18. Dilution analysis for 2-keto-D-[^{14}C] Gluconic acid

(i) Potassium 2-keto-D-gluconate (0.471 g.) was dissolved in an aliquot part of the self-decomposition products (19.04 μC) in water (25 ml.). The solution was allowed to stand for 1 hr. potassium 2-keto-D-gluconate was crystallised as described for potassium D-gluconate. The fall in activity with recrystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	7	8	9	10	m.p.
Counts/min.	-	62	45	42	33	30	27.6	20.0	20.5	20.0	151°C

The final specific activity (1.43 μC/g.-atom of carbon) indicated that 0.09% of the self-decomposition product was 2-keto-D-[^{14}C] gluconic acid.

(ii) A solution of 2-keto-D-gluconic acid in water (10 ml.), obtained by treatment of calcium 2-keto-D-gluconate (0.634 g.) with Amberlite IR-120 [H^+] resin, was added to an aliquot part of the self-decomposition product (13.87 μC) in water (15 ml.). The solution was allowed to equilibrate for 18 hr. To obtain a partial purification, the solution was then treated with De-Acidite FF (carbonate) resin (15 g.). The acid was desorbed by treatment with 3% ammonium carbonate solution (50 ml.) The solution was freed from cations by treatment with Amberlite IR-120 [H^+] (10 g.) and then neutralized with KOH solution to pH 7.0. Potassium 2-keto-D-gluconate was recrystallised as described for potassium D-gluconate. The fall in activity with recrystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	7	8	9	10	m.p.
Counts/min.	-	-	-	-	75	69	64	52	50	51	151°C

It has since been found[49] that De-Acidite FF causes decomposition of 2-keto-D-gluconic acid. This would account

for the low yield from the resin but would not alter the carrier dilution result as the dilution was made before resin treatment. The final specific activity (2.98 μC/g.-atom of carbon) indicated that 0.38% of the self-decomposition product was 2-keto-D-[^{14}C] gluconic acid or a derived lactone.

Expt. 19. Dilution analysis for D-[^{14}C] Glucuronic acid

D-Glucurone (0.994 g.) was dissolved in an aliquot part of the self-decomposition product (15.09 μC) in water (10 ml.). The solution was allowed to equilibrate for 20 hr., evaporated to a syrup and ethanol (1 drop) added. The syrup was seeded with a trace of D-glucurone and crystallised. The product did not possess constant specific activity after 11 recrystallisations. The fall in activity with recrystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	7	8	9	10	11
Counts/min.	±	-	588	148	74	22	12	-	9	8	6

The final specific activity (0.45 μC/g.-atom of carbon) indicated that the maximum amount of D-[^{14}C] glucuronic acid or lactone in the self-decomposition product was 0.1%.

Expt. 20. Dilution analysis for D-[^{14}C] Arabinose

D-Arabinose (0.544 g.) was dissolved in an aliquot part of the self-decomposition product (19.16 μC) in water (20 ml.) and, after evaporation, purified by chromatography on 10 sheets of Whatman No. 3 paper in solvent (a). D-[^{14}C] arabinose was eluted from the strips cut from the chromatograms and freeze-dried. The material was crystallised by dissolving in boiling methanol (4 ml.), adding isopropanol (10 ml.) and refrigerating. The fall in activity with recrystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	m.p.
Counts/min.	-	85	77	71	70	71	159°C

The final specific activity (4.58 μC/g.-atom of carbon) indicated that 0.43% of the self-decomposition product was D-[^{14}C] arabinose.

Expt. 21. Dilution analysis for D-[^{14}C] Arabonic acid

(i) Preparation of Potassium D-Arabonate

The method of Berezovski and Rodionova[85] was slightly modified. D-Arabinose (2 g.) was stirred into a

mixture of bromine (0.9 ml.) and water (10 ml.). The mixture was kept in the dark with occasional stirring for 48 hr. Air was then drawn through the solution to remove excess bromine. The solution was neutralized to pH 7.0 with KOH and evaporated under reduced pressure to ca. 4 ml. Ethanol (10 ml.) was added and potassium D-arabonate precipitated in 5 min. Further addition of ethanol increased the yield. It was necessary to recrystallise the material again to free it from KBr. The purified product (1.7 g.) possessed an $[\alpha]_D^{20}$ in acid solution of +18.8° equilibrating to +46° (Ohle and Berend give $[\alpha]_D^{20}$ + 18.6° → 48.6°.[86]) The compound decomposed between 180°C and 220°C. No m.p. could be found in the literature.

(ii) Potassium D-arabonate (0.813 g.) was dissolved in an aliquot part of the self-decomposition product (23.95 μC) in water (20 ml.). The solution was allowed to stand for 1 hr. The salt was recrystallised as described above. The fall in activity with recrystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6	7	8	9	10
Counts/min.	-	-	-	28	25.4	25	13.5	11	10	10.5

The final specific activity (0.74 μC/g.-atom of carbon) indicated that 0.06% of the self-decomposition product was D-[^{14}C] arabonic acid.

(iii) A solution of D-arabonic acid in water (10 ml.), obtained by treatment of the potassium salt (0.517 g.) with Amberlite IR-120 [H^+], was added to a solution of the self-decomposition product (8.33 μC) in water (10 ml.). The solution was allowed to stand overnight and then concentrated to ca. 3 ml. D-arabonolactone was separated by paper chromatography using solvent (a). This compound was chosen because the acid very readily lactonized and more than half of the material migrated as the lactone in solvent (a). The lactone spot was also well separated from all other acids, lactones and unchanged glucose. The eluted lactone solution was neutralized and the potassium salt crystallised as described above. The fall in activity with re-crystallisation was as follows:-

Recrystallisation	1	2	3	4	5	6
Counts/min.	±	10.2	9.5	6.0	6.1	6.0

The final specific activity (0.45 μC/g.-atom of carbon) indicated that 0.07% of the self-decomposition product was D-[^{14}C] arabonic acid or a lactone.

Expt. 22. Dilution analysis for D-[^{14}C] Mannitol

D-Mannitol (1.974 g.), purified by recrystallisation from 95% aqueous methanol, was dissolved in an aliquot part of the self-decomposition product (4.75 μC) in 90% boiling aqueous methanol and crystallised by slow cooling. Six recrystallisations yielded non-radioactive D-mannitol (m.p. 166°C).

Expt. 23. Dilution analysis for [^{14}C] Oxalic acid

Oxalic acid (0.5 g.) was dissolved in an aliquot part of the self-decomposition product (4.75 μC) in boiling water (0.5 ml.) and crystallised by cooling. After six recrystallisations its specific activity (0.002 μC/g.-atom of carbon), represented a maximum concentration, in the mixture, of 0.0005%, and was below

the limit of accurate determination.

D-Fructose and D-glucitol (as the hexacetate) were also used in dilutions but could not be crystallised to constant specific activity.

Expt. 24. Examination of the Polymeric component (R_G, 0) of the self-decomposition product of D-[^{14}C] Glucose

This component, remaining immobile during paper chromatography (component 1 in Fig. 2 and Table 4), was eluted with boiling water (Expt. 10). Radiochromatograms of the eluate in solvent (a) revealed slow moving impurity and impurity moving with the same R_G value D-glucose (1.0). The material was therefore re-chromatographed using solvent (e), after which it was found to be pure [solvent (a)]. Sulphuric acid was added to portions of the eluate to obtain 0.05 N- and 0.5 N-H_2SO_4 solutions. The solutions were then kept at 100°C for 2.5 hr., neutralized with barium carbonate and concentrated. No radioactivity could be detected on chromatograms either by means of X-ray film or Geiger-Müller counter. The isolation of the polymer was repeated. A portion of the aqueous eluate

(2 ml.) and Amberlite IR-120 [H^+] (0.5 g.) were heated in a sealed tube at 100°C for 24 hrs.[45] Subsequent radiochromatograms [solvent (a)] showed that no hydrolysis had occurred.

Expt. 25. Separation of the self-decomposition products of D-[^{14}C] Glucose and experiments on the individual components

D-[^{14}C] Glucose from one tube (Expt. 11), of ca. 50 μC of radioactivity, was chromatographed on one 8" wide sheet of Whatman No. 1 paper using solvent (a). After detection (X-ray film, 24 hr. exposure) the strips of components 1 to 12 (Table 4, Fig. 2) were cut out and eluted. Elution was carried out with water, firstly in air, when evaporation merely concentrated material at the lower tip of the strip. The final elution was carried out in a water-saturated atmosphere. It was found that 90% of the radioactivity was eluted in the first 3 drops under these conditions. Six drops were collected.

(i) Component 1 (see Expt. 24).

(ii) Slow moving material (components 2, 3 and 4). These were allowed to stand in aqueous solution for 48 hr. and then chromatographed in solvent (a). The original

components appeared on the radiochromatogram with some material moving with the same R_G values as D-gluconolactone (1.6) and D-arabonolactone (1.9). It was apparent that a clear separation of the acids had not been obtained in the initial chromatogram, due to the similarity of their R_G values.

Components 2, 3 and 4 were analysed by ionophoresis in phosphate buffer [Expt. 3(ii)] with D-gluconic, 2-keto-D-gluconic and D-arabonic acids as reference compounds. Components 2 and 3 migrated with D-gluconic and 2-keto-D-gluconic acids (M_{GA} values , 1.0); component 4 appeared to be a mixture of two components of M_{GA} values 1.0 and 1.1, the latter being that of D-arabonic acid.

(iii) The D-Glucose component (No. 5). This component was found to be chromatographically pure in phenol water solvent [solvent (f)].

(iv) Component 6 co-chromatographed with D-arabinose in solvents (a), (d), (e) and (f).

(v) Components 7, 8, 9 and 10 migrated during phosphate ionophoresis [Expt. 2(ii)] with M_{GA} values of 1.0. Each component, however, contained some neutral

material. Component 9 was resolved further into 3 fractions by borate ionophoresis. These fractions possessed M_G values of 1.12, 0.95 and 0.80 respectively. D-Arabonic acid and D-erythrose (prepared by electrolysis of D-arabonic acid solution[87]) possessed M_G values of 1.10 and 0.80 respectively. Authentic D-erythrose (obtained by the action of ammonia on 1:1-diethyl-sulphonyl-D-erythro-3.4.5 trihydroxy-pent-1-ene) was later shown to have the same M_G value as the material prepared by electrolysis.

(vi) Components 7, 8 and 9, when chromatographed in solvent (a), produced the original spots and also spots in the aldo-hexonic acid region (R_G, ca.0.2).

(vii) Component 12 did not migrate and was shown to be neutral by non-migration during phosphate ionophoresis. These facts suggested that the component may be levo-glucosan (1.6 anhydro-β-D-glucopyranose), however the R_G of an authentic specimen (ca. 2.9) was definitely quite different from that of component 12 (3.5).

Expt. 26. Two dimensional chromatography-ionophoresis of the products of self-decomposition of D-[^{14}C] Glucose

Self-decomposed D-[^{14}C] glucose was chromatographed

using solvent (a). The paper was then subjected to borate ionophoresis in a direction at right angles to the solvent flow. Exposure to X-film revealed the presence of 37 components. The radiogram is shown in Fig. 5. The distribution of radioactivity was measured [Expt. 4(i)] and the results shown in Table 10.

A similar experiment using phosphate ionophoresis clearly revealed the distribution of acidic material along the chromatogram (Fig. 6).

Table 10. Two dimensional analysis of the products of self-decomposition of D-[^{14}C] Glucose

Component No.	R_G	M_G	Radioactivity after 26 months (% of total)	Rate of formation (% year)
1-A	0	0	0.64	0.30
1-B	0.1	0.3	0.11	0.05
2,3-A	0.1	1.0	2.14	0.99
2,3-B	0.1	1.2	1.46	0.67
2,3-C	0.1	1.4	0.07	0.03
2,3-D	0.1	1.5	0.04	0.02
2,3-E	0.1	2.0	0.02	0.01
Trail of 4-A	0.3	ca.0.7	0.44	0.20
4-A	0.3	0.95	0.89	0.41
4-B	0.3	1.4	0.1	0.005
4-C	0.3	1.5	0.03	0.01
5-A	1.0	0	0.30	0.14
5-B	1.0	0.85	0.30	0.14
5-C	1.0	1.0	86.4	6.3*
5-D	1.0	1.9	0.03	0.01
6-A	1.3	0.3	0.09	0.04
6-B	1.3	0.7	0.47	0.22
7-A	1.5	0.3	0.06	0.03
7-B	1.5	0.9	0.44	0.20
7-C	1.5	1.0	3.19	1.47
7-D	1.5	1.9	0.04	0.02
8-A	2.1	0.1	0.04	0.02
8-B	2.1	0.4	0.06	0.03
8-C	2.1	0.9	0.32	0.15
8-D	2.1	1.0	0.70	0.32
8-E	2.1	1.2	0.08	0.04
9-A	2.6	0.4	0.11	0.05
9-B	2.7	0.7	0.59	0.27
9-C	2.6	0.7	0.25	0.12
9-D	2.6	1.2	0.11	0.05
10-A	2.9	0.3	0.06	0.03
11-A	3.1	0.5	0.05	0.02
11-B	3.1	0.6	0.05	0.02

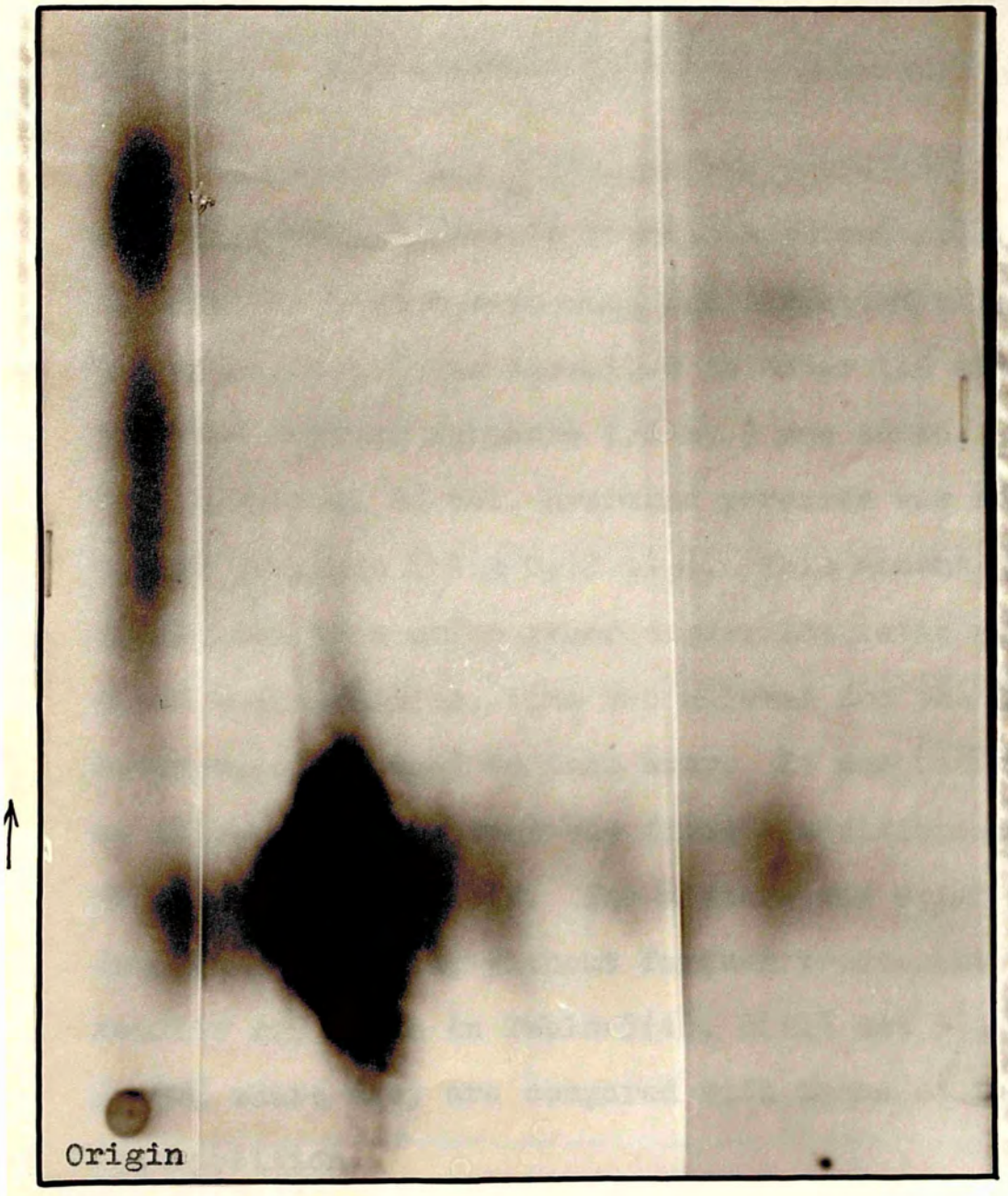
Table 10. (Continued)

Component No.	R_G	M_G	Radioactivity after 26 months (% of total)	Rate of formation (% year)
11-C	3.1	0.9	0.15	0.07
11-D	3.1	1.0	0.04	0.02
12-A	3.3	0.1	0.12	0.06
12-B	3.2	0.3	0.08	0.04
12-C	3.5	0.9	0.02	0.01

* Rate of decomposition

Figure 6.

Chromatography/Phosphate Ionophoresis of the Products of Self-decomposition of D-[^{14}C] Glucose.



PRELIMINARY EXPERIMENTS ON THE PRODUCTS OF OXIDATION OF D-GLUCOSE WITH FENTON'S REAGENT

Expt. 27. Oxidation of D-Glucose with Fenton's reagent

In every case D-glucose was oxidized by the following method (simple fractions or multiples of the amounts stated were used for different experiments). D-Glucose (1 g.) was dissolved in water (10 ml.) and hydrated ferrous sulphate (30 mg.) was added. To this solution, 20 vol. hydrogen peroxide was added in ten portions (10 x 0.32 ml.). This amount was equivalent to a molar glucose:peroxide ratio of 1:1. After each addition, time was allowed for the deep amber colour formed to fade away. It was found necessary to allow 4 hrs. between the first 4 additions and 1 hr. between the others. The mixture was examined chromatographically without further treatments. The results are given in Tables 5(i), 5(ii) and 5(iii) [p.102] where they are compared with those of the self-decomposition.

Expt. 28. Direct chromatographic comparison of the Self-decomposition Products of D-[^{14}C] Glucose and those of the Fenton oxidation of D-Glucose

Paper chromatography in solvent (b) of a mixture of the two systems, exposure to X-ray film and spraying with acetonic silver nitrate/ethanolic sodium hydroxide, revealed 8 coloured spots on the paper chromatogram. Six of these had R_G values similar to those of 6 of the 7 components present in the self-decomposition products which were detected on the radiochromatogram (Table 11).

Table 11

Component no.	R_G Values	
	Self-decomposition	Fenton oxidation
1	0	0
2	0.70	0.60
3	0.78	0.77
4	1.00	1.00
5	1.22	1.20
6	1.37	1.37
7	1.56	1.57
8	-	1.70

Expt. 29. Oxidation of Sucrose and Fructose with Fenton's reagent

The method used was that of Expt. 27. Both compounds reacted much more rapidly than glucose. It was necessary to allow only 15 min. between the first two additions of peroxide and 5 min. between the remainder in the fructose oxidation. The resulting mixtures were chromatographed using solvents (a) and (d). The results are shown in Tables 12 (i) and (ii) where they are compared with those of D-glucose.

Table 12. Chromatography of the products of oxidation of D-glucose, D-fructose and sucrose with Fenton's reagent

Table 12 (i) - R_G values using Solvent (a)

Glucose	Fructose	Sucrose
0	0	0
-	-	0.16
0.26	0.28	0.28
0.34	-	0.36*
1.0	1.0	1.00
1.23	1.20*	1.25*
1.80	1.94	1.70
	2.45	

Table 12 (ii) - R_G values using Solvent (d)

Glucose	Fructose	Sucrose
0	0	0
-	0.14	0.11
0.19	0.20	0.20
0.25 /x	0.26 /	0.26 /
0.34	-	0.32
0.62	-	0.65*
1.00	1.00	1.00
1.2	1.2*	1.2*
1.45	1.4	-
1.6	1.6	1.6
1.9	-	1.9
2.1	2.1	-
2.5	-	2.3
2.6	-	-

/ pink with p-anisidine reagent [Expt. 1(ii)]
* blue with urea-phosphoric acid and α-naphthol reagents [Expt. 1(iv) and (v)]
x orange when sprayed with acidic molybdate solution.

Expt. 30. Chromatographic analysis of the component with R_G 1.0 [Solvent (a)] from the oxidation of D-Glucose, D-Fructose and Sucrose with Fenton's reagent

The components (R_G 1.0) were eluted from chromatograms and re-chromatographed using solvent (f). The eluates from the glucose and sucrose oxidations each yielded two components of R_F 0.39 (glucose) and 0.25 (glucosone)[62]. The eluate from the fructose oxidation gave mainly one spot of R_F 0.25 (glucosone). Some material was also present in the region R_G 0.2 in all cases. This was probably acidic material. In later experiments, on the use of molybdate ion (see Part II), it was found that these three components migrated during molybdate ionophoresis [Expt. 3(i)] with M_S values of 0.80. The eluate from the fructose oxidation gave an immediate yellow precipitate with phenyldrazine acetate in the cold.

Expt. 31. Determination of the amount of acid produced in the Fenton oxidation of D-Glucose

D-Glucose (10 g.) was oxidized (Expt. 27) and the resulting solution made up to 250 ml. An aliquot

(10 ml.) required 22.5 ml. of 0.056 N-NaOH for neutralization using methyl red-methylene blue indicator. If it is assumed that the acid produced was a monocarboxylic six-carbon acid, 56.7% of the glucose had been oxidized to such a compound.

Expt. 32. The U.V. absorption spectra of D-Glucose oxidized with Fenton's reagent compared with that of pure 2-keto-D-Gluconic acid

(i) A solution of oxidized D-glucose (Expt. 31) which had been stored at 0°C for one week was made alkaline. The solution turned dark brown, presumably due to the formation of colloidal iron compounds. Therefore, before any U.V. work could be accomplished, it was necessary to free the solution from iron. Part of the stock solution (100 ml.) was passed down a column of Amberlite IR-120 [H^+] resin (1.5 x 20cm.) at a flow rate of 1 drop/sec. This treatment was repeated and the resulting solution made up to 200 ml. of "iron-free stock". Part of this solution (1 ml.) was made up to 50 ml. An aliquot (2 ml.) was made alkaline by the addition of N-NaOH (0.2 ml.) and examined in

a U.V. spectrometer in a 1 cm. cell. The concentration of this solution was 0.4 g. of oxidized glucose per litre. The resulting U.V. absorption curve is shown in Fig. 7. The curve in acid solution was also determined as described above using $\underline{\underline{N}}$-$H_2SO_4$ (0.2 ml.) in place of $\underline{\underline{N}}$-NaOH (Fig. 7). Control experiments showed that glucose, sulphuric acid and sodium hydroxide did not absorb in the range 200-500 mμ.

$$\lambda_{max.}\text{(alkali) } 300 \text{ m}\mu - \lambda_{max.}\text{(acid) } 279 \text{ m}\mu$$

$$= 21 \text{ m}\mu.$$

cf. The results of Haworth et al.[23]

$\underline{\underline{L}}$-ascorbic acid - 20 mμ.

mannosaccharolactone - 34mμ.

(ii) Calcium 2-keto-$\underline{\underline{D}}$-gluconate (10 mg.) in water (2 ml.) was shaken with Amberlite IR-120 [H^+] (100 mg.) until it dissolved. The solution was filtered from the resin and made up to 4 ml. One half of this solution was made alkaline with $\underline{\underline{N}}$-NaOH (0.2 ml.) and the U.V. absorption measured. While increasing the light wavelength from 210 to 400 mμ, a continuous upward drift of readings was noted. This was thought to be due to the

Figure 7.

The U.V. Absorption Curves of the Fenton Oxidation Product of D-Glucose in Acid amd in Alkali.

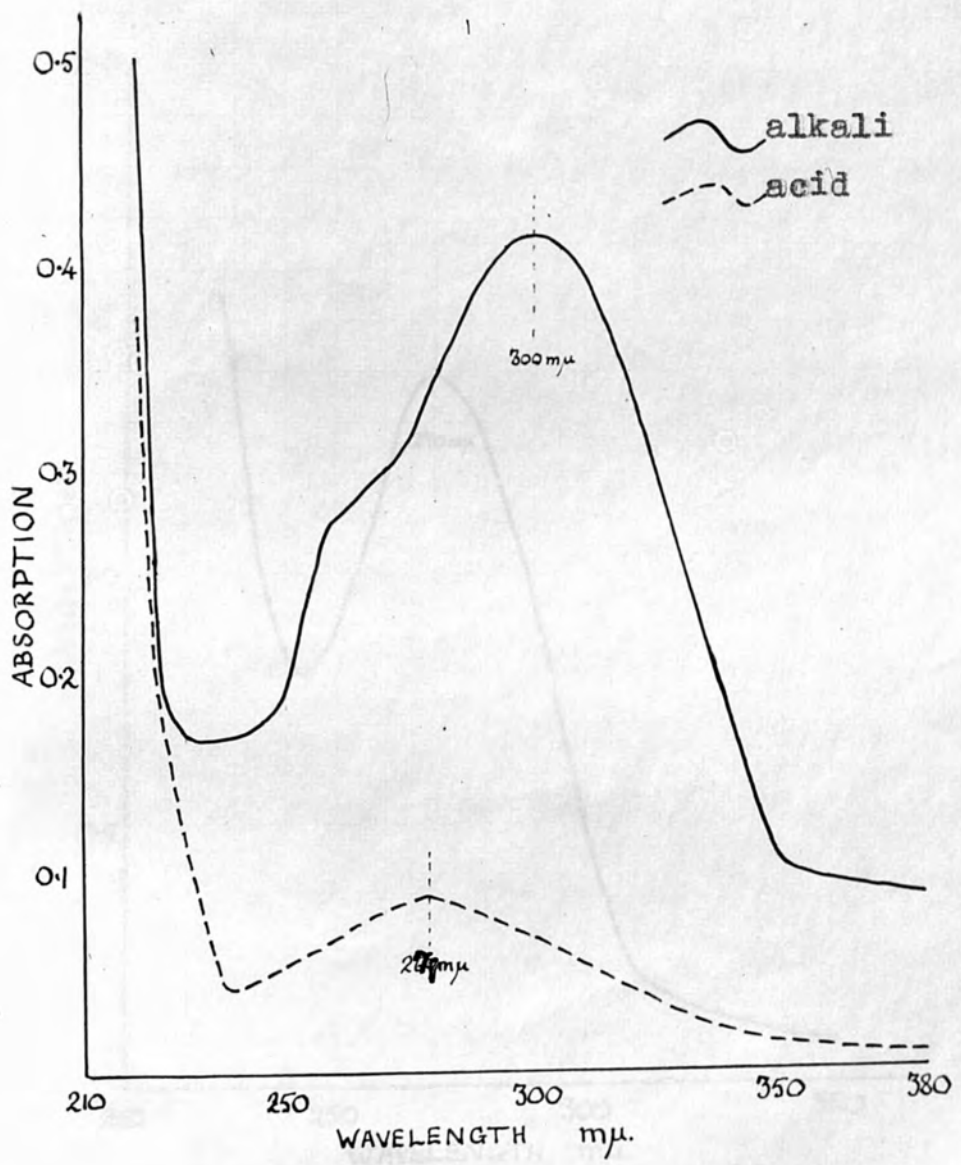
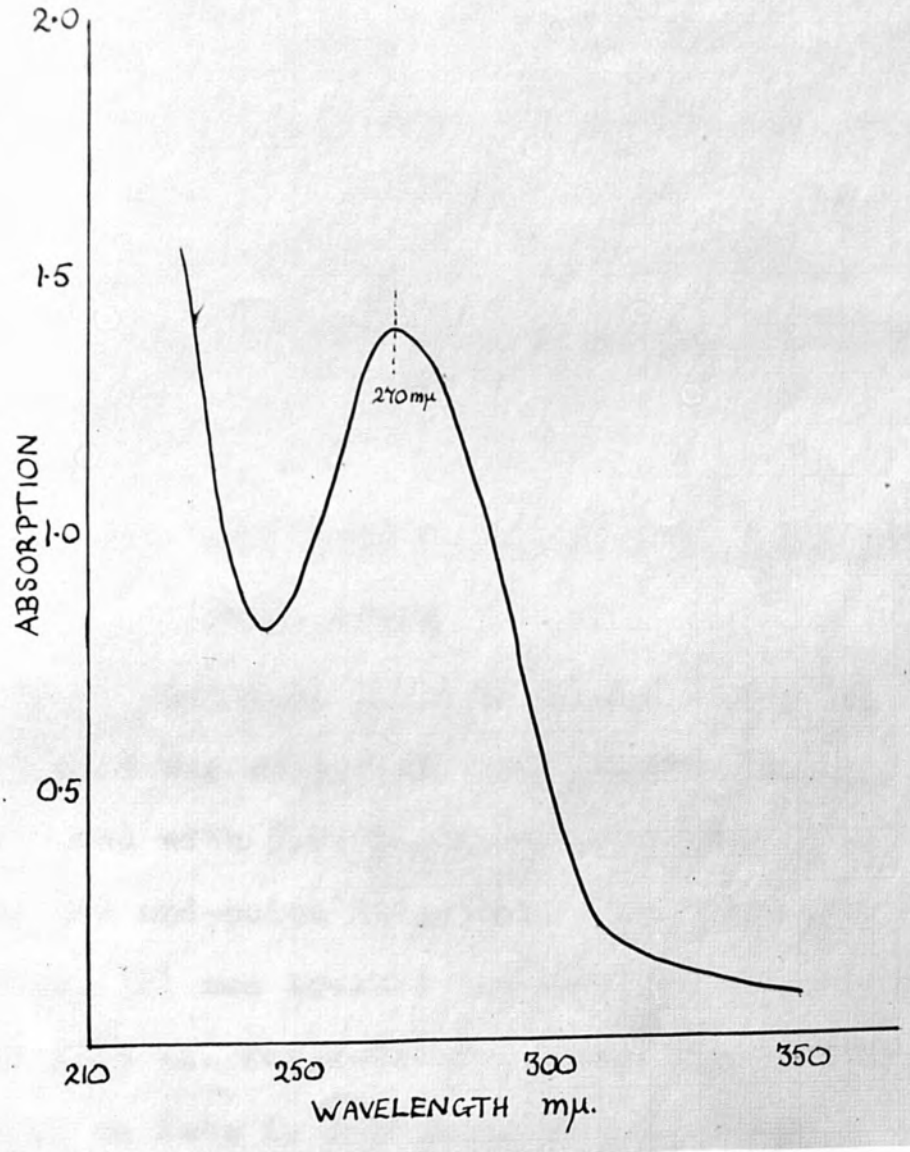


Figure 8.

The U.V. Absorption Curve of 2-keto-D-Gluconic Acid in alkali.



alkali catalysed conversion of the keto-acid to an ene-diolic compound.[63] Readings were taken as quickly as possible while both increasing and decreasing the wavelength. The curve of the average values is shown in Fig. 8. The $\lambda_{max.}$ at 270 mμ coincides with the "shoulder" in the oxidized glucose curve at 267 mμ(Fig. 7). In acid solution, no absorption could be detected at the concentration available.

Expt. 33. The reaction of oxidized D-Glucose solution with Iodine

Oxidized glucose solution from Expt. 31 (50 ml.) was acidified with $\underline{\underline{N}}$-$H_2SO_4$ (10 ml.) and titrated with 0.01 $\underline{\underline{N}}$-iodine using starch glycollate for the end-point detection. The iron-free stock (Expt. 32) was treated identically. Titres were 2.6 and 1.36 ml. respectively. The starch-iodine blue was found to fade in 5-10 min. in both cases. The titrations were continued intermittently for 24 hr. Further additions of iodine were not made because it was found that $\underline{\underline{D}}$-glucose (2 g.) in water (50 ml.), made acidic with $\underline{\underline{N}}$-$H_2SO_4$, decolorized 0.5 ml. of 0.01 $\underline{\underline{N}}$-iodine in

3 days. Titres were 19.9 and 9.5 ml. respectively. When based on L-ascorbic acid it was calculated that 3.7% of the glucose was oxidized to ~~that~~ such a compound.

Expt. 34. Determination of the amount of Aldosone in the oxidized Glucose solution

D-Glucose (1 g.) was oxidized (Expt. 27). Barium carbonate (2 g.) was added to the resulting solution and the mixture shaken for 1 hr. Insoluble barium salts were removed by centrifuging. An equal volume of acetone was added to the supernatant and the resulting flocculent grey precipitate centrifuged down. The acetone was then removed by distillation, the solution concentrated to 4 ml. and decolorized by warming with charcoal. Phenylhydrazine (2 g.) in glacial acetic acid (1.5 ml.) and water (15 ml.) was added to the solution. A red precipitate was formed immediately. This was filtered off after 10 min. and dried at room temperature in vacuo, over P_2O_5, for 48 hr. The solid (250 mg.) became dark red on drying. Calculating on the assumption that the precipitate consisted only of D-glucosazone, the amount of D-glucosone from 1 g. of

D-glucose was 124 mg. (12.4%). The red precipitate was recrystallised twice from hot ethanol. Yellow crystals with m.p. 204°C were obtained. An admixture with an authentic sample of D-glucosazone (m.p. 207°C) melted at 205°C. The $[\alpha]_D^{20}$ (-77°) was that of D-glucosazone.[88] A control reaction with D-glucose (1 g.) showed that under these conditions no solid material was produced.

Expt. 35. Determination of the amount of Carbon Dioxide produced during the Fenton oxidation of D-Glucose

D-Glucose (1 g.) was oxidized in CO_2-free water (Expt. 27) in a stream of CO_2-free nitrogen. At the completion of the oxidation, the solution was warmed to 40°C for 10 min. All the CO_2 evolved was trapped as Na_2CO_3 in the absorption vessel of the combustion apparatus (Expt. 6), and precipitated, dried and weighed as barium carbonate. The amount of barium carbonate produced (32 mg.) was equivalent to 0.49% of the glucose decomposing to give CO_2.

Expt. 36. Determination of Formaldehyde produced in the Fenton oxidation of D-Glucose

D-Glucose (10 g.) was oxidized (Expt. 27) and

the reaction mixture steam distilled at 8 cm. Hg. pressure. The formaldehyde in the distillate was estimated by the chromotropic acid method.[65] The total yield from 10 g. of glucose was 3.2 mg. (0.03%). The experimental details of this type of determination are given in Part II where the same method was used with more positive results.

CARRIER DILUTION ANALYSIS OF THE PRODUCTS OF OXIDATION OF D-[^{14}C] GLUCOSE WITH FENTON'S REAGENT

Expt. 37. Preparation of D-[^{14}C] Glucose samples and oxidation with Fenton's reagent

D-[^{14}C] Glucose, obtained from the Radiochemical Centre, Amersham, was found to be less than 1.0% impure by chromatography. Aliquots of this sample were diluted with B.D.H. "Analar" D-glucose in water, freeze-dried and twice recrystallised from methanol/isopropanol. The specific activities of the resulting materials were determined. In this way 500 mg. of D-[^{14}C] glucose (1229 μC/g.-atom of carbon) and 300 mg. (1165 μC/g.-atom of carbon) were obtained. Several solutions of D-[^{14}C] glucose (*ca.* 100 mg.) were oxidized with Fenton's reagent (Expt. 27). The additions of hydrogen peroxide were made with an "Agla" micrometer syringe.

Expt. 38. A summary of the carrier dilution analyses of the mixture produced in Expt. 37.

A carrier compound was dissolved in each solution and allowed to equilibrate for 24 hr. D-Glucose, potassium D-gluconate, D-mannitol and oxalic acid were isolated and

recrystallised as described for the self-decomposition analyses. Potassium 2-keto-D-gluconate, potassium D-arabonate and D-arabinose were initially purified by chromatography on Whatman No. 3 paper in solvent (e) (acids) and solvent (a) (arabinose), and then further purified by recrystallisation as described above. The details of the analyses are shown in Tables 13 and 14.

Table 13

Details of the carrier dilution analysis of the mixture produced by the Fenton oxidation of D-[^{14}C] Glucose

Carrier	W_G (mg.)	S_o (μC/ g.-atom of carbon)	W_c (g.)	S_i (μC/ g.-atom of carbon	Conc. (%)
D-Glucose	100	1229	3.998	12.24	40.12
Potassium D-Gluconate	100	1229	0.980	17.25	11.68*
Potassium 2-Keto-D-Gluconate	98.7	1165	0.503	6.29	2.32*
D-arabinose	96.9	1165	0.500	1.10	0.49
Potassium D-arabonate	99.0	1165	0.502	3.13	1.11*
Oxalic acid	100	1229	0.500	2.51	1.02
D-Mannitol	100	1229	2.000	0	0

W_G = weight of D-[^{14}C] glucose oxidized
S_o = specific activity of " " " "
W_c = weight of carrier compound added
S_i = specific activity of carrier compound isolated.

* calculated as the free acid.

Table 14. The fall in activities (counts/min.) with recrystallisation

Carrier	No. of recrystallisations											
	1	2	3	4	5	6	7	8	9	10	11	12
D-Glucose	184	-	165	155	164							
Potassium-D-Gluconate	398	245	228	245	240							
Potassium-2-Keto-D-Gluconate	-	107	100	94	93							
D-arabinose	-	18	17	15	15							
Potassium-D-arabonate	-	-	-	-	-	-	80	72	67	59	51	52
Oxalic acid	-	138	48	42	44	42						
D-Mannitol	-	-	-	0.4	0.2							
D-Glucurone	170	81	58	NOT CONSTANT								

D-Glucurone could not be crystallised to constant activity with the quantity of carrier available.

Expt. 39. Examination of the Component from the Fenton oxidation of D-Glucose which was immobile during chromatography

Conditions were found for the fractional precipitation of this compound from the reaction mixture with

acetone. A chromatographically pure sample was obtained from a solution of oxidized D-glucose (20 g.) in water (260 ml.) by the addition of acetone (1.2 l.). The solid was centrifuged down, dissolved in water and freeze-dried. A brown powder (40 mg.) was obtained. A solution of the compound gave only one spot (R_G, 0) on chromatography. Analysis showed that it contained 17.9% or iron. A solution of the product did not yield the characteristic blood red colour with KCNS solution which ferric iron yields. The material dialysed through cellophane. On standing in water at 0°C for ca. 2 months, a small amount of precipitate deposited from solution. This precipitate gave a blood red coloration after solution, and warming in conc. HNO_3 (1 drop). Chromatography in solvent (a) revealed impurities with R_G values of 0.2, 1.0 and a trace at 1.8 (silver nitrate spray).

Expt. 40. Attempt at a separation of Ene-diol containing compounds by ionophoresis of their Molybdate Complexes

Oxidized D-glucose (Expt. 27), L-ascorbic acid, 2-keto-D-gluconic acid and D-gluconic acid were subjected to ionophoresis in 0.2 M-molybdate solution. All

compounds, however, behaved as in phosphate buffer, because of their inherent acidity. The more extensive work on molybdate ionophoresis reported in Part II showed that the idea may be sound if the compound is neutral. D-Glucosone was found to migrate, possibly due to enolization of the keto group.

Expt. 41. Attempt at a chromatographic method for the detection of di-keto groups

Reference solutions (3%) were spotted on paper (Whatman No. 1) and dried. The compounds were then overspotted with one spot of a phenylhydrazine acetate solution (3%). The spots were dried after 1 min. Solvent (a) was found to be the most satisfactory solvent for subsequent chromatography. The effect of N-methyl phenylhydrazine and of o-phenylene diamine (in ethanol) was also investigated. Component 5 of the self-decomposition product was also chromatographed with the latter reagent. Chromatograms were sprayed with silver nitrate-sodium hydroxide reagent. The results are given in Tables 15, 16 and 17 overleaf.

Table 15 - Phenylhydrazine

Compound	R_F values of resulting spots
D-Glucose	0.15 (glucose); 0.62; 0.85 (unchanged reagent)
2-keto-D-Gluconic acid	0.02 (acid); 0.30; 0.85 (unchanged reagent)

Table 16 - N-methylphenylhydrazine

Compound	R_F values of resulting spots
D-Glucose	0.15 (glucose); 0.70; 0.90 (unchanged reagent)
2-Keto-D-Gluconic acid	0.02 (acid); 0.25; 0.90 (unchanged reagent)

Table 17 - o-phenylenediamine

Compound	R_F values of resulting spots
D-Glucose	0.15 (glucose); 0.40; 0.80 (unchanged reagent)
2-Keto-D-Gluconic acid	0.02 (acid); 0.50; 0.80 (unchanged reagent)
Component 5	0.15; 0.40*; 0.80 (unchanged reagent)

* detection with X-ray film.

Expt. 42. Determination of the amounts of Water in freeze-dried samples of D-Glucose and D-Glucitol

Two samples of D-glucose (1.147 g. and 0.243 g.) were dissolved in water (10 ml. each) and freeze-dried. The weights of the freeze-dried samples (1.248 g. and 0.269 g.) showed that they contained 8.12% and 9.79% of water respectively.

Three samples of D-glucitol (1.0145 g., 0.211 g. and 0.114 g.) were dissolved in water (10 ml. each) and freeze-dried. The weights of the freeze-dried samples (1.002 g., 0.220 g. and 0.143 g.) showed that they contained 7.10%, 2.64% and 2.01% of water respectively.

EXAMINATION OF THE PRODUCTS OF SELF-DECOMPOSITION OF D-[^{14}C] GLUCITOL AND A COMPARISON WITH THOSE OF THE OXIDATION OF D-GLUCITOL WITH FENTON'S REAGENT

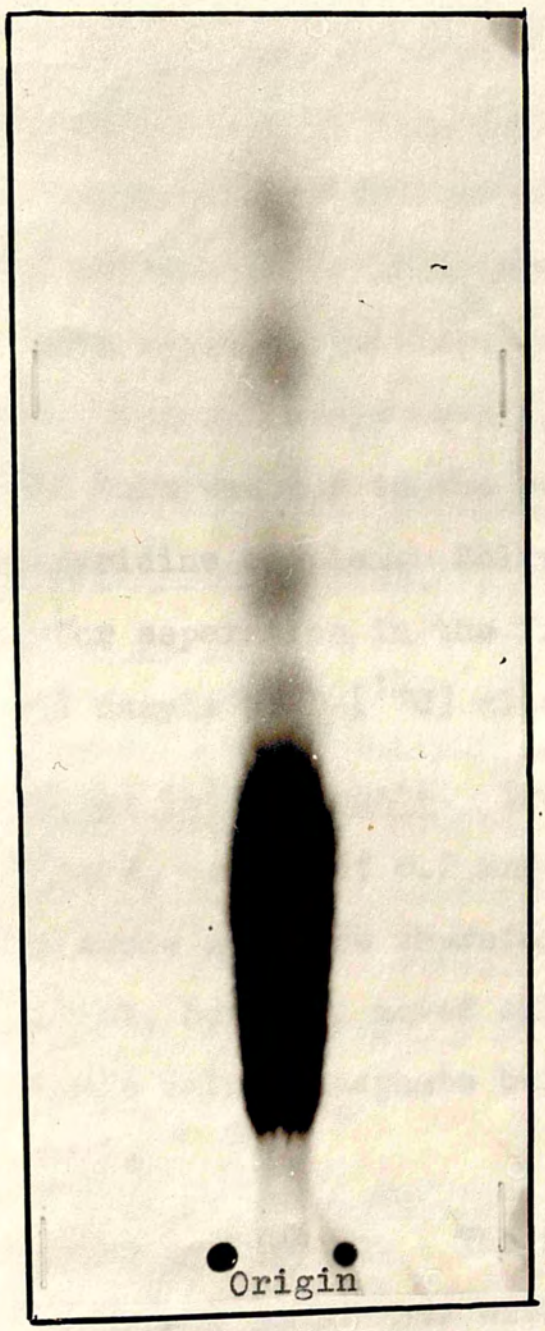
Expt. 43. Chromatographic analysis of D-[^{14}C] Glucitol and estimation of the Rate of self-decomposition

D-[^{14}C] Glucitol (specific radioactivity, 7.43 mC/m.mole) was chromatographed, six months after preparation, using solvent (a). A radiochromatogram revealed impurities with R_G values of 0.2 (trace) and 2.6, the unchanged D-[^{14}C] glucitol having an R_G value of 1.0. A radiochromatogram prepared using solvent (e) revealed impurities with R_G values of 0.2 (trace), 2.1 and 2.3. A similar separation using solvent (g) revealed 6 well-defined spots with R_G values of 0.2 (trace), 0.5 (glucitol), 0.90, 1.05, 1.44 and 1.66. The distribution of radioactivity on this chromatogram was measured and the impurity found to constitute 1.7% of the total.

Another sample of D-[^{14}C] glucitol, one year after preparation, was shown by this method to contain 3.5% impurity. The same 6 components were present (Fig. 9).

Figure 9.

Radiochromatogram of the Products of Self-decomposition of D-[^{14}C]Glucitol.



Two dimensional chromatography/ionophoresis analyses

Solvent (g), though excellent for the one-dimensional separation of impurities, was not suitable for use in conjunction with ionophoresis techniques. Both borate and phosphate ionophoresis, after chromatography in this solvent, resolved D-glucitol into two components of approximately equal quantity. It was thought that this was due to the partial formation of a glucitol-pyridine complex. Solvent (a) was used, therefore, for separation in the first dimension. The one-year-old sample of D-[^{14}C] glucitol was used.

(i) Solvent (a)/Phosphate. Traces of material (0.38%), with R_G values of 0.2 and 2.5, migrated towards the anode and were therefore acidic. The bulk of the material, however, moved only by endosmosis. A repeat analysis using phosphate buffer at pH 10.0 gave the same result.

(ii) Solvent (a)/Borate. The main impurity (R_G, 2.6) was resolved into 3 components with M_G values of 0.37, 0.64 and 0.78. Assay of radioactivity showed that the ratios of these components were ca. 3:5:4. The acidic materials moved as in part (i) above.

(iii) Solvent (a)/Molybdate. Components with R_G values of 1.0 and M_S values of 0 and 1.1 suggested the presence of small amounts of glucose and gulose in the mixture. Faint spots were present in the fructose and sorbose regions but they represented a minute amount of activity (<0.01%) and were not considered further. The main impurity (R_G, 2.6) was resolved into 2 spots with M_S values of 0 and 1.0.

Expt. 44. The oxidation of D-Glucitol with Fenton's reagent and determination of the amount of acid produced

D-Glucitol (1 g.) was oxidized with Fenton's reagent (Expt. 27) and the resulting solution made up to 25 ml. An aliquot of this solution (5 ml.) required 3.3 ml. of 0.056 N-NaOH to neutralize it. This titre corresponded to 16.8% conversion of glucitol to a 6-carbon monocarboxylic acid.

Expt. 45. Chromatographic analysis of oxidized D-Glucitol (Expt. 44)

Chromatography using solvent (a) resolved the mixture into 9 components with R_G values of 0, 0.12, 0.24, 0.35, 1.0, 1.2, 1.54, 1.6 and 1.75. The amount of

material with R_G value <1.0 was very much less than in the D-glucose oxidation product. It was apparent that most of the product appeared in the region with R_G value 1.0 to 1.2. Part of the chromatogram was sprayed with urea phosphate [Expt. 1(v)]. The blue ketose colour developed strongly at R_G, 1.2 and weakly at R_G, 1.0. It was found that, of those used, solvent (f) separated the products most efficiently. The trichloroacetic acid/p-anisidine reagent [Expt. 1(iii)] made possible the simultaneous detection of aldoses and ketoses. The analysis using the above combination is reported in Table 18.

Table 18

Component	R_G value	Colour	Reference compound*	Colour
1	0.64	Faint brown	D-Glucosone(0.65)	Faint brown
2	0.79	Brown	D-Gulose(0.80)	Brown
3	1.03	Brown	D-Glucose(1.0)	Brown
4	1.17	Yellow (in cold)	L-Sorbose(1.17)	Yellow (in cold)
5	1.55	Yellow (in cold)	D-Fructose(1.55)	Yellow (in cold)
6	1.93	Pink	-	-

* R_G values in parenthesis.

Expt. 46. Ionophoresis of oxidised D-Glucitol (Expt. 44)

(i) Borate ionophoresis. A separation of the products could not be obtained.

(ii) Molybdate ionophoresis. The mixture was resolved into 7 components. Reference compounds and specific detecting reagents aided the analysis as reported in Table 19.

Table 19

Component	M_S value	Reference Compound	M_S value
1	0*	D-Glucose	0*
2	0.37 /	L-Sorbose	0.37 /
3	0.60 /	D-Fructose	0.62 /
4	0.83	D-Glucosone	0.87
5	1.0	D-Glucitol	1.0
6	1.14*	D-Gulose	1.15*
7	1.3	D-Gluconic acid	1.3

* Brownish blue on heating to 100°C with p-anisidine reagent.[74]

/ Visible in U.V. light without heating, after treatment with trichloroacetic acid and p-anisidine.[75]

PART II

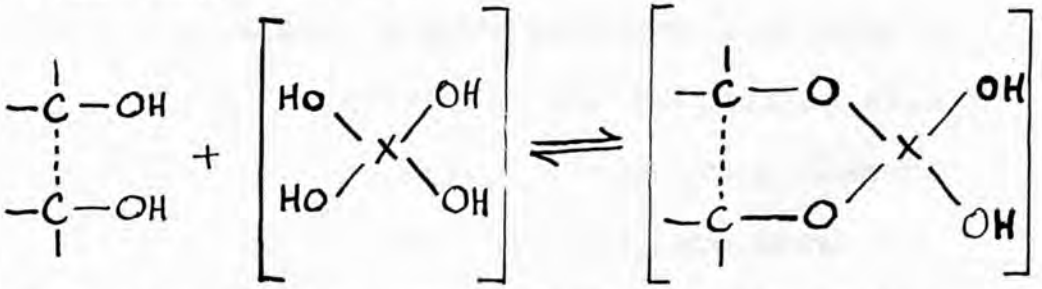
REACTIONS OF CARBOHYDRATES WITH INORGANIC OXY-ACIDS

THEORETICAL

INTRODUCTION

Many elements form oxy-acids which are capable of reversible complexing with polyhydroxy compounds. Among these are boron,[89] germanium,[90] tellurium,[91] vanadium, chromium, molybdenum, tungsten and uranium.[92] The acids vary greatly in their tendency to form complexes in this way. The borate ion, for example, forms a strong complex in alkaline solution which is broken down as the solution is neutralised. The molybdate ion behaves in the reverse fashion.

The complexes are cyclic esters[89,90] formed in the following manner:-



The effect of pH on the equilibrium depends on the change of charge in any of the reactions leading to complex formation. For example, if a proton is released in an equilibrium reaction leading to complex formation,

an alkaline medium will favour that reaction, and a complexing will be enhanced.

Complexes possess properties different from those of the parent polyhydroxy compounds and thus their stabilities may be studied by the measurement of changes in these properties. The optical rotations of optically active compounds are changed by complexing and, in the case of the alditols, enhanced to a remarkable degree. The ionic character of the complexes causes them to migrate in an electric field, and hence the technique of ionophoresis has been applied to neutral carbohydrates.[80]

With the advent of modern techniques of separation and analysis of carbohydrates, these complexes are gaining in importance. A more profound knowledge of the stereochemical requirements for complexing will be also of use in biochemistry. Some trace elements, for example, molybdenum and vanadium, are known to exist as their oxy-anions in biological systems. Experiments on the activation and deactivation of enzymes suggest that reversible complexing plays an important part in natural processes.

A limited number of anions, possessing a central atom of a particular size (2.5-3.0 Å) show unique behaviour. The periodate and bismuthate ions[93] are examples of such. Complexes between α-glycols and such ions, once formed, rapidly rearrange with subsequent breakdown of the C-C bond. The periodate ion has become very important in carbohydrate chemistry because of this property.

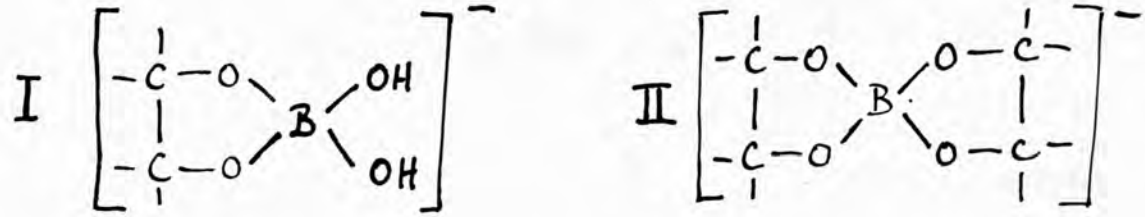
The purpose of the present investigation was to find whether the borate complex was stable enough to change the course of chemical reactions. If oxyanions of this type could change the course of periodate oxidation, for example, the technique would be of use in determining the structures of such complexes. The problem also entailed the possible use of these anions in directing the course of a reaction to enhance the yield of a particular compound. Borate has been used in this capacity in the selective benzoylation of methyl glucoside.[94]

The second section consists of the attempts made to determine the stereochemical requirements for the complexing of polyhydroxy compounds with molybdate

and to develop useful chromatographic and ionophoretic methods for the separation of carbohydrates as their molybdate complexes.

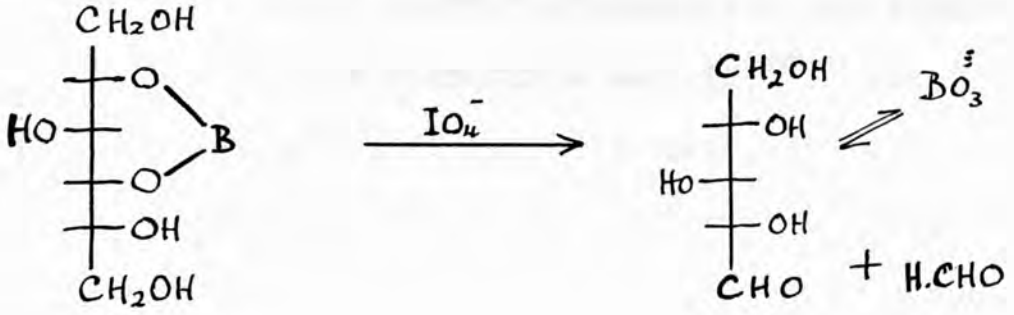
The Borate-Carbohydrate Complex

Migration of neutral sugars, during ionophoresis, occurs at an alkaline pH in the presence of borate ions[95] and results from the formation of the weakly negatively charged complexes (I) and (II).



In acid media, the equilibria, which involve boric acid, lie well to the side of the boric acid. With increasing pH, however, the concentrations of (I) and (II) increase. The effect of this type of complexing on the conductivity of 0.5M boric acid at 25°C has been reviewed by Boeseken.[96] The general interaction of boric acid with polyols has been described by Isbell et al.[97] and by Zittle.[98] Foster[99], by the ionophoresis of sugar derivatives has clearly established that, in the aldehydo-form

of D-glucose, the C(2) and C(4) hydroxyls are the most favoured for complexing. It is clear from Foster's results that the C(2) and C(4) hydroxyls of D-glucitol should be favoured in complexing. If this is the case, and the complex is stable under the conditions of periodate oxidation, the oxidation of D-glucitol in borate should yield L-xylose.



The Mechanism of Periodate Oxidation

Periodate oxidation has been briefly mentioned. It is a technique of immense importance in carbohydrate chemistry and has been very well reviewed.[100,101] Consequently, the scope and experimental details of the reaction will not be discussed. There are, however, certain factors which must be noted as they play an important part in the discussion.

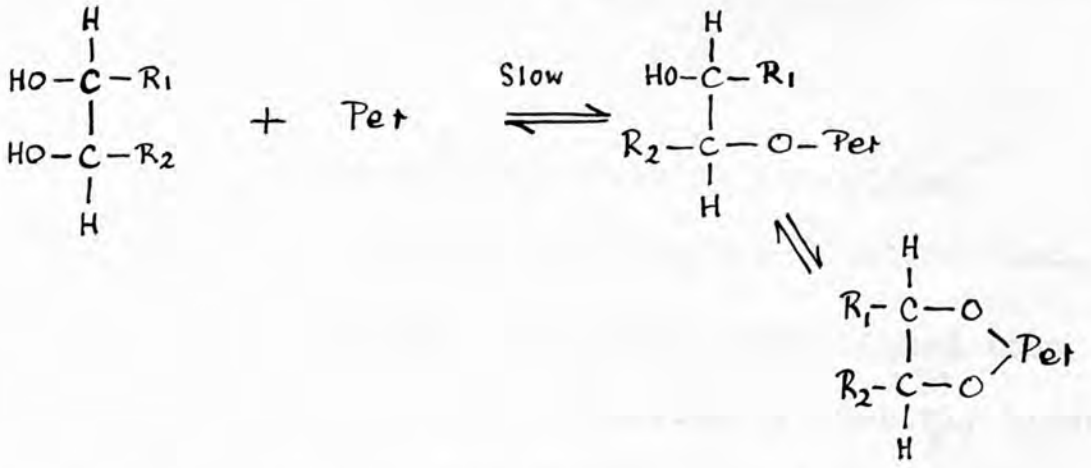
The exact course of periodate oxidation

has not yet been fully established. A cyclic ester intermediate was proposed by Criegee et al.[102] Price et al.[103] enlarged upon this proposal by suggesting an initial rearward approach of the periodate ion to the glycol molecule. Heidt et al.[93] correlated ionic radii and oxidation potentials for the periodate-glycol and the lead tetra-acetate-glycol reactions and correctly predicted the similar reactivity of sodium bismuthate and Ag^{+++}. Duke[104] studied the variation of reaction rate with varying, high concentrations of glycol. His results were interpreted as indicating the presence of an intermediate co-ordination complex whose formation must preceed that of the final products. This work was repeated in more detail by Taylor[105] who suggested either the formation of an inactive complex which was in equilibrium with reactive periodate and glycol or that the formation of the intermediate was a reversible reaction. These ideas have been extended further[106, 107]. Reversible intermediate formation is favoured by Bunton and co-workers[108-111]. From kinetic studies of the oxidation of some open chain glycols, it was possible to calculate both the equil-

ibrium constant for the formation of a cyclic intermediate, and the first order rate constant for its breakdown to products. This simple treatment was applied to the reaction:-

$$\text{Glycol} + \text{Per.} \rightleftharpoons \text{Intermediate} \longrightarrow \text{Products.}$$

The evidence could give no information on the mechanism by which the intermediate is formed. It was noticed[109] that the addition of ethylene glycol to a solution of periodate in the pH range 9-11, at 0°C, gave a change in optical density slow enough to measure but much faster than the overall reaction. It was assumed that this initial change was due to the formation of the intermediate. The utilisation of this change and a consideration of the behaviour of substituted glycols suggested that the formation of the intermediate was a two-stage reaction. The first stage, the formation of a monochelated intermediate, was a slow reaction which was followed by rapid cyclisation.



(Per = Periodate residue)

It is in these early stages of the reaction that the pH of the medium seems to play an important part. This factor will be considered later in the discussion.

The structure of the cyclic intermediate is also, as yet, uncertain. Structures other than the cyclic form have been proposed.[112] In most of these only one of the hydroxyl oxygen atoms is bonded directly to the iodine atom, the other being attached via a hydrogen bond. It is considered[109], however, that the five membered ring is the only structure which explains the effect of changes in the glycol structure on the formation and breakdown of the intermediate. Models of the hypothetical structure were made up[109] with dimensions based on those of ammonium trihydrogen paraperiodate. These models

showed that the ring is probably not planar, but slightly puckered to relieve O-O interaction.

It has been fairly well established from a consideration of the oxidation of sterically hindered compounds[113] that the initial periodate attack is a rearward one. Both stages of the esterification are accompanied by inversion of configuration.[114]

Steric Factors in the Oxidation of Polyols

A polyhydric alcohol possesses a number of hydroxyl groups varying in their spatial relationship to one another. Thus some pairs will be more favourably placed than others for periodate oxidation. If a deficiency of periodate be added to a polyol, the products will be largely those arising from the cleavage of the most readily broken bonds. Hence, a "pattern" of products will emerge, directly related to the ease of cleavage of the various C-C bonds in the molecule. The presence of a complexing ion or molecule in the reaction mixture may alter the availability of certain hydroxyl groups and hence change this "pattern". The D-glucitol-borate complex was examined by this method. Firstly, however, it was

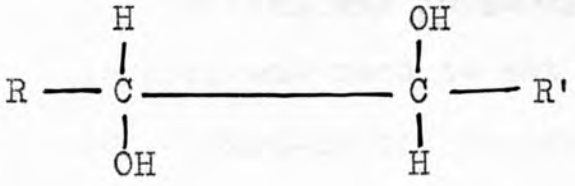
necessary to ascertain the oxidation pattern of polyols under normal conditions.

Qualitative work by Schwarz[115] and semi-quantitative work by Courtois and Guernet[116] clearly showed that the C-C bonds between α-trans (αT) hydroxyl groups (in the Fischer projection formula) were most readily broken by periodate. That is, a deficiency of periodate reacting on D-glucitol produces mainly glyceraldehyde and erythrose. The oxidation of erythritol in this manner yielded more glyceraldehyde than glycol-aldehyde.

i.e. α-glycols are more readily cleaved than α-cis glycols. These workers also found that the products of oxidation were more rapidly attacked than the original molecule. They suggested that "everything takes place as though periodate completed the degradation of the polyol molecule on which it began the attack". The possibility that the products appeared in a form more reactive with periodate was refuted. The same products were found on the successive addition of small amounts of periodate to a polyol as on the bulk addition of the

same quantity.

The periodate oxidation of diastereoisomeric diols has been quantitatively examined.[117] It was found that, with open chain compounds containing vicinal hydroxyl groups, the threo-isomer (αT) was oxidized, more rapidly than the erythro-isomer (αC). The rate of oxidation in a threo series of compounds was decreased by increasing the length of R or R'.



No full quantitative results were available for the partial periodate oxidation of D-glucitol. It was necessary, therefore, to determine the products of oxidation of pure D-glucitol as well as those from the D-glucitol-borate complex.

THE REACTION CONDITIONS

The periodate oxidation of the polyol-oxy-acid complex was carried out under conditions such that the complex possessed maximum stability. These conditions for the borate ion were two-fold. The solution had to be concentrated and the pH at, or above, 10.[97] This entailed oxidation under conditions which might be expected to give some anomalous results. A control reaction, carried out in phosphate buffer of the same pH and molarity, was used to act as a check for anomalous effects. Certain discrepancies have already been found using phosphate buffer.[118] In spite of this, it was felt that similar discrepancies may result in borate buffer and therefore that the conditions would constitute a valid comparison. Therefore, three oxidations were carried out in each case: A - D-glucitol in water; B - D-glucitol in 0.5 M borate buffer (pH 10); and C - D-glucitol in 0.5 M-phosphate buffer (pH 10) [Expt. 48]. Schwarz,[115] using 0.1 mole of periodate to 1 mole of D-glucitol, found that less than 1% of the products were oxidized. To obtain a higher yield of products, 0.25 mole of periodate was used in these experiments. Under these conditions ca. 2% of the periodate was used on the products in the non-

buffered oxidation (Table 21).

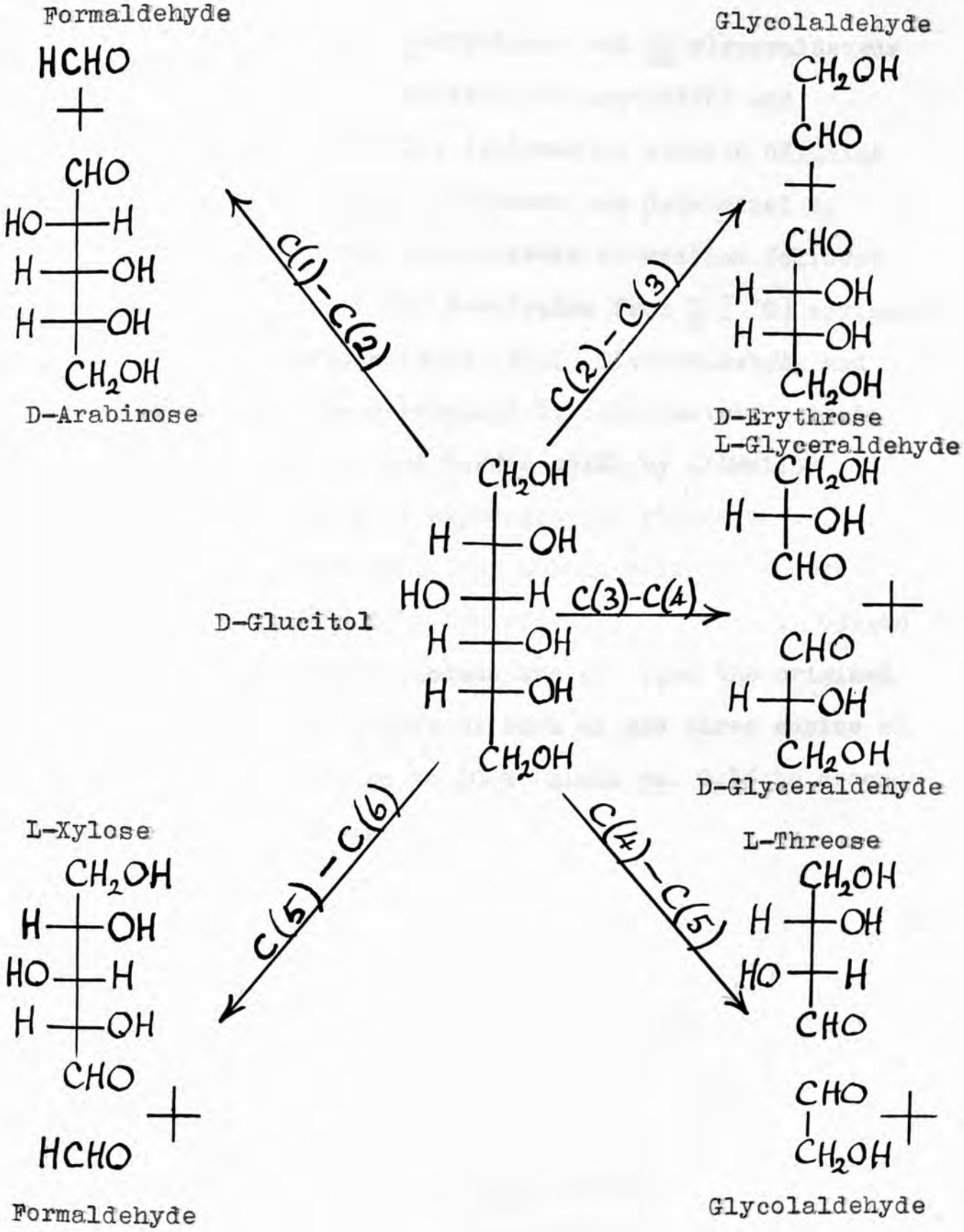
Previous workers[115,116] allowed solutions of periodate reactions to stand for over an hour, when periodate deficiencies were used. It was inadvisable to allow this in the present experiments because most of the products were alkali-sensitive and would have been damaged by prolonged solution at pH 10. The reaction time allowed was 10 minutes. Periodate was shown to have disappeared after 5 minutes (Expt. 48); therefore the problem of incomplete oxidation did not arise. Chromatographic results (Expt. 47) indicated that the borate complex altered the proportions of products. The effect was more noticeable when the oxidation was carried out in 4M, rather than 0.5M, borate buffer.

Analysis of the Products

The possible products from the cleavage of the various C-C bonds are shown in Fig. 10. The yields were determined by a variety of methods. D-[^{14}C] Glucitol and carrier dilution analysis were used to obtain the yields of D-glucitol, D-arabinose and L-

Figure 10.

Products formed on the cleavage of the various C-C bonds of D-Glucitol by Periodate.



xylose (Expt. 49). D-Erythrose and DL-glyceraldehyde were determined by reduction to erythritol and glycerol, respectively, followed by carrier dilution analysis (Expt. 50). L-Threose was determined by chromatographic and ionophoretic separation followed by a comparison of the β-emission from D-[^{14}C] erythrose and L-[^{14}C] threose (Expt. 51). Glycolaldehyde and formaldehyde were determined by colorimetric methods (Expts. 52 and 53) and formic acid, by titration (Expt. 54). Table 20 expresses the yields on a weight per cent basis. The formic acid values are corrected for the fact the molecule contains an oxygen atom derived from periodate and not from the original D-glucitol. The totals of each of the three series of values should add up to 100% (minus ca. 0.3% to account for loss of hydrogen).

Table 20. The weight per cent yields of products of the periodate oxidation of D-glucitol

Product	Conditions		
	A(Control)	B(Borate)	C(Phosphate)
D-Glucitol	74.98	91.20	89.75
D-Arabinose	0.38	0.63	0.39
L-Xylose	0.76	1.75	0.88
D-Erythrose	3.16	0.20	0.53
L-Threose	0.99	0.04	-
DL-glyceraldehyde	13.61	0.75	1.90
Glycolaldehyde	2.79	2.37	2.70
Formaldehyde	0.24	0.27	0.19
Formic acid	0.08	0.69	1.28
TOTALS	96.99	97.90	97.62

The above table serves as a convenient check on the completeness of the analyses. A more convenient expression of the results for a discussion is obtained by calculating the molar yield of a compound from 1 mole of D-glucitol (Table 35.p.242) and hence, the percentage of the added periodate used on producing that compound. Table 21 shows the results expressed thus.

Table 21. The percentage of periodate consumed by the various C-C bonds of D-Glucitol

	C-C bond	Type of glycol	% based on yield of:-	A (Control)	B (Borate)	C (Phosphate)
1	1-2	α	Arabinose	1.84	3.04	1.88
2	5-6	α	Xylose	3.68	8.48	4.28
3	1-2,5-6	α	Formaldehyde	5.84	6.52	4.60
4	2-3	αT	Erythrose	19.84	1.24	3.32
5	4-5	αC	Threose	6.60	0.28	-
6	2-3,4-5	αT + αC	Glycolaldehyde	33.84	28.80	32.76
7	3-4	αT	Glyceraldehyde	55.04	3.04	7.68
8	Oxidation of products		Formic acid	2.04	16.76	31.16

THE PERIODATE OXIDATION OF PURE D-GLUCITOL

Observations from the Experimental Results

The weight percentage yields of the products (Table 20-A) indicate that the analysis was satisfactory within the limits of the methods used. Table 21-A expresses the availability of the various pairs of hydroxyl groups for the periodate reaction. Immediately obvious from the table are the ~~high~~ large amounts of periodate used in producing DL-glyceraldehyde, D-erythrose and glycolaldehyde, i.e. the high rate of cleavage of αT glycol groups. This result is in agreement with other workers.[115-117] The cleavage of αC pairs of hydroxyl groups is unfavoured and α pairs appear to be least attacked (low pentose yield). Hence the rates of cleavage of the three groups are in the order $\alpha T \gg \alpha C > \alpha$. The work ~~115~~ described on p.163 suggests that the order should be $\alpha T \gg \alpha > \alpha C$. The order of α and αC obtained in the present work may be incorrect (because of an unreliable L-threose value). The discrepancy will be discussed after a theoretical treatment of the problem. Formic acid, which could arise only by the oxidation of the products, was present

in very low yield. This fact with the correct D-glucitol value (74.98%, extreme accuracy fortuitous) indicated that an 0.25 molar proportion of periodate caused very little degradation of the products in the control solution, and that the conditions of the experiment were valid.

Summary of the Reaction Mechanism

Before considering stereochemical factors the current ideas on the mechanism of the oxidation will be summarised (see Introduction). It will be assumed that there are three main stages in the reaction.

I. The slow reversible formation, by rearward attack, of a monochelated hexitol-periodate complex, accompanied by inversion of the configuration of the carbon atom involved.

II. A rapid reversible cyclisation, with inversion, to form a more or less planar 5-membered ring.

III. A rapid irreversible decomposition of this intermediate to the final products.

Steric Considerations in the Reaction

It is only in stages I and II that steric factors

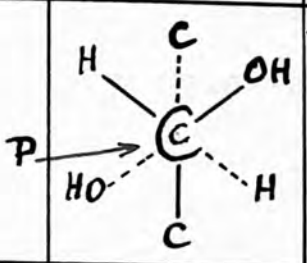
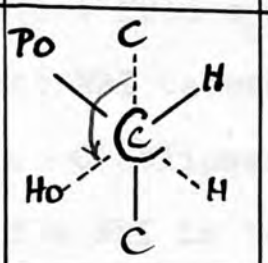
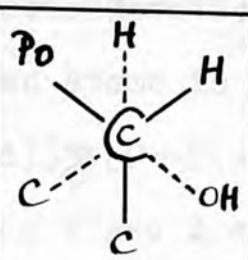
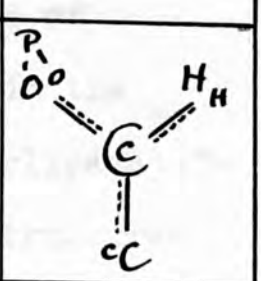
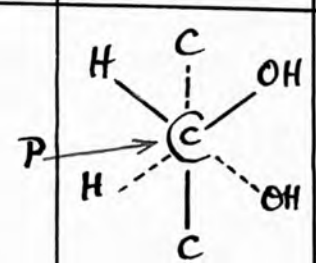
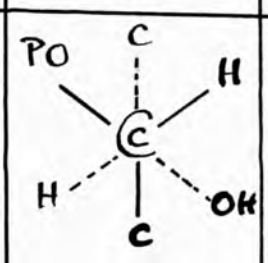
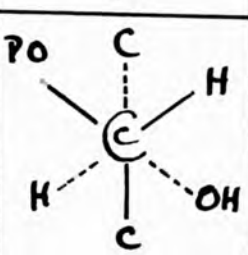
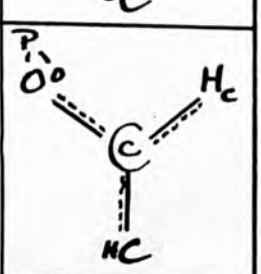
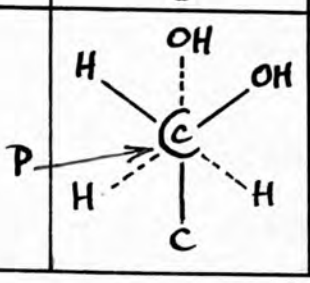
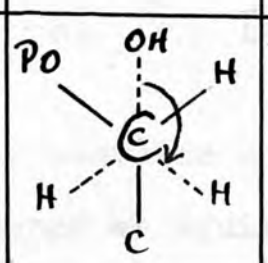
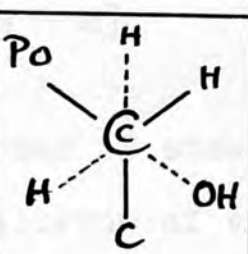
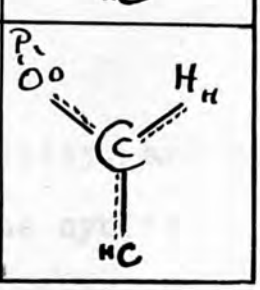
will affect the rate of oxidation as these are the rate controlling stages. As the reaction to form the cyclic intermediate is reversible, the problem is analogous to that of cyclic acetal formation. Barker et al.[119] extended the empirical rules of Hann et al.[120] for cyclic acetal formation with hexitols. These rules were explained on a planar zig-zag structure for the hexitol and took into consideration the amount of steric hindrance against the rotation of C-C bonds to form the acetal structure. Mills[121] is of the opinion that a study of the end product of the reaction is a safer approach to this problem, and to other reversible reactions. This is because deductions based on the conformations of the reactants would only be sound if these conformations and the mechanisms of the reactions were well established. Applied to periodate oxidation, the "end product of reaction" (above) is the cyclic intermediate. Both approaches were applied and the results found to be slightly different, the former approach appearing the more realistic.

Mills took into account only the steric

hindrance in the formed 5-membered acetal ring and suggested that the structure with the greatest number of eclipsed substituents would be least favoured. The empirical approach of Barker et al. has been applied to the present problem with slight modifications involving the various stages of intermediate formation. Stages I and II of the mechanism are split up into three intermediate stages X, Y and Z (Fig. 11). The treatment takes into account the rearward approach of the periodate ion along the line of the C-O bond (W); the conversion of the resulting conformation (X) of the monochelated derivative into conformation Y, which is required for a rearward attack of the periodate on the adjacent carbon atom; and finally, cyclisation to structure Z.

Figure 11.

The intermediate stages in the formation of the cyclic periodate-glycol complex.

Type of Glycol (Fischer formula)	"W" Natural arrangement in Polyol zig-zag chain	"X" Arrangement after initial periodate attack	"Y" Arrangement for Cyclisation	"Z" Arrangement after Cyclisation
∝C				
∝T				
∝				

P = Periodate residue.

The Relative Ease of Cleavage of Glycols with αC-, αT- and α- Hydroxyl groups

(i) A Consideration of Non-bonded Interaction (NBI) in the Cyclic Intermediate, Z (Mills approach)

It is apparent from a consideration of molecular models that NBI between atoms is in the following order: [C---C(eclipsed)] $\gg$ [C---H(eclipsed)] $>$ [H---H(eclipsed)]. The NBI in the three Z structures are:-

αC - [C----C] + [H----H]

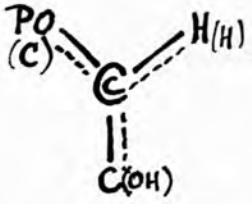
αT - 2[C----H]

α - [C----H] + [H----H]

It is clear that the order of stability, and hence the order of preference at equilibrium of the cyclic intermediate is α $>$ αT $>$ αC. The rates of cleavage will also be in this order. The order is not found experimentally and for a better interpretation of the results, the X and Y structures were examined.

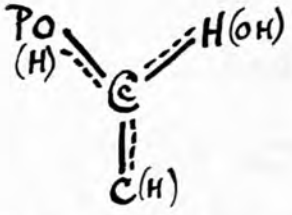
(ii) Steric Hindrance in the Conversion of Conformation X into Y (Barker, Bourne and Whiffen approach)

The monochelated derivative X, produced from αT hydroxyls (W) has the correct conformation for cyclisation, i.e. X is identical with Y, and no additional energy is required before cyclisation can occur. The αC-X derivative, however, requires rotation of 120° about the glycol C-C bond for its conversion to αC-Y. During this rotation (60°)



(a) is produced.

Similarly the α-X structure must rotate 120° around its C-C bond to form the α-Y structure. During this rotation (60°)



(b) is produced.

Clearly the NBI in structure (a) above is much greater than that in structure (b) as it involves the strong [C----O] interaction. Consequently αC cyclisation and cleavage is the least likely of the three possibilities and αT the most. The order of glycol cleavage is then,

$$\alpha T > \alpha > \alpha C$$

This order agrees with experimental work in the literature. The discrepancy between it and the order in the present experiments is probably due to an incorrect L-threose value. This compound could only be determined by comparing paper β-emission from chromatograms with that from another compound. It was the least reliable of the determinations.

The Varying Cleavage Rates of αT-glycol Groups in D-Glucitol

Previous workers in the polyol-periodate field[115-116] have not searched for finer differences between rates of bond cleavage which were necessary for this control to be of any value. Consequently, cleavage rates of αT systems have been considered as more or less equal. The products of αT cleavage of D-glucitol are DL-glyceraldehyde (G) and D-erythrose (E). Assuming that α and αC cleavage is negligible and that attack on C(2), C(3) and C(4), from behind, is equally likely, attack on these three carbon atoms should yield:

(a) C(2) $\xrightarrow{\text{C(2) - C(3) cleavage}}$ E

(b) C(3) $\xrightarrow{\text{C(3) - C(2) and C(3) - C(4) cleavage}}$ $\frac{1}{2}$(2G + E)

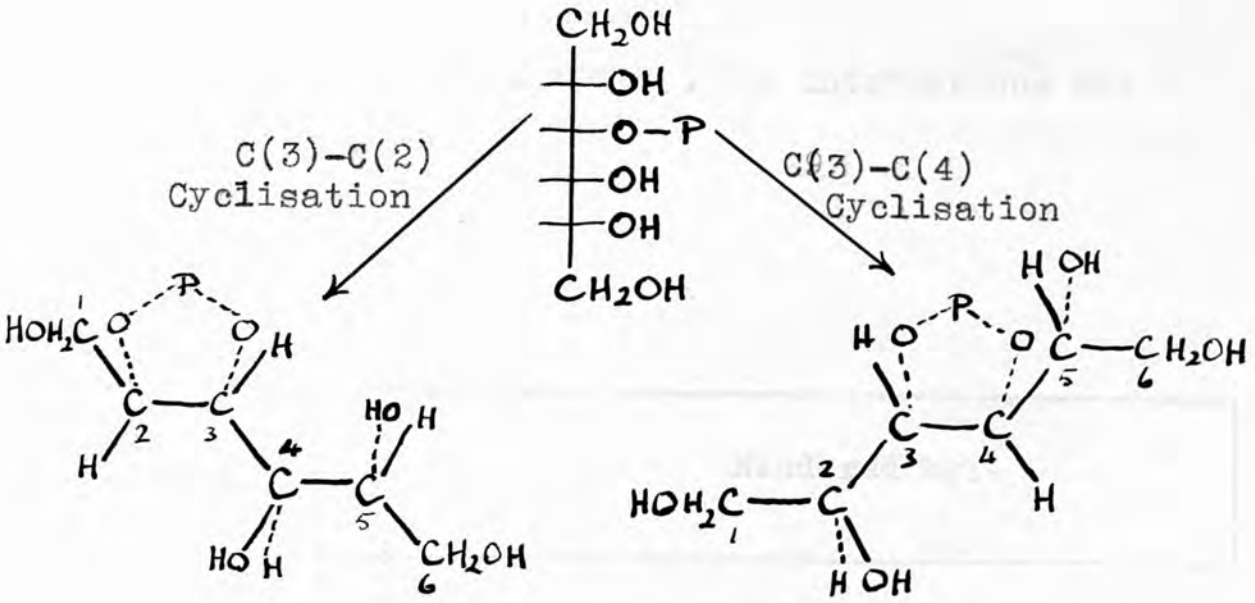
(c) C(4) $\xrightarrow{\text{C(4) - C(3) cleavage}}$ 2G

The sum of products is 3G + 1.5E, i.e. the molar yield of G is twice that of E.

The above treatment assumes that C(3)→C(2) cyclisation and C(3)→C(4) cyclisation are equally likely. The experimental ratio of molar yields (Table 35) is $^{2752}/_{496}$ = 5.55, i.e. the cyclisation to yield glyceraldehyde occurs much more readily than C(3)→C(2) cyclisation. There are other factors which enhance the production of glyceraldehyde. All the factors will be considered in their probable order of importance.

(iii) Steric hindrance from βC oxygen atoms

An initial attack on C(3) leads to either glyceraldehyde or erythrose. A consideration of the stereochemistry of the two cyclic intermediates (A) and (B) affords a reason why glyceraldehyde should preponderate in the products. It is assumed that the molecule adopts a conformation in which the carbon atoms and the terminal oxygen atoms are, when possible, in a planar zig-zag chain. The difference in the free energy change on the formation of intermediates (A) and (B) from the monochelated intermediate



A B

C-C chains approximately in plane of paper; substituents above plane (unbroken lines) or below (broken lines).

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is due to the difference of NBI of O(3) - O(5) and O (4) - O(5), respectively. The distance between O(3) and O(5) is 2.51 Å and that between O(4) and O(5) is 2.83 Å. The combined van der Waals radii of two oxygen atoms is 2.80 Å. [122] It is thus clear that cyclisation involving C(3) and C(4) [B] will occur more readily than that involving C(3) and C(2) [A].

(iv) Steric hindrance to the path of initial attack

The approach line of periodate to C(1) and C(6) (along the line of the C-O bond) may be hindered by substituents on C(3) and C(4) respectively. The approach to other carbon atoms is hindered by substituents on

their adjacent carbon atoms. The interactions are shown in Table 22.

Table 22

Attack on carbon:-	Hindered by:-
1	βH + β OH*
2	H + H or H + OH [from rotn. of C(1)]
3	H + H
4	H + OH
5	H + OH or OH + OH [from rotn. of C(6)]
6	βH + β OH*

* when terminal oxygens are continuation of planar zig-zag.

Carbon (3) is most easily attacked. This factor will support factor (iii) [above] and enhance the yield of glyceraldehyde.

(v) Steric interaction in the planar bimolecular transition complex formed during the periodate attack.[123]

The examination of molecular models indicated that the formation of monochelated derivatives involving C(2) and C(4) is unfavoured. There is considerable steric interaction in the planar transition complexes, caused by the close approach of the βC hydroxyls of C(2) and C(4) when either carbon atom is attacked. This interaction will raise the activation energy of the reaction and reduce its rate accordingly. Hence C(3) is more likely to be attacked than C(2) or C(4) and thus factor (iii) [above] is enhanced.

The Preponderance of L-Xylose over D-Arabinose in the Products of Partial Periodate Oxidation of D-Glucitol

Pentose is produced from D-glucitol by attack on C(1), C(2), C(5), and C(6). A consideration of [O----O] NBI [Factor (iii)] shows that arabinose is the more likely product. This factor is probably swamped by factor (vi) [below].

(vi) Competition in cyclisation from neighbouring carbon atoms

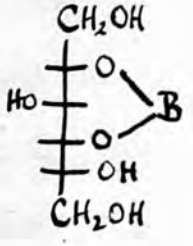
Attack on C(1), C(2), C(5), and C(6) is followed by 6 possible cyclisations: C(1)→C(2); C(2)→C(1); C(2)→C(3); C(5)→C(4); C(5)→C(6); and C(6)→C(5). The first two of these result in the production of arabinose. C(2)→C(3) is an αT cyclisation and has already been shown to occur much more readily than α or αC cyclisations. Thus attack on C(2) is eliminated as a likely route to arabinose. Similarly, attack of C(5) produces more xylose than threose (α cyclisation > αC cyclisation). Table 22 also shows that attack by periodate on C(1) and C(6) is probably less hindered than attack on C(2) and C(5).

THE EFFECT OF BORATE BUFFER ON THE PERIODATE OXIDATION OF D-GLUCITOL

The oxidation in borate buffer produced a very different set of results from that in water (Tables 20 and 21). The phosphate buffer control (column C of tables), however, showed that some of these differences were due to the high pH of the reaction medium. The two effects are now explained and it is hoped to show how they combined to give a very good yield of L-xylose under certain conditions.

The Effect of Borate Ion

It is probable that a monoborate-glucitol complex will have a structure involving principally the C(2) and C(4) hydroxyls.[99] If this complex were 100% stable, periodate would cleave only the C(5) and C(6) bond, producing L-xylose. It was decided to use the pentose ratio, xylose/arabinose, to measure the effect of borate as the yields of these compounds were not affected greatly by high pH (cf. phosphate buffer results).



The yield of xylose, relative to that of

arabinose (Table 23) was increased by the presence of borate. Tables 20 and 21 show that xylose was a major product in the borate buffered reaction.

Table 23

Water	Borate	Phosphate
2.0	2.8	2.27

Hence, the theory that the complex may enhance xylose formation has been verified. The above structure of the borate complex shows that arabinose formation should have been suppressed in this reaction. It was enhanced, however. This cannot be explained on any theory involving oxidation of the products in the control reaction, as it has been established that very little periodate was used in this way. It may be that complexes are formed involving the C(3): C(5) or C(4): C(6) hydroxyl pairs. As the borate-glucitol complex is in equilibrium with the constituents, it is possible, in the statistical sense, to have in the reaction mixture, small quantities of complex which contain free

C(1) and C(2) hydroxyl groups.

The Effect of High pH

Under-oxidation of glucitol occurred in basic solution. Other facts were that glyceraldehyde, erythrose and formaldehyde were suppressed to a low level, and formic acid production enhanced by a factor of ca. 10. It was clear that more oxidation of the products was taking place in the reaction at pH 10 than at pH 7. Since arabinose and erythrose would arise from glucitol and not from the 2:4 borate of glucitol, the ratio of these compounds should be the same in all three reactions. The ratios (0.09 [A], 2.4 [B] and 0.6 [C]) indicate that erythrose must be oxidised further, much more rapidly than arabinose. The same is true of glyceraldehyde. The decrease in the triose + tetrose molar yield from the phosphate to the borate buffered oxidation (0.0184) is approximately accounted for by the increase in pentose (0.0134) in borate.

The reason for the extensive oxidation of products probably lies in the effect of pH on the relative rates of the various stages in the overall reaction. It has been noticed[109] that a high pH decreases the rate of

formation of the cyclic glycol-periodate intermediate. This is presumably due to saponification of the mono-chelated ester intermediate. The result will be a displacement of the equilibrium to the side of free periodate. Products will form more slowly in basic solution but some will appear before all free periodate has been utilised by the glucitol. Periodate has a choice of oxidising either glucitol or products. The yields of the pentoses and experiments designed to find the relative rates of oxidation of pentoses and glucitol in the presence and absence of borate (Expt. 55) suggested that glucitol and the pentoses were oxidised at very similar rates. This is at variance with the ideas of Courtois[116], but the low yields of the smaller fragments are well explained on their findings.

It is also probable that oxidation of the products was enhanced by the "protective"action of borate on glucitol and some of the products. This action would presumably depend on the stability of the complexes which, in turn, may be estimated by their migration rates during borate ionophoresis. The M_G values follow the sequence glucitol = pentose > tetrose >

glyceraldehyde> glycolaldehyde. Thus oxidation of the products would be in this order (reversed) in the presence of borate.

Some products (ca. 2.5%) were not accounted for in the analyses. The action of base could have caused some loss of the smaller fragments by dimerization etc. Hence the true values of glyceraldehyde, erythrose and particularly, formaldehyde, may be a little higher. Formaldehyde may be calculated by combining the molar yields of the pentoses. The calculated value for the borate buffered oxidation (0.0288) is much higher than the experimental yields (0.0163). The phosphate values show similar discrepancies (0.0154 and 0.0115), whereas those of the control oxidation agreed very well (0.0148 and 0.0146). Glycoldehyde was determined by a method involving acid which probably also determined dimerized material. The latter values could have been high because incomplete oxidation of glyceraldehyde etc. could produce glycolaldehyde.

The Utilisation of Periodate

The theoretical utilisation of periodate may be calculated from the most reliable sets of figures from Table 21. The most reliable set of figures is 1,

2, 6, 7 and 8 for each of the oxidations (A, B, and C). The percentage of periodate used in each oxidation is given in Table 24.

Table 24

Control	Borate	Phosphate
96.48	60.12	77.76

The control may be regarded as theoretically correct but, in the buffered reactions, large discrepancies are evident. Part of these may be due to low values caused by destruction of products by base. The difference between borate and phosphate cases, however, suggests that this is not the only effect. Some experiments to check the possibility of periodate consumption by alkaline buffers were performed. Ten moles of periodate were added to one of glucitol under the three conditions. The periodate uptake was measured by the arsenite and iodine method[124] [Expt. 56(i)]. More than the theoretical quantity of periodate was taken up by both of the buffered reactions, the borate

reaction consuming the greater amount. It has been suggested[125] that, under alkaline conditions, di- and tri-sodium paraperiodates form and partially settle out, and hence escape determination. Partial precipitation could account for the incomplete oxidation of glucitol but Hartman[126] could not confirm that the periodate-arsenite reaction was not quantitative.

Oxidation of glucitol with 5 moles of periodate was attempted [Expt. 56(ii)]. Chromatography of the products indicated that much material was incompletely oxidized in the borate buffered oxidation (Fig. 19). It is, hence, fairly certain that periodate is partially immobilised by borate in such a way that it is incapable of glycol oxidation.

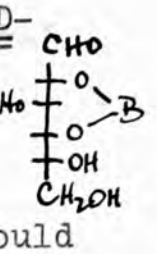
THE PREPARATION OF L-XYLOSE

The percentage of borate complex increases with concentration of solution. It was conceivable, therefore, that the yield of L-xylose could be increased by working in concentrated borate buffer. The wastage of periodate on the products and by interaction with borate would also increase. This was counteracted by the addition of larger quantities of periodate. The optimum conditions were found (Expt. 57) by chromatography of the products of various reactions. The best yield of xylose was ca. 40% (Fig. 20). Attempts to crystallise the product directly failed, probably because of contamination by large quantities of glyceraldehyde. It should be possible to purify the material by column chromatography.

APPLICATION OF THE PERIODATE TECHNIQUE TO OTHER COMPLEXES

The D-Glucose-borate Complex

Foster[99] postulated that the most favoured D-glucose-borate complex has the structure -



An attempt was made to oxidize the complex. The production of xylo-trihydroxyglutaric aldehyde would

be evidence in favour of Foster's structure. D-Arabinose, however, was produced (Expt. 58). A control reaction yielded a fast-moving pink spot on chromatography (p-anisidine spray) which, on short treatment with alkali, yielded arabinose. This must have been 4-O-formyl-D-arabinose, reported by Warsi and Whelan[127] on the partial periodate oxidation of D-glucose. It is concluded that the concentration of glucose-borate complex is very low. It has been found[128] that a very large molar excess of borate was necessary (ca. 45) to obtain the maximum increase in the optical rotation of a glucose-borate solution.

The oxidation of D-mannitol could not be influenced by borate (Expt. 59).

The D-Glucitol-molybdate Complex

This was examined with more success (Expt. 60). A chromatogram of the products of a control and an oxidation in the presence of 3 moles (ratio) of molybdate is shown in Fig. 21. L-Xylose formation was clearly depressed and D-arabinose, unaffected. This indicates that the C(1) and C(2) hydroxyls are least affected by molybdate complexing.

MOLYBDATE - CARBOHYDRATE COMPLEXES

GENERAL ASPECTS

Chromate, molybdate and tungstate solutions have been shown to complex under acidic conditions with organic compounds containing hydroxyl groups.[129] The remainder of Part II is devoted to a study of molybdate complexes with polyhydroxy compounds. Honnelaitre[130] has reviewed this field up to 1925. The first effect noticed was the ability of acidic molybdate solution to increase the optical rotation of a solution of mannitol. Gernez[131], Tanret,[132], Honnelaitre[130], Frèrejacque[133], Richtmeyer and Hudson[129] and, recently, Barker et al.[134] used this effect to study the molybdate-mannitol complex. The only positive result that this type of work has produced is that two moles of molybdate combine with one of mannitol to form an acid-stable complex which is decomposed by the addition of alkali. A parallel line of research was based on the increase in pH when mannitol was added to neutral sodium molybdate solutions.[130] This developed into an examination of the complexing by conductimetric and potentiometric methods.[130,135-139] These two tech-

niques have yielded similar results on the composition of complexes. No idea has been gained, however, of the exact structures of complexes by either method.

This complexing has found relatively few applications. The enhancing of polyol rotations has been developed quantitatively,[129] and Plško[138] has attempted to use the pH increase when sodium molybdate and polyhydroxy compounds are mixed to determine sugars. The complexing has been used to alter the rates of elution and enhance the separation of oligosaccharides from a charcoal column.[134]

Ionophoresis of carbohydrates was reported briefly by Frahn and Mills[140] who described the behaviour of sorbitol, mannitol, dulcitol, ribose and fructose at pH 5.6. Whelan also developed the method to a limited extent.[141]

The purpose of the work now reported was to extend the investigations of Barker, Bourne and Ward with a view to (a) utilising the complexing further in the analysis, characterisation and structural examinations of carbohydrates and (b) elucidating the nature of the complexes.

THE EFFECT OF MOLYBDATE ON THE OPTICAL ROTATION OF SOME POLYOLS

The specific rotation of an optically active polyol is greatly increased by the addition of molybdate in acid solution. It is assumed, in these studies, that this increase is proportional under given conditions to the amount of complexing. The optically active polyols available (Expt. 65) were D-mannitol, D-glucitol, 2-deoxy-D-glucitol, D-arabitol, cellobiitol and melibiitol.

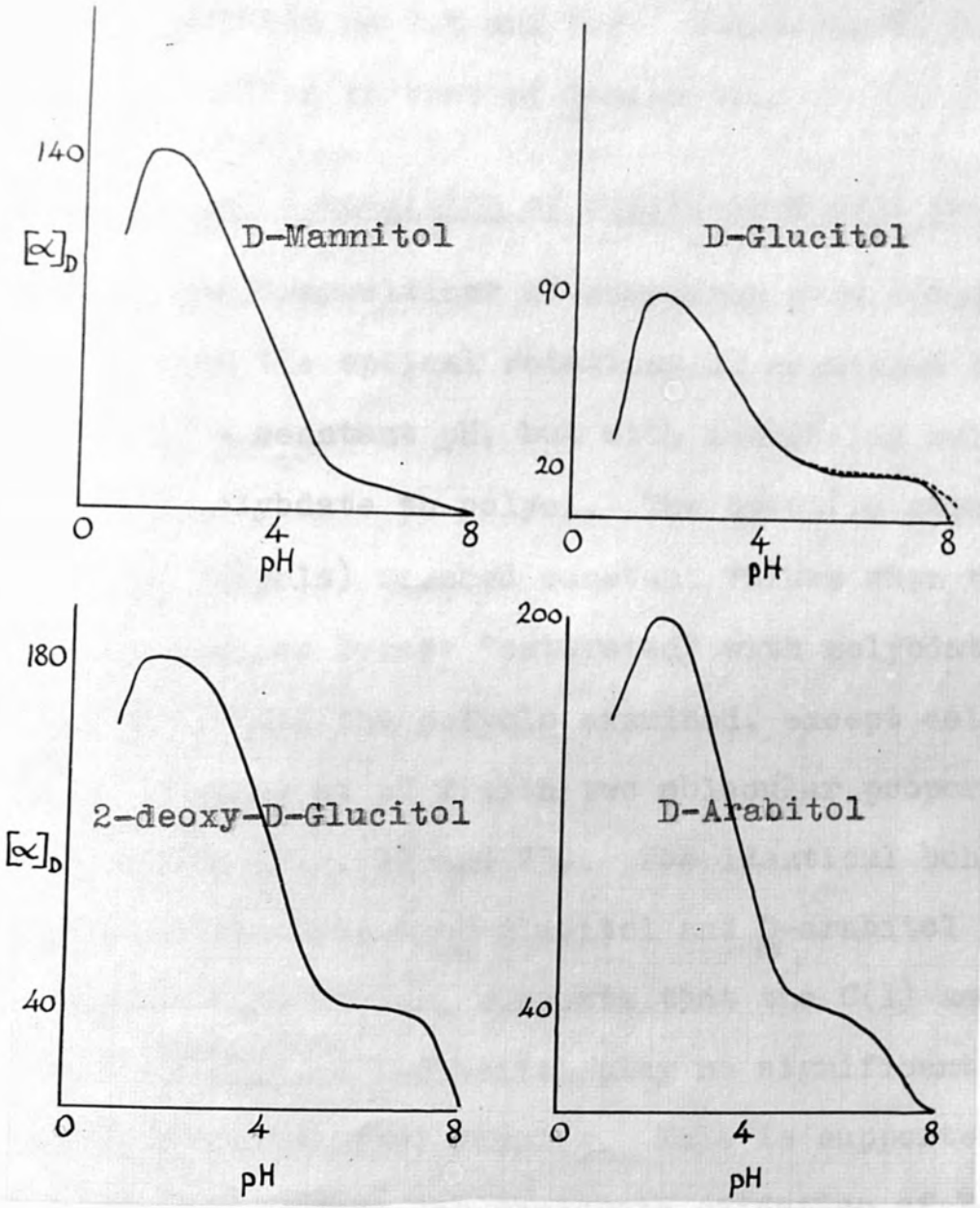
The Effect of pH on the Specific Rotations of Polyols in the presence of Molybdate

The effect of varying the pH of solutions of D-mannitol, D-glucitol, 2-deoxy-D-glucitol and D-arabitol in molybdate was followed polarimetrically (Expt. 61). Complexing appeared to be strongest at pH 2 in all cases. At pH values below 2, a sharp drop in the optical rotation of the solution indicated decreased complexing. This was not reported by previous workers. It is probably due to condensation of the molybdate to a polymer[142] incapable of complexing with hydroxyl groups.

The behaviour of D-glucitol, 2-deoxy-D-glucitol

Figure 12.

The effect of pH on the optical rotation of some Polyols Molybdate solution.



and D-arabitol differed from that of D-mannitol (Fig. 12) in that complexing commenced at pH 8. The specific rotations then rose sharply and remained approximately constant between pH 7.5 and 5.0. Behaviour below pH 5 was similar to that of D-mannitol.

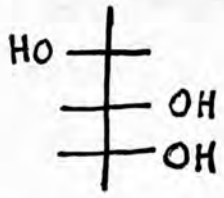
The Molecular Composition of Molybdate-Polyol Complexes

The compositions of complexes were determined by measuring the optical rotations of solutions of the polyols at a constant pH, but with increasing molar ratios of molybdate to polyol. The specific rotations (based on polyols) reached constant values when the polyol molecules became "saturated" with molybdate (Expt. 62). All the polyols examined, except cellobiitol, complex, at pH 2, with two molecular proportions of molybdate (Fig. 22 and 23). The identical behaviour of D-glucitol, 2-deoxy-D-glucitol and D-arabitol in the experiments so far, suggests that the C(1) and C(2) hydroxyl groups of D-glucitol play no significant part in the molybdate-glucitol complex. This is supported by evidence gained from the periodate oxidation of D-glucitol in the presence of molybdate (Expt. 60). The constitution of the melibiitol complex shows that the C(6) hydroxyl also probably plays no part in the moly-

bdate-glucitol complex. The behaviour of cellobiitol shows that the C(4) hydroxyl of glucitol is concerned in complexing.

The variation of pH, within the range where complexing occurs, had no effect on the composition of the complex of glucitol (Expt. 63).

The "step" in pH/$[\alpha]_D$ curves of glucitol, 2-deoxy-glucitol and arabitol seems to be a property of the group :-



Mannitol also possesses this group. It arises twice in the four secondary hydroxyl groups. However, unlike the other compounds, the specific rotation of the mannitol complex exactly doubles as the molybdate-polyol ratio is raised from 1 to 2. This suggests that the second mole of molybdate complexes in exactly the same way as the first, i.e. on identically spaced hydroxyl groups, and that all four are involved. The "step" may be caused by different sets of hydroxyl groups being available for complexing at different pH values. This, in turn, could be caused by a change in the molybdate ion, due to a change of charge or to the fact that molybdate condenses progressively as the pH of its solution is

lowered.[142]

The Stability of the Molybdate-Polyol Complexes

The complexes were shown to be much more stable than borate complexes. The latter are decomposed by dilution of their solutions, as evidenced by a fall in specific rotation.[95] The molybdate complex, however, showed no change in specific rotation on dilution (Expt. 64). A molybdate-mannitol complex was precipitated with ether and isopropanol but always possessed the same composition as the added constituents. It seemed that a type of co-precipitation was effected.

MOLYBDATE COMPLEXING AND PAPER CHROMATOGRAPHY

An organic solvent containing boric acid will complex with hexitols and slow down their rate of chromatographic movement. Thus a separation of hexitol and hexose is possible. The greater stability of the molybdate complex suggested that it could be used in a similar technique. It was not possible to dissolve molybdate in organic solvents, therefore two other techniques were attempted. Chromatography on molybdate-impregnated paper yielded neither reproducible nor useful results.

Chromatography of the pre-formed complexes in the usual solvents proved more successful (Expt. 66). The partial decomposition of complexes resulted in a series of spots on the final chromatogram (Table 38). The unchanged polyol was present on the chromatograms with free molybdic acid on the origin line. Between these two spots were a number of spots corresponding to different numbers of moles of molybdate complexing with the polyol. Results recently obtained by conductimetric measurements[139] support the validity of the above method. Both methods have shown that mannitol, erythritol and

glycerol complex two, one and no, moles of molybdate respectively.

IONOPHORESIS OF CARBOHYDRATES IN MOLYBDATE SOLUTION

Rotational studies have shown that complexing is most favoured at pH 2. Ionophoresis, therefore, had to be carried out at a pH low enough to assure sufficient complexing but high enough to allow reasonable ionization of the complexes. A pH of 5.0 to 5.5 was found to be optimum. Other workers in the field[140,141] also used this range.

The initial ionophoretograms revealed that, while glucose did not migrate, sorbitol (D-glucitol) migrated rapidly towards the anode. Sorbitol was thus used as a standard for the comparison of migration rates of various compounds (hence the MS value). Glycerol was used as a non-migrating marker for endosmosis corrections because it reacted rapidly with the silver nitrate spray reagent. Details are given in Expt. 67. The results are given in Tables 25,26,27. Compounds have been classified as follows, to facilitate the interpretation of their behaviour:-

A. "Flexible acyclic compounds" - those in which the distances between hydroxyl groups may be easily varied by rotation about C-C bonds (e.g. hexitols)

[Table 25].

B. Cyclic compounds with conformational isomerism of the Cl⇌1C type.[143] The lower aldoses have been included in this section for convenience (Table 26).

C. The Acetals - in which the flexibility of Group A compounds has been reduced by the formation of an acetal ring (Table 27).

D. Compounds with unambiguous, ridgidly fixed structures (Fig.15).

The term, non-complexing, in the following tables refers to any compound with M_S value < 0.1.

* denotes slight streaking from the origin towards the anode.

Table 25. M_S values of Group A Compounds

Complexing	M_S	Non-Complexing
D-Glucitol	1.0	3-O-methyl-D-Glucitol
2-deoxy-D-Glucitol	1.0	2.3-di-O-methyl-D-Glucitol
Dulcitol	1.0	Glycerol
6-deoxy-D-Galactitol	1.0	Ethane-1.2-diol
D-Mannitol	1.0	Butane-2.3-diol
1-deoxy-D-Mannitol	1.0	" " 1.4- "
2-O-methyl-D-Mannitol	1.0	" " 1.3- "
5.6-di-O-methyl-D-Mannitol	1.0	Propane-1.2-diol
D-Arabitol	1.1	" " 1.3- "
Ribitol	1.1	Pentane-1.5- "
Xylitol	1.1	Hexane-1.6- "
Erythritol	1.0	2-ethylhexane-1.3-diol
D-Threitol	0.5	2-methylpentane-2.4-diol
		Pentaerythritol
Reduced oligosaccharides		
See Table 29.		

Table 26. M_S values of Group B Compounds

26(i) The Aldoses and Derivatives

Complexing	M_S	Non-Complexing
D-Mannose	0→0.9	α- and β-D-Glucose*
L-Rhamnose	0→0.6	2-deoxy-D-Glucose
D-Gulose	1.1	3.0-methyl-D-Glucose
D-Talose	0.7	2.3.4-tri-O-methyl-D-Glucose
		2.3.6-" " " "
D-Lyxose	1.1	2.3.4.6-tetra-O-methyl-D-Glucose
D-Ribose	0.4	Methyl α-D-Glucoside
Methyl-α-D-riboside	0.1	Phenyl glucoside
		Catechol glucoside
Heptoses:-		3.4-di-O-methyl-D-Mannose
D-glycero-		Methyl-α-D-mannoside
D-ido-	1.0	" " -β- " " "
D-gala-	0.4	Methyl-α-L-rhamnoside
L-gala-	0.36	D-Galactose*
D-gulo-	1.1	D-Arabinose*
D-allo-	0.9	D-Xylose*
L-manno-	0.8	Methyl-α-andβ-D-arabinosides
L-gluco-	0.14	Methyl-α-and β-D-Lyxosides
		2-deoxy-D-Ribose
		Methyl-β-L-Glucoheptoside
D-Erythrose	0.9	Methyl-α-D-xylo-fructo-furanoside
	0.6	1.2-dideoxy-D-Arabinose
		D-Glyceraldehyde
		Glycol aldehyde
		Common disaccharides with D-glucose reducing group

26(ii) The Inositols and Related Compounds

Complexing	Ms	Non-Complexing
muco-Inositol	0.1	scyllo-Inositol
meso-Inositol	0.2	Quercitol
allo-Inositol	0.33	Mytilitol
epi-Inositol	1.1	Pinitol
		Quebrachitol
		l-vibo-Quercitol
		scyllo-Quercitol
		Cyclohexanetriol 13/2*
		Cyclohexanetetrol 134/2*
		" " 123/4*
		" " 14/23*

26(iii) Ketoses and Derivatives

Compound	Ms
D-Fructose	0.5
L-Sorbose	0.3
D-Glucosone	0.9
D-Mannoheptulose	0.35
D-Glucoheptulose	1.0
Leucrose	0.4
Turanose	0.14

Table 27. Group C

None of the following compounds migrated during ionophoresis
2.4-O-methylene-D-Glucitol
1.3-O-methylene-D-Mannitol
2.4-" " " " " "
3.4-" " " " " "
2.5-" " " " " "

The group D compounds are discussed in a later section.

The Behaviour of Group A (Table 25)

Flexible carbon chains with three or more adjacent hydroxyl groups (except glycerol) complex with molybdate. Experiments with various glycols indicate that two hydroxyl groups are insufficient for molybdate complexing.

The Behaviour of the Pentoses, Hexoses, Heptoses and Derivatives

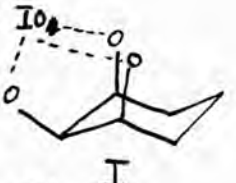
Aldoses tend to form very weak complexes (evidenced by slight streaking of compounds from the origin). This weak complexing is prevented by fixing the sugar in the ring form, e.g. by glycoside formation. It is inferred that the weak complexing is with the small amount of aldehydo-form in solution, as is the case in borate buffer.[99] Certain aldoses, however, complex very strongly with molybdate. It is not possible to attribute the complexing to the aldehydo-forms as unique structural characteristics do not present themselves. An exception to this is D-ribose. In aqueous solution, ca. 8% of ribose is known to be in the aldehydo-form.[144] The rapid migration of D-lyxose cannot be explained on this

basis (see Table 28).

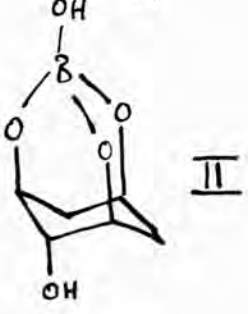
Table 28. The Amount of Aldehydo-Pentose in Solution compared with the M_S value

Pentose	M_S	Aldehydo-form[144] (%)
D-Xylose	0.1	0.17
D-Arabinose	0.1	0.28
D-Lyxose	1.1	0.40
D-Ribose	0.4	8.50

Barker and Shaw[91] noted the rapid uptake of one mole of periodate by D-ribose to form a fairly stable complex. It was suggested that pyranose rings containing a cis-cis-1:2:3-triol system of hydroxyl groups will form a "tridentate" complex (I). A similar structure has been postulated by Angyal and McHugh[145] for the complex between scyllo-quercitol and borate (II). In this case, β-cis-hydroxyl groups are involved.



I



II

An examination of the conformations of the aldopyranoses revealed that those pentoses and

hexoses which complex strongly with molybdate could possess the necessary cis-cis-1:2:3-triol arrangement (Fig. 13). Indeed, none of the non-complexing compounds in Table 26 (i) could adopt a structure which would contain a cis-cis-1:2:3-triol system. The participation of the glycosidic hydroxyl group is necessary, except in the cases of talose and ribose, for this theory. Hence a more reasonable explanation of the non-complexing of the methyl glycosides listed is forthcoming. The non-complexing of 2-deoxy-D-ribose and 3:4 di-O-methyl-D-mannose fits in with this theory.

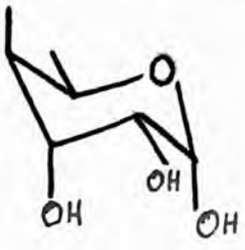
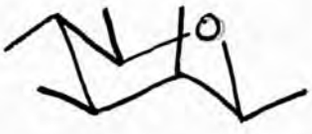
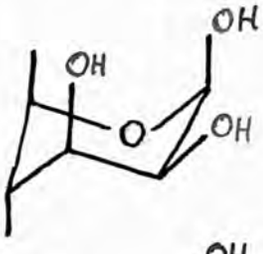
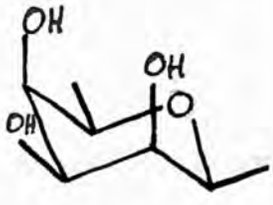
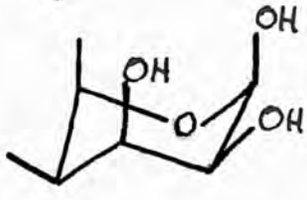
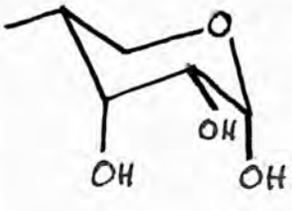
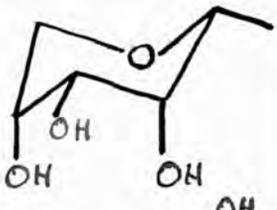
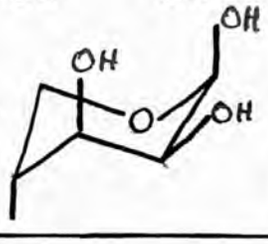
The heptoses which migrated appreciably all contained the cis-cis-1:2:3-triol system except the D-ido-, D-gala- and L-gala- derivatives [Table 26(i)]. These compounds, however, possess hydroxyl groups which are capable of forming the structure by rotation about external C-C bonds [C(4), C(6) and C(7)-hydroxyls].

The Behaviour of the Inositols

Results of the ionophoresis of the inositols may be interpreted on the "tridentate" theory. Of the

Figure 13

"Tridentate" Complex formation in some Aldoses

Sugar	Conformations of Pyranose Forms		M_s value
	Preferred	Non-preferred	
α-D-Gulose			1.1
β-D-Mannose			0→0.9
β-D-Talose			0.7
α-D-Ribose			0.4
β-D-Lyxose			1.1

eleven inositols tested, three complexed appreciably. The migration rates of these compounds can be related to the amounts of the conformations present containing the cis-cis-1:2:3-triol grouping.

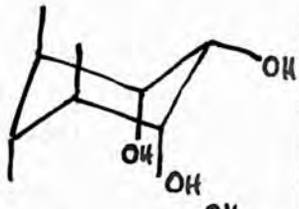
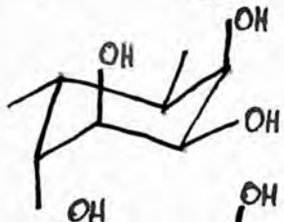
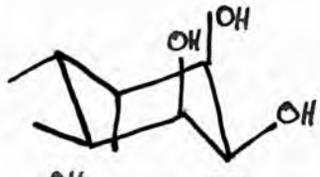
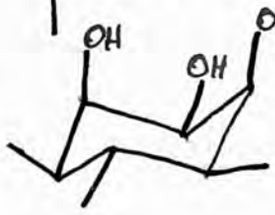
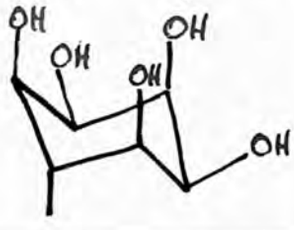
The inositols can be regarded as each existing in either of two conformations (Fig. 14). These will be termed the A and B forms (as the terms C1 and 1C are not applicable). meso-Inositol, in form A, possesses the "tridentate" arrangement. Conformation A, however, is very unfavourable as five of the six hydroxyl groups are in axial positions. Consequently, meso-inositol exists almost exclusively in the B form which cannot complex with molybdate.

The A and B forms of allo-inositol are coincident and hence equally favourable. The structure possesses one axial hydroxyl group which is not involved in complexing. This would introduce steric hindrance into the complex and make it less stable than epi-inositol.

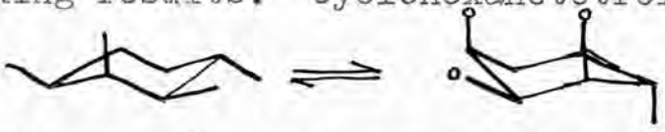
epi-Inositol will exist primarily in the A form, in which four of the six hydroxyl groups are equatorial. Hence, the more favoured conformation possesses the "tridentate" arrangement and strong

Figure 14

"Tridentate" Complex formation in some Inositols

Inositol	Conformation		M_s value
	A	B	
scyllo-			0.05
muco-			0.10
meso-			0.20
allo-			0.33
epi-			1.10

complexing results. Cyclohexanetetrol 123/4



only possesses the arrangement in its unfavoured conformation and therefore will not complex strongly.

These arguments require the assumption that the conformational "freezing" ability of molybdate is too weak to overcome the interaction between large axial substituents.

The Behaviour of the Tetroses

The relative migration rates of D-erythrose (0.9) and D-threose (0.6) are similar to those of erythritol (1.0) and threitol (0.5). This suggests that the tetroses complex in the open chain form and that the C(1)-hydroxyls of the tetritols are not needed in the complexing, as would be expected if three hydroxyl groups were involved.

The Behaviour of Ketoses

The nature of the molybdate-ketose complex is very uncertain. There is insufficient information available at present. The following types of complexing could be involved:-

(i) Complexing in the open chain form.

(ii) Slight enolisation of the keto-group and complexing of the resultant ene-diol with molybdate.

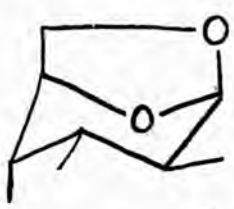
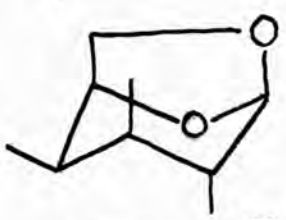
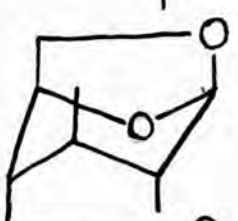
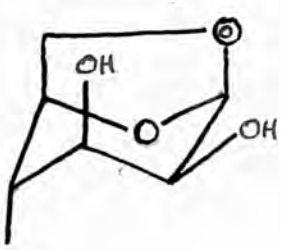
(iii) Tridentate arrangements can be found on examination of molecular models of furanose ring forms.

The Behaviour of the 1.6-anhydro-β-D-aldopyranoses

These are valuable compounds as they present a limited number of hydroxyl groups possessing a steric relationship unchanged by conformational isomerism. The mannose derivative, only, migrated during ionophoresis (Fig. 15). The only unique feature of this molecule is the "tridentate" relationship of the C(2), C(3) and ring oxygens. The only explanation that comes to mind is that a "didentate" complex forms and is stabilized by hydrogen bonding between a molybdenum hydroxyl and the anhydro ring oxygen. There is no evidence from other compounds to refute this suggestion.

Figure 15.

The Conformations of some Anhydropyranoses.

Compound	Conformation	M_s
1:6anhydro- -D-		
altro-		0
galacto-		0
gluco-		0
gulo-		0
manno- pyranose.		0.5

Predictions

1.6-anhydro-β-D-allopyranose would complex strongly with molybdate and migrate during ionophoresis with an M_S value of ca. 1.0. D-Aldose would probably have an M_S value of ca. 0.6 (intermediate between that of ribose and talose). β-(and probably α-)Methyl-D-taloside should migrate rapidly (ca. 1.0). If the compounds become available these predictions will be tested.

UTILISATION OF MOLYBDATE-CARBOHYDRATE COMPLEXES

Perhaps the most striking feature that has emerged from the experiments is the specificity of molybdate in its ability to complex with carbohydrates. It offers, therefore, a valuable complementary technique to ionophoresis in borate and other electrolytes.[146] As an illustration of this, borate and molybdate ionophoretograms of the products of the Fenton oxidation of D-glucitol are displayed in Fig. 16.

Other possibilities, including some reviewed and attempted by Ward[147], are:-

(i) the use of molybdate in conjunction with charcoal columns[134] (cf. Borate[148])

(ii) the above method (i) could be applied to monosaccharides using the highly active "Ultrasorb C" charcoal.[149]

(iii) separations of carbohydrates involving the molybdate form of a basic resin (cf. Borate[150]).

Specific Uses of Molybdate Ionophoresis

The various separations possible by molybdate ionophoresis will be evident on a perusal of Tables 25 and 26 (pp. 204-6).

Figure 16.

Ionophoresis of the Products of the Fenton Oxidation of D-Glucitol.

BORATE

A = Glucose
B = Sorbose
C = Fructose

MOLYBDATE

D = Glucitol
E = Gulose
F = Gluconic acid

X = Oxidation product

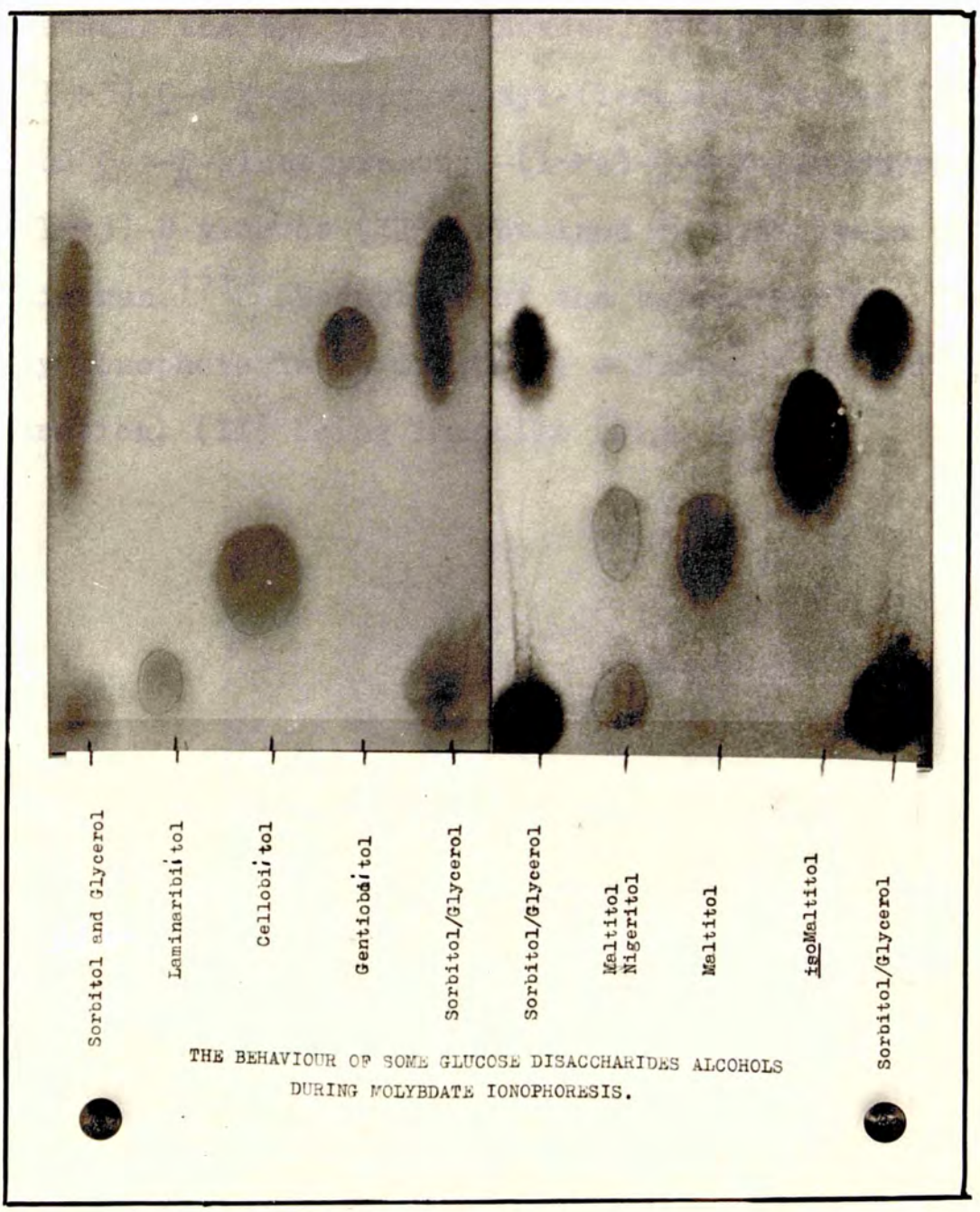
Differentiation between the Linkages at the Reducing Ends of Oligosaccharides

3-O-Substituted D-glucitol will not complex with molybdate. Each molecule of 4-O-substituted D-glucitol will form a complex containing one molybdenum atom and each molecule of 2-deoxy-D-glucitol and 6-O-substituted D-glucitol, a complex containing two molybdenum atoms. Hence, since glucosides do not complex, the M_S values of O-glycosyl D-glucitols, obtained by the reduction of oligosaccharides with glucose as the reducing end group, fall into three well-defined groups, i.e. with 1:3-, 1:4-, and 1:2- or 1:6-glycosidic linkages. An efficient separation of these groups was possible by ionophoresis (Fig. 17 and Table 29).

Table 29. M_S Values of some Reduced Oligosaccharides

Compound	Linkage	M_S value
Sophoritol	β-1:2	0.9
Nigeritol	α-1:3	0
Laminaribiitol	β-1:3	0
Maltitol	α-1:4	0.4
Cellobiitol	β-1:4	0.4
Lactitol	β-1:4	0.4
Isomaltitol	α-1:6	0.8
Gentiobiitol	β-1:6	0.8
Melibiitol	β-1:6	0.8

Figure 17.

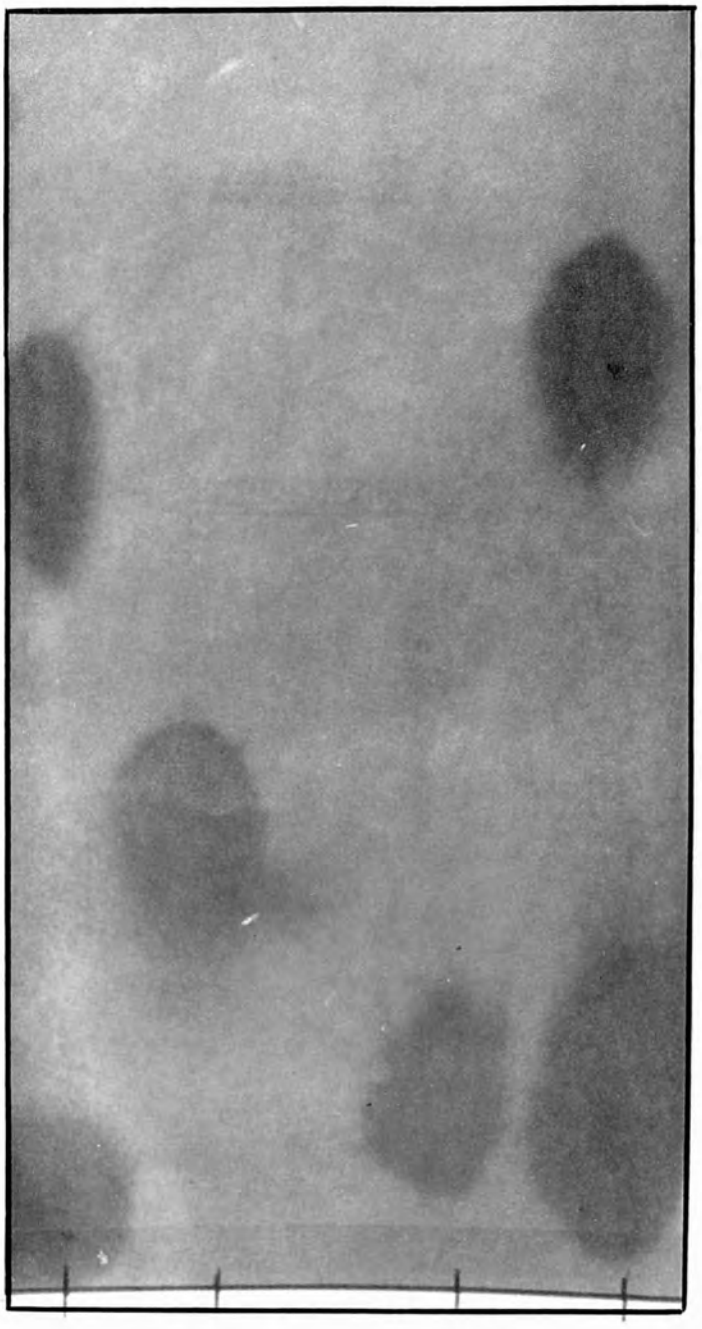


THE BEHAVIOUR OF SOME GLUCOSE DISACCHARIDES ALCOHOLS DURING MOLYBDATE IONOPHORESIS.

The technique was used to distinguish between the two trisaccharides, O-α-D-glucopyranosyl-(1→3)-O-α-D-glucopyranosyl-(1→4)-D-glucose (I) and O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→3)-D-glucose (II), obtained on hydrolysis of nigeran.[151] Reduction of the trisaccharides followed by ionophoresis in molybdate effected a marked separation, (II) being immobile (Fig. 18).

Figure 18.

Molybdate Ionophoresis of "Trisaccharide I" and "Trisaccharide II". (after reduction).



"I" "II"

PART II

EXPERIMENTAL

Expt. 47. Chromatographic evidence for the effect of borate on the periodate oxidation of D-glucitol

D-Glucitol (0.182 g., 1 m.mole) was dissolved in water (2 ml.) and boric acid (0.247 g., 4 m.mole) added. The solution was adjusted to pH 10.3 by the addition of N-NaOH solution. Sodium periodate (0.107 g., 0.5 m.mole) in water (2 ml.) was added, the solution stirred, and the pH change with time noted (Table 30).

Table 30

Time (secs.)	0	10	20	30	45	60	90	120
pH	10.3	9.95	9.90	9.85	9.80	9.79	9.76	9.75

(A similar reaction mixture was checked for periodate by spotting samples on a paper soaked in KI solution. Iodine was liberated by periodate for 2 min. after the initial addition.) After 10 min., the solution was shaken with Amberlite IR-120 [H^+] (5 g.) for 10 min. The resin was filtered off and boric acid removed from the filtrate by evaporation to dryness three times with dry methanol. Other methods of

borate removal, e.g. precipitation as the silver salt and removal with basic resins, were found to be much less efficient.

A control reaction using the same quantities, but without boric acid and sodium hydroxide, was carried out. The products were chromatographed using (d) [Expt. 1] and water-saturated methyl ethyl ketone. The latter solvent separated tetroses from glyceraldehyde. Chromatograms were sprayed with p-anisidine hydrochloride and silver nitrate reagents (Expt. 1). D-Arabinose and L-xylose were present in increasing quantities in the borate buffered oxidation. Glyceraldehyde and tetrose formation was greatly suppressed.

When the reactions were carried out in borate at pH 6.5 the above effects were not noticeable. Oxidations in 4 M buffer at pH 10 showed a greater effect than those in 0.5 M buffer.

Expt. 48. The conditions of oxidation for quantitative determinations

The oxidations were carried out in 0.5 M-buffer. The amount of D-glucitol (usually 72 mg.) dissolved in the borate buffer (pH 10.6) and phosphate

buffer (pH 10.1) was such that the molar ratio of buffer to glucitol was 3:1. The pH of the final solutions was 10 in both cases. D-Glucitol in water was used as a second control reaction. D-[^{14}C] Glucitol was used for a number of quantitative determinations.

An amount of standard periodate solution, equivalent to 0.25 mole with respect to D-glucitol, was added to the solutions. After exactly 10 min., Amberlite IR-120 [H^+] (10 g. per g. of glucitol) was stirred into the buffered oxidation mixtures and the solutions agitated for 15 min. A description of the precise quantities involved in the radioactive experiments is given in the collective Table (p.232). The products were separated and determined in various ways as described below.

Expt. 49. The determination of D-glucitol, D-arabinose and L-xylose

D-Glucitol, D-arabinose and L-xylose (ca. 250 mg. of each) were added to the resin "acidified" mixture of oxidised D-[^{14}C] glucitol (Expt. 48). The resin was filtered off, and, after standing overnight, borate was removed by distillation with methanol. Phosphate ion was removed by precipitation with $Ba(OH)_2$

solution. The solution was then evaporated to 4 ml. and applied in strips to 8 sheets of Whatman No. 3 paper. Chromatograms were developed with solvent (d) for 20 hr. The three compounds (R_G values, 1,0, 1.2 and 1.4) were eluted with water and the solutions freeze-dried.

D-Glucitol was converted to the hexacetate as follows:-

D-Glucitol (250 mg.) was mixed with anhydrous sodium acetate (0.2 g.) and refluxed in acetic anhydride (2 ml.) for 2 hr. The resulting mixture was poured into water and neutralised with sodium bicarbonate (3 g.). Extraction with chloroform (2 x 10 ml.) and removal of solvent yielded the product which was recrystallised from hot ethanol (1 ml.). The specific activities of the resulting samples were determined by combustion (Expt. 6). The material possessed constant activity during 3 recrystallisations.

The [^{14}C]-pentoses were converted to their phenylosazones. Pentose (250 mg.) in water (2 ml.) was heated on a boiling water bath for 15 min. with phenylhydrazine (0.5 ml.) in acetic acid (1.5 ml.). The resulting solution was cooled and the osazone

precipitated by the addition of water (4 ml.). The products of both pentoses were recrystallised twice from water and twice from benzene, when they possessed m.p's. 164°C and constant specific activity.

Expt. 50. The determination of DL-glyceraldehyde and D-Erythrose

D-[^{14}C] Glucitol was oxidized (Expt. 48) and, 10 min. after the addition of periodate, potassium borohydride (200 mg.) was added to each solution and the reduction[152] allowed to proceed overnight. The reason for reducing the product was three-fold.

1. Erythritol and glycerol were more readily available for dilution analysis than the corresponding aldehydes.

2. Reduction destroyed the optical activity of glyceraldehyde and the D- and L-forms could be measured together.

3. Part of the alkali damage of products was minimised, e.g. enol forms reduced to the alcohol and were thus included in the final values.

After ca. 12 hr., Amberlite IR-120 [H^+] (2 g.)

and glycerol and erythritol (ca. 250 mg. of each) were added to the solution. Boric acid was removed after filtering off the resin. The solution was then applied to 8 sheets of Whatman No. 3 paper and the paper developed with solvent (a) for 16 hr. Pure [^{14}C] glycerol and [^{14}C] erythritol (R_G values ca. 2.0 and 3.0) were eluted and freeze-dried. [^{14}C] Glycerol was converted to the tribenzoate by solution in pyridine (2 ml.) and the addition of benzoyl chloride (0.8 ml.). The resulting mixture was allowed to stand for 2 hr. Water (2 ml.) and $\underline{\underline{N}}$-$H_2SO_4$ (4 ml.) were added and the precipitated oily product centrifuged down. The oil was washed twice with water and then dissolved in ethanol (2 ml.). Water was added dropwise until the solution became turbid. Crystals very quickly formed after seeding with a trace of pure material. The second, third and fourth recrystallisations yielded crystals of constant specific activity and m.p. 72°C.

[^{14}C] Erythritol was treated exactly as above, but the oil was recrystallised from pyridine/water, with seeding. The tetrabenzoate (m.p. 185°C) possessed constant activity during the second, third and fourth

recrystallisations.

NOTE:- care was required when combusting the above compounds as they tended to burn explosively.

The details of experiments 49 and 50 are given in the following Table 31.

Table 31. Practical details of carrier dilution analyses

SERIES	COMPOUND SOUGHT	Wt. OF [^{14}C]-GLUCITOL USED (mg.)	SP.ACTIVITY OF [^{14}C] GLUCITOL μC/g. atom of C	CONCENTR-ATION OF BUFFER
A				
OXID^{N}. OF GLUCITOL IN WATER	Glucitol	72	3163.77	-
	Arabinose	"	"	-
	Xylose	"	"	-
	Erythrose	"	"	-
	Glyceraldehyde	"	"	-
B				
OXID^{N} IN BORATE BUFFER pH 10.0	Glucitol	"	"	0.5 M
	Arabinose	"	"	"
	Xylose	"	"	"
	Erythrose	"	"	"
	Glyceraldehyde	"	"	"
	Glucitol	"	"	0.25 M
	Arabinose	"	"	"
	Xylose	"	"	"
C				
OXID^{N}. IN PHOSPHATE BUFFER pH 10.0	Glucitol	400	821.00	0.5 M
	Arabinose	72	3163.77	"
	Xylose	"	"	"
	Erythrose	"	"	"
	Glyceraldehyde	"	"	"
	Glucitol	"	"	0.25 M

* Wt. of the alcohol added

MOLAR EXCESS OF BUFFER OVER GLUCITOL	MOLAR RATIO PERIODATE/ GLUCITOL	Wt. OF CARRIER (mg.)	SP.ACTIVITY OF ISOLATED CARRIER μC/g.atom of C	Wt. OF PRODUCT (mg.)SOUGHT
-	0.25	250	561.90	53.990
-	"	254	3.43	0.276
-	"	341.4	5.03	0.548
-	"	242.3*	29.40	2.273
-	"	254*	117.52	9.799
3	"	247	664.40	65.659
"	"	233	6.14	0.453
"	"	226	17.54	1.260
"	"	256*	1.77	0.143
"	"	242.3*	7.06	0.542
"	0.125	249.4	679.10	68.165
"	"	259.4	3.83	0.314
"	"	247	11.26	0.882
"	0.25	724.7	272.00	359.00
"	"	275.1	3.24	0.282
"	"	195.5	10.25	0.635
"	"	255.3*	4.71	0.381
"	"	242.3*	17.66	1.360
"	0.125	242	653.00	62.140

Expt. 51. The determination of L-Threose

D-[^{14}C] Glucitol (20 mg.) in water (1 ml.) was oxidized by the addition of 0.24 M sodium periodate solution (0.113 ml.) from an "Agla" syringe. A second oxidation was carried out in borate (0.6 ml.) and treated in the usual manner (Expt. 48). Tetroses were separated from other products by chromatography using water-saturated methyl ethyl ketone as solvent. The eluted tetroses were subjected to ionophoresis in 0.1 M molybdate solution (pH 5.0) at 750.v.for 3 hr. The p-anisidine spray reagent and U.V. light revealed the positions of the tetroses on the ionophoretogram. The spots were cut out and the β-emission estimated by direct counting [Expt. 4(i)]. The results are shown in Table 32.

Table 32

Oxidation in	Tetrose	Activity (c/min.)	Ratio
Water	Erythrose	693	3.2
	Threose	216	
Borate	Erythrose	222	4.4
	Threose	50	

The yields of threose were then calculated

from the known erythrose value.

Expt. 52. The determination of Glycolaldehyde

Glycolaldehyde was prepared via tartaric acid and dihydroxy maleic acid exactly as described by Powers et al.[153] A batch of crystalline material (5 g.) was obtained (m.p. 95°C).

The colorimetric method of Dische and Borenfreund[154], with a slight modification, was suitable for the estimation. These workers examined the colour reaction of all the organic components in the present mixture except the tetroses. Absorption curves were drawn and published. It is clear from the curves that measurement of the optical density at 680 mμ would give a value which would contain, at the most, ca. 3% error due to glyceraldehyde. Measurements were hence made using 608 filters in a Hilger "Spekker".

Experiments to check the interfering action of ions and tetroses

A standard solution of glycolaldehyde (1 mg./ml.) was prepared. The colour reaction with diphenylamine was examined in the presence of erythrose, phosphate,

borate, iodate and formate. Iodate was found to interfere seriously.

Removal of iodate

The weak base resin, Amberlite IR-45 removed iodate from solution, 95% of the glycolaldehyde was also removed. Precipitation with barium hydroxide solution and shaking with barium carbonate were found to be inefficient. Finally Amberlite IRA-400 (strong base), in its acetate form, was shown to effect 100% removal of iodate without affecting the glycolaldehyde concentration.

Glycolaldehyde was shown, by ionophoresis, to complex with borate ion. Therefore, to avoid possible loss by absorption on the resin as the borate complex, the borate reaction mixture was swamped with glycitol before passage down the resin column. Borate ion would, in this way, be used up by preferential complexing with glucitol.

The determination of Glycolaldehyde in the reaction mixtures

D-Glucitol (910 mg.) was dissolved in (i) water, (ii) borate, (iii) phosphate (30 ml.). Sodium periodate (268 mg.), as a 0.403 M solution, was added to each

mixture. Reactions (ii) and (iii) were adjusted to pH 5.0 with Amberlite IR-120 [H^+] after 10 min. One tenth of each solution was shaken with fresh resin (5 g.) for 15 min. to completely remove cations. D-Glucitol (0.5 g.) was added to the borate reaction mixture. The solutions were then passed down columns (1 x 12 cm.) of Amberlite IRA-400 [acetate] at 1 ml./min. and the eluates made up to 100 ml. with column washings. Aliquot parts (2 ml.) of the resulting solutions were measured for glycolaldehyde as follows[131]:-

A 100% solution of trichloroacetic acid (0.4 ml.) was added to the solution under test (2 ml.). Recrystallised diphenylamine (1% in ANALAR acetic acid - 5 ml.) was added. The solution was shaken and heated on a boiling water bath for 30 min. After cooling, the optical density was measured in a Hilger "Spekker" using 608 filters. The results are given in Table 33. The total volumes of the solutions were each 100 ml., thus the final column of Table 33 represents the weight of glycolaldehyde from 91 mg. of glucitol and hence the percentage yields are as follows:-

Aqueous control (A) - 2.79%

Borate buffer (B) - 2.37%

Phosphate buffer (C) - 2.70%

Table 33.

Solution	"Spekker" reading	Concentration (mg./100 ml.)
Standard 1	487	3.33
" 2	318	2.50
" 3	259	2.0
" 4	112	1.0
" 5	46	0.5
A	356) 330) 346	2.54
B	287) 282) 284	2.16
C	324) 332) 328	2.46

Expt. 53. The determination of Formaldehyde

Formaldehyde, after steam distillation from the mixtures was determined with chromotropic acid.[65] D-Glucitol (0.91 g.) was oxidized under the three conditions (A, B, and C) and the reaction mixtures steam distilled

until two batches of ca. 300 ml. had been collected. An acetone/solid CO_2 trap was used to avoid loss of formaldehyde. B.D.H. Formalin solution (2 ml.) was made up to 50 ml. and standardised by the "Dimedone" method. Five standards were obtained by dilution of this solution.

Chromotropic acid (0.5 g.) was dissolved in water (50 ml.). A mixture of two parts of conc. H_2SO_4 and one part of water (200 ml.) was added to this and the solution used immediately (light sensitive). This solution (9 ml.) was added to aliquot parts of the test solutions (2 ml.). The mixtures were heated for 30 min. in a boiling water bath. After cooling, the optical densities were measured with an "Eel" absorptiometer using a 606 filter. The results are given in Table 34 and correspond to final yeilds of:-

Aqueous control	-	0.243%
Borate buffer	-	0.265%
Phosphate buffer	-	0.190%

Table 34

Sample	Absorption reading	Concentration of HCHO(γ/ml.)	Volume of distillate (ml.)
Standard 1	6.86	7.72	-
" 2	3.52	3.86	-
" 3	1.79	1.93	-
" 4	0.90	0.97	-
" 5	0.51	0.48	-
A I	5.40	6.00	325
II	0.91	0.90	290
B I	6.95	7.75	262
II	1.33	1.40	272
C I	3.96	4.40	310
II	1.25	1.35	260

Expt. 54. The determination of Formic acid

Formic acid was removed from the mixtures by distillation and estimated by titration with standard alkali. It was found necessary to follow each determination with valid blank reactions. Titration was carried out to pH 6.5 using a pH meter for end-point detection.

Series A D-Glucitol (0.91 g.) in water (30 ml.) was oxidized with sodium periodate (268 mg.). After 15 min., the solution was made up to 50 ml. and 10 ml. aliquots titrated with 0.01 N-NaOH. The average titre was 2.1 ml. A blank reaction was carried out as follows:- ethylene glycol (0.31 g.) in water (50 ml.) was oxidized with sodium periodate (268 mg.). Titration as above gave a reading of 1.6 ml. The glycol was not acidic, hence the real titre for formic acid was 0.5 ml. and the weight per cent yield from glucitol 0.13%.

Series B In this series, it was necessary to remove the formic acid from the large excess of buffer. This was accomplished by acidifying the solution and distilling out the acid. Distillation to 0.25 of the original volume and addition of water back to that volume was repeated 3 times. The recovery of formic acid was 99%.

D-Glucitol (0.91 g.) in 0.5 M borate buffer (30 ml.) was oxidized with sodium periodate (268 mg.). After 10 min., the pH of the solution was taken to 5.0 with Amberlite IR-120 [H^+]. The resin was filtered off and the solution made up to 50 ml. Aliquot parts (10 ml.) were taken, and saturated aqueous $KHSO_4$ (5 ml.)

added, and the solution distilled to ca. 3 ml. Water (15 ml.) was added and the distillation repeated. The distillates were combined after a third distillation and required 5.6 ml. of 0.01N-NaOH to neutralise them. A blank reaction using ethylene glycol (0.31 g.) required 1.4 ml. Hence the true titre was 4.2 ml. and the yield of formic acid, 1.06%.

Series C The B series oxidation was repeated using 0.5 M phosphate buffer in place of borate. The corresponding titres were 9.2 ml. and 1.4 ml. and, hence, the real titre was 7.8 ml. This corresponded to a formic acid yield of 1.97%.

Table 35. Summary of the yields of products (m.moles) from D-Glucitol (1 m.mole)

Product	Yields		
	Control-A	Borate-B	Phosphate-C
D-Glucitol	0.7498	0.9120	0.8975
D-Arabinose	0.0046	0.0076	0.0047
L-Xylose	0.0092	0.0212	0.0107
D-Erythrose	0.0496	0.0031	0.0083
L-Threose	0.0165	0.0007	-
DL-Glyceraldehyde	0.2752	0.0152	0.0384
Glycolaldehyde	0.0846	0.0920	0.0819
Formaldehyde	0.0146	0.0163	0.0115
Formic acid	0.0051	0.0419	0.0779

Expt. 55. The relative rates of periodate oxidation of D-Glucitol and Pentoses

Part of a mixture of D-glucitol and D-xylose (1 m.mole of each) was applied to the origin line of chromatograms. The remainder was oxidized with periodate (1 m.mole). The products were chromatographed on the same paper as the control (above). Chromatograms were developed with solvent (d) for 20 hr. They were then sprayed with the silver nitrate reagent and, while still wet, the intensities of the glucitol and xylose bands were measured with an "Eel" scanner. A similar oxidation was carried out in borate buffer. The experiment was repeated using arabinose in place of xylose. The relative changes of the compounds was found by measuring the area under the absorption curves under the various conditions

Glucitol: xylose ratios

Aqueous solution	Control - 0.56
	Oxidation - 0.32
Borate Buffer	Control - 0.67
	Oxidation - 0.55

Glucitol: arabinose ratios

Aqueous solution	Control - 1.26
	Oxidation - 1.08
Borate Buffer	Control - 1.63
	Oxidation - 1.26

In all cases the glucitol/pentose ratio decreases on oxidation, i.e. glucitol is oxidized more rapidly than pentose.

Attempts to study the rates of oxidation of the smaller fragments were unsuccessful because glucitol gave high yields of those products on oxidation and the chromatograms were too complicated.

Expt. 56. The consumption of periodate by alkaline buffers

(i) D-Glucitol (45.5 mg.) in water (12 ml.) was oxidized with 0.268 M sodium periodate (10 ml.). The periodate remaining after 30 min. was measured by the addition of 0.102 N arsenious oxide solution (40 ml.), KI (0.5 g.) and $NaHCO_3$ (2 g.). The arsenite remaining was titrated after 15 min. with 0.096 N iodine.

The experiment was repeated using glucitol in the presence of a 3 molar excess of borate and of phosphate buffers, and with a 10 molar excess of borate buffer (all at pH 10). The results are shown in Table 36.

Table 36

Glucitol used (m.mole)	Molar excess of buffer	Periodate used (m.mole)	I_2 titre (ml.)	Periodate uptake (m. mole)	Theoretical uptake (m. mole)	Periodate "lost" (m. mole)
0.25	-	2.5	12.45	1.240	1.24	0
0.25	3(borate)	2.5	13.80	1.304	1.24	0.0664
0.25	3(phosphate)	2.5	13.05	1.270	1.24	0.030
0.25	10(borate)	2.5	13.85	1.307	1.24	0.067

If the theoretical uptake is taken as 1.24 m.mole, the per cent excess uptake is

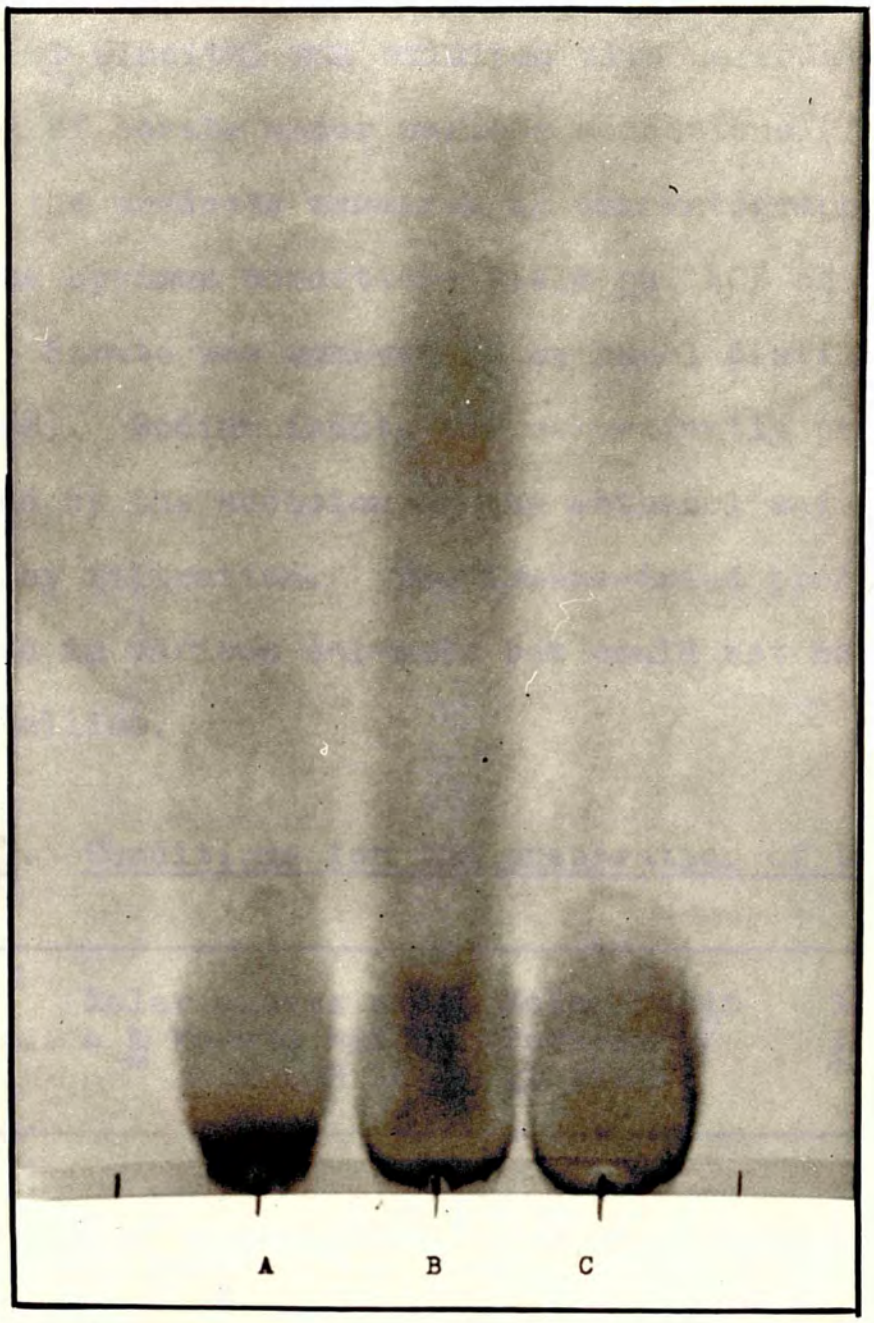
Aqueous control - none
Borate (3M) - 5.16%
Phosphate - 2.42%
Borate (10 M) - 5.40%

A repeat experiment yielded iodine titres of 11.4, 12.8, 12.3 and 13.0 ml. respectively.

(ii) D-Glucitol (1 m.mole) was oxidized with periodate (5 m.mole) in water, borate and phosphate. Glycolaldehyde was determined (Expt. 52) but none found. The deionized solutions were chromatographed [solvent (a)]. The result (Fig. 19) showed that, whereas the water and phosphate oxidations were almost complete, the borate oxidation was far from so.

Figure 19.

The oxidation of D-Glucitol (1 mole) by Periodate (5 mole) in A, aqueous solution; B, Borate buffer; C, Phosphate buffer.



Expt. 57. The preparation of L-xylose

D-Glucitol was oxidized with periodate in the presence of borate under various conditions (Table 37) and the products examined by chromatography (Fig. 20). The optimum conditions yield ca. 40% of L-xylose. Borate was removed by methanol distillation (Expt. 48). Sodium iodate was conveniently precipitated by the addition of the methanol and was removed by filtration. The freeze-dried product was dissolved in various solvents but could not be induced to crystallise.

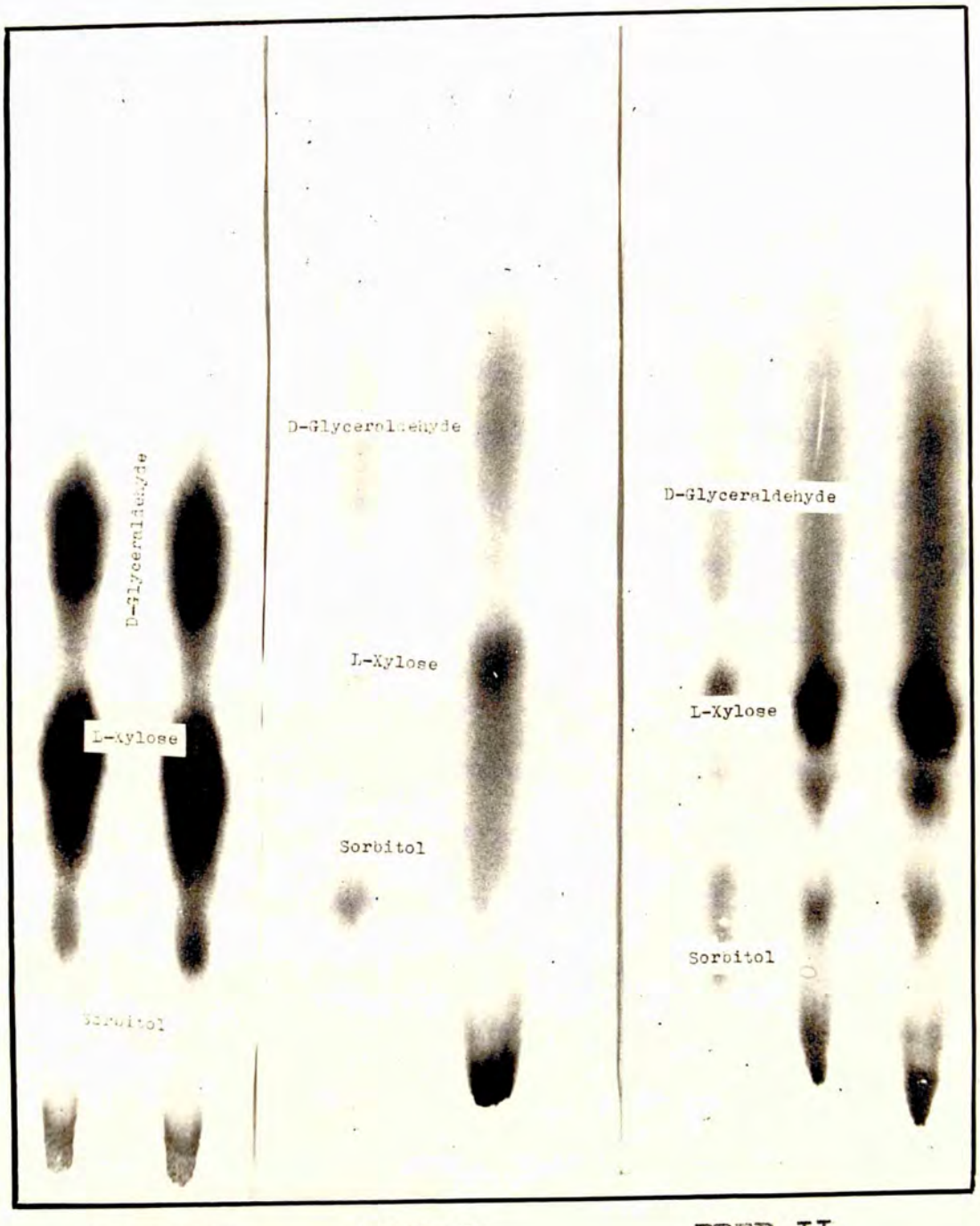
Table 37. Conditions for the preparation of L-Xylose

Preparation No.	Molar excess of 4 M Borate buffer (pH 10)	Molar ratio Periodate/ glucitol	% yield of L-xylose *
I	3	2.0	20
II	5	2.0	30
III	5	2.5	40
IV	5	3.0	30

* estimated visually from chromatograms.

Figure 20 .

Chromatograms illustrating the yields of L-Xylose.



PREP.III. PREP.I. PREP.II.

Expt. 58. The periodate oxidation of D-Glucose in the presence of borate

D-Glucose (0.90 g.) and boric acid (1.28 g.) were dissolved in water (10 ml.). The pH of the solution was adjusted to 10 by the addition of N-NaOH. Sodium periodate (0.107 g.) in water (10 ml.) was added. Borate was removed after 10 min. in the usual manner (Expt. 48). A control reaction (without borate) was carried out. The products were chromatographed [solvent (d)] and detected by spraying with p-anisidine. In the control reaction ca. 80% of the glucose was destroyed yielding traces of glyceraldehyde and erythrose but mainly (ca. 50%) a pink spot of R_G value 1.7, which was probably 4-O-formyl-D-arabinose.[127] It was eluted from the paper and yielded arabinose on mild treatment with alkali. The borate buffered reaction produced ca. 30% arabinose and traces of tetrose and triose. A large amount of glucose (ca. 60%) remained unchanged.

Expt. 59. The periodate oxidation of D-Mannitol in the presence of borate

D-Mannitol (0.91 g.) was oxidized with periodate (1.07 g.) in the presence of boric acid (1.96 g.) at pH 10, as described in Expt. 58. The molar ratios were

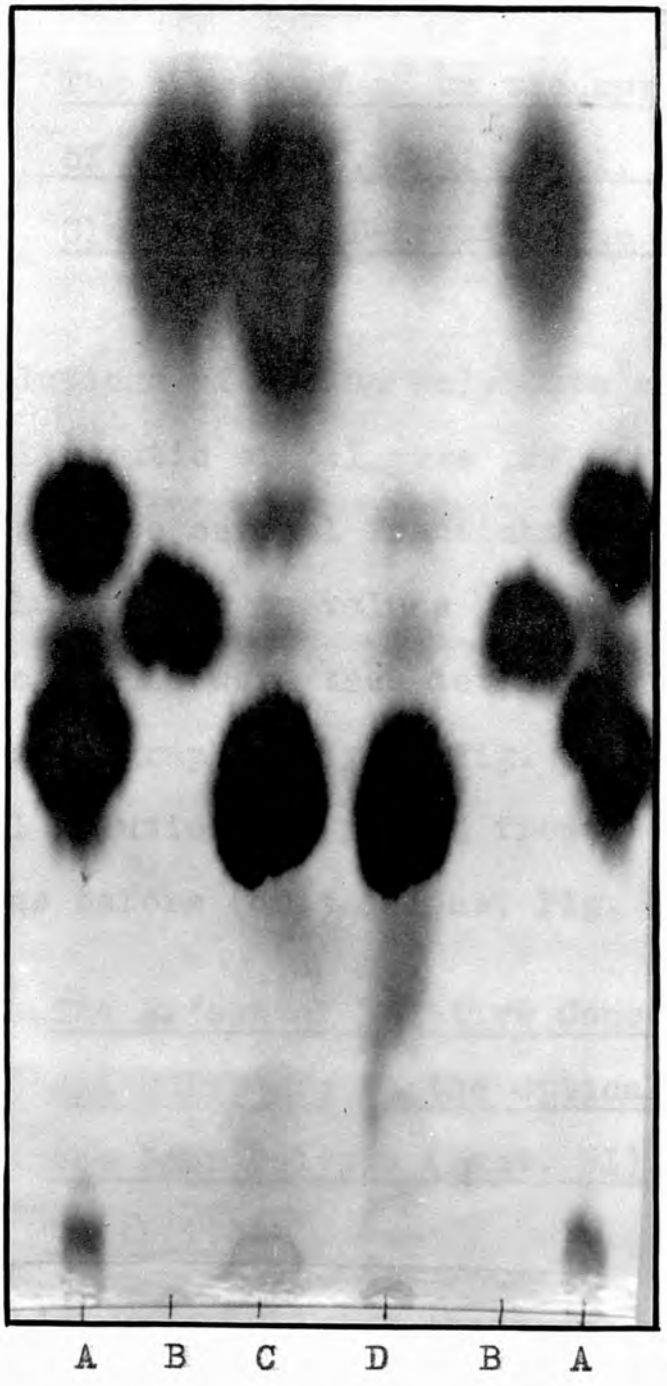
1:6:1 respectively. Examination of the products (Expt. 58) and comparison with a control reaction revealed no significant differences. The main products were glyceraldehyde and arabinose.

Expt. 60. The periodate oxidation of D-Glucitol in the presence of Molybdate at pH 2.0

D-Glucitol (0.91 g.-5 m.mole) and sodium molybdate (3.63 g.-15 m.mole) were dissolved in water (10 ml.) and the pH adjusted to 2.0 with N-H_2SO_4. Sodium periodate (0.54 g.-2.5 m.mole) in water (10 ml.) was added. After 10 min. an aliquot part of the solution ($^1/20$th) was treated with Amberlite IR-120 [H^+] (2 g.) and then passed down a column (10 x 1 cm.) of Amberlite IRA-400 [acetate]. The resulting solution was freed from acetic acid by repeated evaporation and chromatographed [solvent (d)]. A control reaction, in which no molybdate was present, was also chromatographed (Fig. 21).

Figure 21.

Periodate oxidation of D-Glucitol in the presence of Molybdate.



A = Glucitol
Xylose

B = Arabinose
Glyceraldehyde

C = Control
oxidation

D = Oxidation in
presence of
Molybdate.

THE EFFECT OF PH AND RELATIVE CONCENTRATION ON THE OPTICAL ROTATION OF POLYOLS IN MOLYBDATE SOLUTION

Expt. 61. The Effect of pH on the optical Rotations of D-Mannitol, D-Glucitol, 2-deoxy-D-Glucitol and D-Arabitol in Molybdate Solution

Solutions of sodium molybdate and the polyol in the molar ratio of 3:1 were prepared. The solution was divided into several fractions and the pH adjusted with sulphuric acid to values between 8 and 1. The optical rotations were then determined. The results are expressed graphically in Fig. 12. The pH of the D-glucitol solution was raised from 1 to 9 and the rotations measured as before (dotted line, Fig. 12).

Expt. 62. The Effect of Relative Concentration of Polyol and Molybdate on the Optical Rotations of the Four Polyols (Expt. 61), and Cellobiitol and Melibiitol

Solutions containing the same amount of polyol and increasing amounts of sodium molybdate, were prepared and the pH adjusted to 2 with sulphuric acid. The specific rotation (based on polyol) was determined for

each solution. The results were expressed graphically by plotting the $[a]_D$ against the molar ratio of molybdate to polyol (Fig. 22 and 23).

Figure 22.

The effect of relative concentration of Molybdate and Polyol on the Optical Rotation of the solution at pH 2.

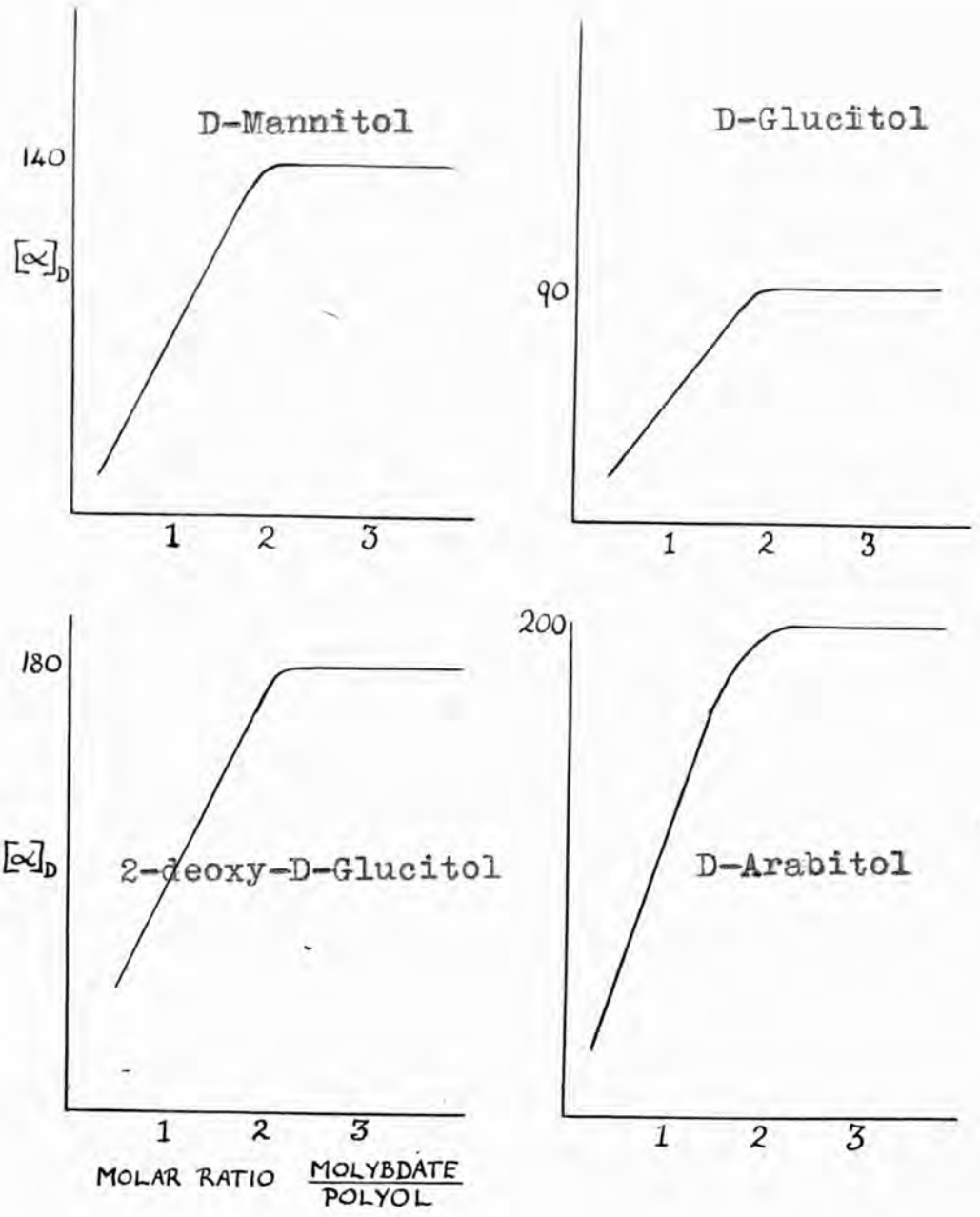
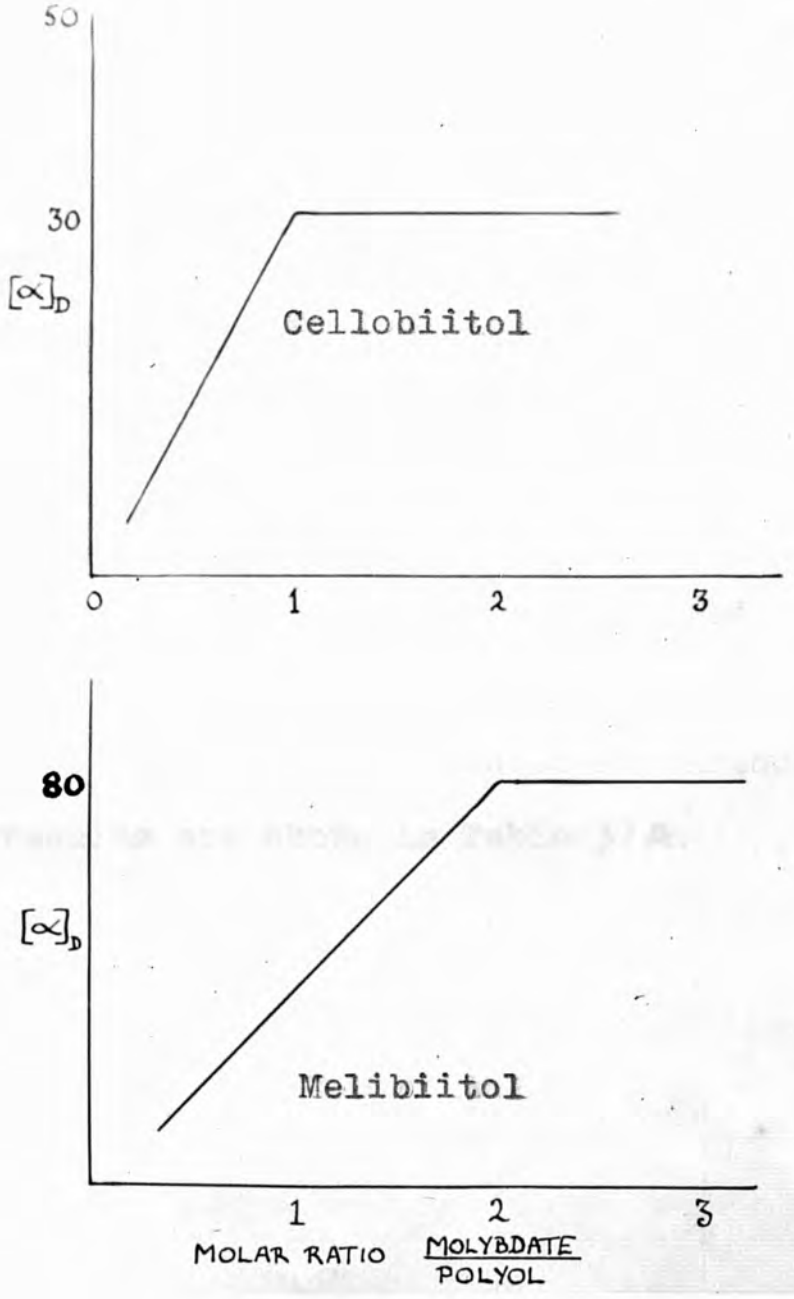


Figure 23.

As Fig. 22.



Expt. 63. The Effect of pH on the Glucitol-Molybdate Complex

Expt. 62 was repeated at pH 4 and 6. The $[\alpha]_D$ reached a lower constant value but the composition of the complex remained unchanged.

Expt. 64. The Effect of Dilution on the Mannitol-Molybdate Complexes

A solution of D-mannitol (1 mole) with (i) 1 mole and (ii) 3 moles of sodium molybdate was prepared. The pH was adjusted to 2 with sulphuric acid. The specific rotation was measured at various dilutions. The results are shown in Table 37A.

Table 37A

Molar ratio* 2		Molar ratio* 1	
Concn. of Mannitol (g./100 ml.)	$[\alpha]_D^{24}$	Concn. of Mannitol (g./100 ml.)	$[\alpha]_D^{24}$
6.06	136.3	3.0	65.0
4.05	144.0	2.3	66.1
3.03	133.3	1.5	67.0
1.51	143.2	0.75	68.2
0.76	141.3		
0.38	140.0		
0.05	147.0		

* $\frac{\text{Molybdate}}{\text{Mannitol}}$

Expt. 65. The Reduction of Sugar to Sugar Alcohol

A number of samples used in rotational studies and ionophoresis experiments were prepared by the reduction of the more readily available aldoses. The following description is representative of all these reductions. The amount of starting material varied from 2 g. (arabinose) to 1.5 mg. (sophorose).

2-deoxy-D-Glucose (0.5 g.) was dissolved in water (10 ml.) and potassium borohydride (0.5 g.) in water (10 ml.) added. The solution was allowed to stand overnight. Amberlite IR 120 [H^+] (5 g.) was added and shaken for 15 min. After filtering, water was removed by distillation under reduced pressure and boric acid removed by distillation to dryness with dry methanol (5 ml.), three times, under reduced pressure. If a known weight of material was required, an aliquot part was oxidized with periodate and the resulting formaldehyde determined with dimedone.

Expt. 66. Chromatography of Pre-formed Molybdate Complexes

Complexes were formed by solution of polyol in water with sodium molybdate and adjusting the pH to 2 with acid resin. Solutions were then spotted on untreated paper and chromatograms developed with solvents (a), (d), (e) or the organic phase of a mixture of n-butanol, acetic acid, and water (4:1:5) [solvent (h)]. The silver nitrate spray was used to locate polyol containing spots and aqueous catechol (5%), the molybdate containing spots. The results are given in Table 38.

Table 38. R_G values of Molybdate Complexes

Solvent	Parent Compound	R_G Value	Silver nitrate reagent detected spots at:-		
	D-Glucitol	1.0	0.90	0.50	0.20
	D-Mannitol	1.1	0.95	0.56	0.20
(a)	Dulcitol	0.96	0.94	0.56	0.36
	Erythritol	2.2	2.20	0.80	
	Glycerol	3.2	3.20		
	D-Glucitol	1.0	1.00	0.74	0.30
	D-Mannitol	1.0	1.00	0.70	0.30
(b)	Dulcitol	0.9	0.90	0.54	0.20
	Erythritol	2.3	2.30	1.61	
	Glycerol	3.2	3.20		
Solvent (d) - poor results of no value					
	D-Glucitol	1.0	0.87	0.60	0.50
	D-Mannitol	1.1	0.90	0.70	0.50
(h)	Dulcitol	1.1	1.00	0.70	0.50
	Erythritol	1.7	1.70	1.20	
	Glycerol	2.2	2.20		

Expt. 67. Ionophoresis in Molybdate Solution

Electrolyte - sodium molybdate (25 g.) was dissolved in water (1200 ml.). The pH of the solution was adjusted to pH 5.0 by the addition of conc. H_2SO_4. Ionophoretograms were prepared by applying a voltage of 10-20 v/cm. for 2-3 hr. Compounds were detected by spraying with silver nitrate reagent, p-anisidine reagent, or by spraying the paper with 0.1 $\underline{\underline{N}}$ acid and heating at 100°C for 15 min. The latter treatment caused all compounds containing primary hydroxyl groups to appear as bluish-green spots.

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