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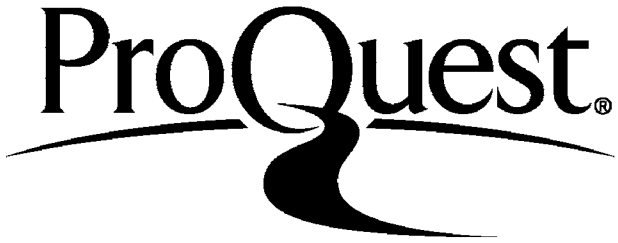

1.

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Über im Pile bestrahltes Cystin.

Durch die Beobachtung, daß bei der Reaktion $^{34}S\ (n, \gamma)\ ^{35}S$ zwei γ-Quanten in entgegengesetzten Richtungen abgegeben werden können, die Rückstoßenergie des ^{35}S-Kernes in einigen Fällen also Null sein kann, wurden BALL, SOLOMON und COOPER[1]) veranlaßt, die Umwandlung des im Cystinschwefel enthaltenden ^{34}S-Isotops durch direkte Bestrahlung des Cystins mit thermischen Neutronen durchzuführen. Durch Bestrahlung von Cystin im Oak-Ridge-Pile erhielten sie ein Produkt mit einer Aktivität von 5 μC. Gegenüber der erwarteten Aktivität von 7 mC sind dies nur 0,07%. Die Autoren stellten von diesem Cystin verschiedene Derivate her, die alle die gleiche molekulare Aktivität zeigten, und folgerten hieraus, daß der radioaktive Schwefel des bestrahlten Präparates Cystinschwefel sein müsse.

Wir haben Cystin untersucht, das im Pile von HARWELL in England mit gebremsten Neutronen bestrahlt worden war, und konnten die Befunde von BALL, SOLOMON und COOPER nicht reproduzieren.

Die Aktivierung des Cystinschwefels erfolgte ohne Rücksicht auf seine chemische Bindung und in gleicher Weise wie die des elementaren Schwefels. Daß die radioaktive Substanz in dem bestrahlten Präparat das ^{35}S-Isotop ist, ließ sich sicherstellen, indem der Schwefel quantitativ in $BaSO_4$ überführt und dessen Aktivität mit der des eingesetzten Cystins verglichen wurde. Kohlenstoff, Stickstoff, Wasserstoff und Sauerstoff wurden hierbei nicht radioaktiv.

Das mehrmals umgefällte Präparat wurde in Benzylcystein übergeführt, dieses in Cystein gespalten und letzteres zum Cystin oxydiert[2]). Dadurch sollte das bestrahlte Präparat von radioaktiven Verunreinigungen befreit werden. Die Aktivitätsmessungen zeigten, daß hierbei die radioaktive Substanz vom Cystin vollständig abgetrennt wird. Mehr als 55% des radioaktiven Schwefels konnten aus der Mutterlauge als Sulfat ausgefällt werden.

Die Umwandlung des stabilen Schwefelisotops ^{34}S, das im natürlichen Schwefel des Cystins mit 4,18% enthalten ist, in das radioaktive Schwefelisotop ^{35}S nach einem (n, γ)-Prozeß durch direkte Bestrahlung des Cystins mit gebremsten Neutronen im Pile gelingt also nicht, ohne daß der gesamte an der Kernreaktion beteiligte Schwefel infolge seiner großen Rückstoßenergie aus der Cystinmolekel herausgeschleudert wird. Inwiefern dabei die Molekel in anderer Weise umgewandelt wird, ist noch ungeklärt.

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Aachen, Organisch-Chemisches Institut der Rheinisch-Westfälischen Technischen Hochschule.

MARIA LIPP und HELMUT WEIGEL.

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2.

[^{14}C]-CELLULOSE FROM *ACETOBACTER ACETIGENUM*

By E. J. Bourne and H. Weigel

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It has been proved recently in two laboratories[1, 2] that the highly-swollen water-insoluble polysaccharide produced by growing cultures of *Acetobacter acetigenum* (T. K. Walker's strain) is a cellulose. We now report studies of the utilization of DL-[*carboxy*-^{14}C]-lactic acid by the organism and of the ultimate distribution of the labelled atoms in the cellulose structure.

The DL-[*carboxy*-^{14}C]-lactic acid was prepared from acetaldehyde and sodium [^{14}C]-cyanide in the presence of an acidic Amberlite resin, which served both to take up the cations and to catalyze the hydrolysis of the cyanide group. The crude product contained [^{14}C]-formic acid, which was removed as hydrogen and [^{14}C]-carbon dioxide by treatment with a palladium catalyst on a barium sulphate carrier, as described by Paal and Poethke.[3] The DL-[*carboxy*-^{14}C]-lactic acid was isolated as its zinc salt in a yield of 65—70%, based on sodium [^{14}C]-cyanide. The possibility that any significant isotopic exchange had occurred during this synthesis was dismissed when it was shown that 98·6% of the ^{14}C was liberated as [^{14}C]-carbon dioxide, during treatment of the DL-[^{14}C]-lactic acid with sodium metaperiodate.

The organism was grown at 29° for 27 days on a medium (500 c.c.) containing NH_4^+, K^+, Mg^{++}, Ca^{++}, Fe^{++}, Zn^{++}, PO_4^{---}, SO_4^{--}, CO_3^{--}, DL-alanine, L-asparagine, DL-valine, L-leucine, DL-*iso*leucine, L-glutamic acid, *p*-aminobenzoic acid, nicotinic acid, calcium DL-pantothenate, riboflavin, biotin, and D-glucose (concentrations 0·0004—0·1%), commercial ammonium lactate (4%), and DL-[*carboxy*-^{14}C]-lactic acid (160 μc). The pH was maintained at 5·8 by periodic additions of lactic acid.

The [^{14}C]-cellulose produced (233 mg.) was purified by washing well with water, dissolving in cuprammonium solution, precipitating with acid, and washing again. Paper chromatographic analysis of a hydrolysate showed only one radioactive spot, having an R_F value identical with that of a glucose reference spot. An aniline hydrogen phthalate spray revealed only the same spot. The hydrolysate was mixed with D-glucose (950 mg.) and purified by crystallization, to give [^{14}C]-D-glucose with a specific activity of 139·9 μc/mole.

The distribution of the radioactivity in the [^{14}C]-D-glucose was determined by converting each individual carbon position into barium carbonate. The method employed was essentially that described by Bevington, Bourne and Turton,[4] and was based on glycol-cleavage of potassium D-gluconate, D-glucose phenylosotriazole and 6-*O*-benzoyl-D-glucose phenylosotriazole. The results, shown in Table I, revealed that the carbon

Table I

Distribution of ^{14}C in glucose

Carbon position of glucose	Specific activity in μc/mole carbon	Percentage distribution
1–6	139·9*	100
1	2·6	2
2	16·6	12
3	50·2	36
4	51·7	37
5	15·4	11
6	1·1	1

* μc/mole glucose

chain of the glucose was labelled symmetrically. Positions 3 and 4 were three times as active as positions 2 and 5; positions 1 and 6 carried only traces of activity. Such a distribution suggests strongly that the glucose units of the cellulose arise by the fusion of two three-carbon fragments. Experiments are in progress to provide further evidence on this point, and to correlate the pathways of carbohydrate metabolism in this organism, and in the starch-synthesizing *Polytomella coeca*,[4] which do not involve photosynthesis, with those followed during the production of cellulose and starch by plants.

The authors are indebted to Prof. M. Stacey, F.R.S., and Prof. M. Lipp for their interest, to Dr. J. C. Bevington for providing facilities for the activity assays, to Dr. T. K. Walker for details of the medium used, and to the Kulturministerium des Landes Nordrhein-Westfalen for the award of a scholarship to one of them (H. W.).

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3.

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Synthesis of Dextran Sulphate labelled with Carbon-14 and Tracer Experiments in the Rat

PREVIOUS experiments[1,2] with the heparinoid anticoagulant, dextran sulphate, in experimental animals and man have established that between 40 and 50 per cent of a single intravenous dose of dextran sulphate of a molecular size suitable for clinical use was excreted in the urine in 48 hr.

Autoradiographs of the tissues of animals given dextran sulphate of similar molecular characteristics labelled with sulphur-35 suggested that the portion of the dose unaccounted for by urinary excretion was broken down within the body but that the ^{35}S-sulphate residue was retained in the general body pool of ester sulphate since, as with the administration of $Na_2{}^{35}SO_4$ [3], radioactivity was demonstrable in gastric mucin, skin and, to a lesser extent, in cartilage (Walton, unpublished work). This redistribution of the labelled sulphate residue rendered difficult the interpretation of the results obtained in terms of the distribution of dextran sulphate in the tissues. Accordingly, further experiments have been undertaken using dextran sulphate labelled in the dextran part with carbon-14.

^{14}C-Dextran was prepared by inoculating an aqueous buffered medium containing yeast extract and ^{14}C-sucrose (3·76 gm., 3·3 mc.) with *Leuconostoc mesenteroides* and incubating the mixture for 3 days at 25° C. ^{14}C-Dextran and protein were precipitated with ethanol. The precipitate was redissolved and freed from protein by treatment with trichloracetic acid yielding 1·162 gm. ^{14}C-dextran with a specific radioactivity of 22·39 mc./mole of carbon.

The ^{14}C-dextran so obtained was partially hydrolysed with $N/1$ sulphuric acid and fractionated by the addition of successive increments of acetone. A fraction (232 mgm.) of viscosity comparable to that used for the preparation of clinical dextran sulphate was selected for sulphation by treatment with chlorosulphonic acid in pyridine as previously described[4].

The ^{14}C-dextran sulphate (413 mgm.) (found: C, 20·40; H, 2·15; S, 16·85. $[C_6H_{8\cdot16}O_5\ (SO_3Na)_{1\cdot84}]_n$ requires C, 20·59; H, 2·35; S, 16·86 per cent) had a specific radioactivity of 22·39 mc./mole carbon or 0·45 μc./mgm. and an anticoagulant activity of

Table 1. Labelled Carbon Contents of Respired Carbon Dioxide and of Tissues of Rat following Injection of 5 mgm. (2·25 μc.) of ^{14}C-Dextran Sulphate in Experiment lasting 48 hr.

Source	Weight (gm.)	Carbon content (per cent)	Specific radio-activity (μc./mole carbon)	Total radio-activity (μc.)	Concentration of injected radio-activity (per cent)
Carbon dioxide respired	125·674	6·085	0·13	0·08	3·55
Liver	13·366	13·35	1·19	0·18	8·0
Spleen	0·650	9·15	1·00	0·005	0·22
Lungs	2·810	9·08	0·19	0·004	0·18
Kidneys	2·423	23·20	1·68	0·08	3·55
Muscle (sample)	1·120	9·73	0·17	—	—
Total musculature	123·94*	—	—	0·17	7·56
Bone (femur)	0·86	5·62	0·15	—	—
Whole skeleton	14·80*	—	—	0·01	0·44

Total radioactivity accounted for : 23·50 per cent of dose.
* Average figure derived from published data, ref. 10.

13 units/mgm. Paper chromatography revealed results similar to those obtained with the dextran sulphate previously employed[2].

A male albino rat weighing 273 gm. was injected intravenously via the tail-vein with a solution of ^{14}C-dextran sulphate (5 mgm., that is, 2·25 μc. and dose equivalent to 18·3 mgm./kgm./body-weight) in saline and placed immediately in a closed-circuit apparatus[5], allowing collection of the respired air. The rat was maintained thus for 48 hr., being allowed access to food and water *ad libitum*. The expired carbon dioxide was collected in 3 *N* sodium hydroxide and converted into barium carbonate. Aliquots were used for the determination of specific radio-activity.

At the end of the experimental period the animal was killed by a blow on the head, various organs were removed, weighed and portions homogenized by grinding under liquid air. Other portions were submitted to histological examination which confirmed that the injected material, like previously tested examples of dextran sulphate for clinical use[6], was not segregated in reticulo-endothelial cells.

^{14}C-Dextran and ^{14}C-dextran sulphate were burned in a stream of oxygen free of carbon dioxide, and aliquot parts of the homogenized tissues were treated with boiling chromic acid combustion fluid[7]. The carbon dioxide produced in each case was converted into barium carbonate and the radioactivity determined by the infinitely thick disk method[7–9] using

an end-window counter and a sample of poly-(^{14}C-methyl) methacrylate supplied by the Radiochemical Centre, Amersham, as a standard source for barium ^{14}C-carbonate. The results tabulated are corrected for background and paralysis time of the counting equipment.

Excretion of carbon dioxide labelled with carbon-14 in the expired air indicated complete oxidation of a portion of the administered ^{14}C-dextran sulphate. The rate at which this occurred appeared to be somewhat slower than the rate of excretion of labelled carbon dioxide from ^{14}C-dextran in rats since 3·55 per cent of the injected dose of ^{14}C-dextran sulphate was accounted for after 48 hr. in the present experiment as compared with 8–10 per cent of ^{14}C-dextran[11] in the same period. The radioactivity of the expired air together with that found in the organs examined accounted for 23·50 per cent of the dose administered. Assuming urinary excretion of a further 40–50 per cent in the first 48 hr. as previously found[1,2] about 30 per cent remained unaccounted for and presumably was distributed in the body fluids and tissues not sampled.

The distribution of radioactivity in the tissues suggested that the major sites of retention (and therefore possibly of metabolism) of the material were in the liver and in muscle (8 per cent each), as has also been found for ^{14}C-dextran[11]. High retention in the liver has previously been found for other labelled polysaccharide sulphates (namely, 7·2 per cent for cellulose ^{35}S-sulphate[12] and 9·0 per cent for one preparation of low molecular weight xylan ^{35}S-sulphate[13]). The significance of the relatively high radioactivity found in the kidneys in the present experiment (3·55 per cent) is less clear since this might be accounted for partly by this organ being the one chiefly concerned in the excretion of dextran sulphate[2] and therefore probably containing ^{14}C-dextran sulphate in process of excretion in the urine.

The sample of dextran sulphate used for the experiments described underwent decomposition before further biological experiments could be carried out. It seems likely that decomposition was initiated by self-irradiation and continued autocatalytically under acidic conditions. Details of this work will be published elsewhere.

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4.

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SELF-DECOMPOSITION OF COMPOUNDS LABELLED WITH RADIOACTIVE ISOTOPES

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IT is quite evident that before an isotopically labelled compound can be profitably used its purity must be known. This will normally be determined following the preparation of the material and, if it is purchased, the information will be provided by the commercial supplier. However, if the material is not used at once, it may undergo significant decomposition under the influence of its own radiation, and there are some aspects of this problem of which we feel users of tracer compounds are insufficiently aware. It is true there is a substantial literature on radiation chemistry generally, but most of it is concerned with irradiation of solutions rather than of pure organic substances, while the actual phenomenon of radiation self-decomposition has been dealt with in relatively few papers[1–5]. The object of this article is to analyse the problem with particular reference to carbohydrates and to present some further quantitative information.

It is important to distinguish in what follows between a chemical and a radiochemical impurity, and to be clear what is meant by the latter. We define a radiochemical impurity as being any radioactive species other than the declared isotope in the stated position(s) of the given compound. In passing, we may note that it commonly happens that a small quantity of radiochemical impurity can be critical whereas a comparable amount of inactive impurity is unimportant.

Types of Self-decomposition

The decomposition of a compound labelled with a radioactive isotope can be due to one or more of four effects:

(i) Primary (internal) radiation effect, that is, the decomposition or transformation of a molecule caused

by the disintegration of one of its unstable atomic nuclei. This atom, transformed into another element, might or might not be eliminated from the molecule. However, in either case, providing the daughter nuclei are stable, this will only give rise to a radiochemical impurity if the molecule contains a further radioactive atom.

(ii) Primary (external) radiation effect, that is, the decomposition or transformation of a molecule by interaction with a nuclear particle. If the molecule is a labelled one, this will contribute to the radiochemical impurity.

(iii) Secondary radiation effect, that is, the decomposition or transformation of a molecule of the labelled compound due to its reaction with reactive species, for example, free radicals, produced as a result of primary radiation decomposition. The reactive species may not necessarily be produced from the labelled compound but from its environment, for example, residual water in freeze-dried samples.

(iv) Chemical effect, that is, the decomposition or transformation of the radioactive material caused by chemical reactions which are not connected with radiation. Clearly, this is a hazard faced by any chemical compound, but it may be more probable in the case of a labelled one for several reasons. First, in an effort to obtain a good yield of material of high specific radioactivity, the highest chemical purity is not always achieved, and it is often difficult or expensive to determine this purity. The impurities may then react with the compound. Further, the small amount—possibly only a few micrograms—of radioactive material which is commonly synthesized or distributed by suppliers may suffer chemical decomposition in unexpected ways which would not normally be observed with larger quantities. It could be due, for example, to the atmosphere above a solid, or non-inertness of the container.

Magnitude of the Radiation Effects

The magnitude of the primary (internal) effect can be calculated in any particular case from the available data. Commonly it is a greater practical concern to know the amount of radiochemical impurity produced, and this can be calculated if the pattern of labelling is known. An example of this is given in the subsequent section on macromolecules.

One of the factors influencing the amount of decomposition due to primary (external) and secondary effects is the fraction of its own radiation energy absorbed by the labelled material. For a pure β-emitter

distributed over a relatively large area in a layer of even thickness l (cm.) this fraction, F, is given by:

$$F = \frac{pl}{2r}\left(1{\cdot}5 + \ln\frac{r}{pl}\right)$$

where p is the density (mgm. cm.$^{-3}$) and r is the mean range of the particles, expressed in mgm.cm.$^{-2}$ units. In this it is assumed that $r \not< pl$ and that all particles have the energy corresponding to their mean energy. A more exact value may be obtained for definite values of p and l using a known beta-spectrum, but the error is generally small. No allowance is made for back-scatter from the container or for edge effects, though for a given area these latter will, of course, be of increasing significance with increasing energy of the β-particle. The calculation involves determination of the average range of a β-particle by a double integration, the first for the solid angle around a point in a layer of the material, and the second for the contribution of all layers throughout the thickness of the solid.

The magnitude of decomposition (per cent) due to the primary (external) radiation effect is $\{1 - \exp(-F\epsilon a s_0 t.\ 6{\cdot}14 \times 10^{-16}\}\ 100$, where F is the fraction of its own radiation energy absorbed by the compound, ϵ is the mean energy of the radiation in electron-volts, a is the number of molecules irreversibly damaged per 100 eV., s_0 is the initial specific radioactivity of the compound in curies/mole, and t is the time in seconds. For compounds labelled with relatively short-lived isotopes, the term t in this expression should, of course, be replaced by $1/\lambda\ \{1 - \exp(-\lambda t)\}$, where λ is the radioactive decay constant.

Remedial Measures

It is clearly desirable to reduce the magnitude of decomposition of labelled compounds to a minimum. Setting aside the decomposition due to the primary (internal) radiation effect as being beyond control, we can consider the possible remedies for the other three types of decomposition.

Considering decomposition due to the chemical effect first, it is evident that the highest possible degree of freedom from harmful impurities is desirable, consistent with the requirements for specific radioactivity. Storage under vacuum is a common and useful precaution. Attention should also be paid to the inertness of the storage vessels, particularly for liquids or freeze-dried syrups. In our experience the alkalinity of normally washed 'Pyrex' glass is detrimental to the

stability of carbon-14 carbohydrate syrups. Storage at as low a temperature as possible can also help by reducing the rate of otherwise unavoidable chemical reactions.

The two fundamental methods for reducing the magnitude of primary (external) radiation decomposition are dispersion over a large area and dilution.

As already pointed out, the fraction of its own radiation energy absorbed by a radioactive compound distributed over a relatively large area is dependent on the thickness and density of the layer. Thus, the principle of such dispersion is to ensure that the nuclear particle escapes from the body of the radioactive material with the minimum interaction on the way. This method has been widely utilized in storage of labelled compounds—particularly for small quantities at high specific radioactivities—but frequently the material may need to be distributed over an area too large to be practical in order to reduce the self-irradiation to a satisfactory level.

The object of dilution is to interpose inactive molecules in the path of the radiation. This can be achieved either by adding the inactive form of the compound itself or by introducing other substances. The former avoids the necessity of subsequent isolation of the radioactive compound, but as it involves a drop in specific radioactivity it is not always acceptable.

It is not possible to suggest a universal method which will prevent the decomposition due to secondary radiation effects. However, it is evident that either dispersion over an area or dilution with the substance itself will reduce this type of decomposition, but the effect of dilution with a foreign substance will depend on the nature of that substance. On the credit side it will absorb radiation which might produce radioactive impurities, and in certain cases it may terminate chain reactions which would otherwise result in substantial decomposition following the radiolysis of a few radioactive molecules[6,7]. Conversely, if unwisely chosen, it may produce, by interaction with the radiation, reactive species which will interact with the labelled compound, and so do more harm than good. It is important to appreciate how wide is the range of susceptibilities to radiation for different compounds[4]. Storage of radioactive materials at low temperatures will usually reduce the decomposition due to secondary radiation effects, but will not, in general, completely prevent it. A high standard of chemical purity, already stressed for another reason, may also reduce this decomposition, since, although the impurity may not be harmful in itself, the products of its interaction with the nuclear particles may be. An

example of such an impurity is water in the case of carbohydrates.

Observations on Carbon-14 Carbohydrates

The stabilities of samples of uniformily labelled [^{14}C]-sucrose and D-[^{14}C] glucose with relatively high specific radioactivities are shown in Table 1. The compounds were prepared by photosynthesis, purified by ion-exchange and paper chromatography and finally crystallized. No radiochemical impurity was detected in either case by reverse isotope dilution analysis, and their chromatographic radiochemical purities were both found to be 99·9 per cent. After being dispensed from aqueous solutions, samples were stored in the freeze-dried form or uniformly distributed on Whatman No. 3 paper. In either case they were contained in 'Pyrex' tubes which had been filled with water and autoclaved for 2 hr. at 15 lb./sq. in. to remove surface alkalinity. The tubes of those samples which were stored *in vacuo* were evacuated to a pressure of 0·01 mm. mercury for several hours before being sealed in order to reduce the moisture content to a minimum.

The radiochemical impurities produced during storage were determined by scanning of paper chromatograms. Spots with R_F values corresponding to those of glucose and fructose were the principal impurities in the case of sucrose.

Many other experiments covering a wide range of specific radioactivities have shown that, under given storage conditions, the amount of decomposition is proportional to the specific radioactivity, provided that the substance is not allowed to decompose too far.

A comparison of tubes 7–10 suggests that, in at least the first three of these, secondary radiation effects take a major role in the decomposition. This is perhaps not surprising, since freeze-dried glucose will retain non-bonded water as 'impurity'. Sucrose, on the other hand, can with efficient freeze-drying techniques be obtained anhydrous, and the small difference in magnitude of decomposition between tubes 1 and 2 suggests that in these cases decomposition is to a great extent due to primary radiation effects. This view is supported by the relatively high calculated value for the apparent energy required to disrupt one molecule (24 eV. for tube 2). By the same criterion the most stable freeze-dried glucose sample (tube 10) is probably still greatly susceptible to decomposition due to secondary radiation effects.

It is clear from the high $G(-M)$ values for tubes 3–6, 11, 12, that such decomposition as does occur when radioactive sugars are distributed on paper is due to

Table 1. SELF-DECOMPOSITION OF [^{14}C]-SUCROSE AND D-[^{14}C] GLUCOSE

Compound	Tube No.	Storage conditions: Form	Temp.	Pressure	Impurity observed (per cent)	Initial decomposition per year per mc./mM (per cent)	Average mass per unit area (freeze-dried) (mgm./cm.^{2})	Area of paper (cm.^{2}) (*d*)	Energy absorbed by sugar or sugar syrup ($F \times 100$) (per cent) (*e*)	$G(-M)$ value (*f*)
Sucrose (*a*, *b*)	1	Freeze-dried	Room	Vac.	16·4	0·071	0·35	—	16	4·6
	2	Freeze-dried	−80° C.	Vac.	15·1	0·065	0·35	—	16	4·2
	3	On paper	Room	Atm.	15·7	0·068	—	21	0·3	234
	4	On paper	−80° C.	Atm.	4·9	0·020	—	21	0·3	68
	5	On paper	Room	Vac.	2·4	0·010	—	21	0·3	33
	6	On paper	−80 °C.	Vac.	1·8	0·007	—	21	0·3	25
Glucose (*c*)	7	Freeze-dried	Room	Atm.	18·9	0·77	0·2	—	10	79
	8	Freeze-dried	−80° C.	Atm.	6·0	0·23	0·2	—	10	23
	9	Freeze-dried	Room	Vac.	13·1	0·51	0·2	—	10	53
	10	Freeze-dried	−80° C.	Vac.	3·6	0·133	0·2	—	10	14
	11	On paper	Room	Vac.	1·2	0·044	—	47	0·05	900
	12	On paper	−80° C.	Vac.	0·7	0·026	—	47	0·05	526

(*a*) Each tube of sucrose contained 500 μc. in 1·16 mgm. sucrose (149 mc. per m.mole; abundance of carbon-14 19·5 per cent) and was stored for 88 weeks. Calculation shows that primary (internal) radiation decomposition contributed 0·05 per cent to the observed impurity.

(*b*) Other experiments with samples of freeze-dried sucrose have shown that at −80° C. decomposition is considerably increased if the sugar is stored under atmospheric pressure instead of being sealed under vacuum. The difference is even greater with samples held at room temperature.

(*c*) Each tube of glucose contained 100 μc. in 0·43 mgm. glucose (42 mc. per m.mole ; abundance of carbon-14 11·0 per cent) and was stored for 34 weeks. Calculation shows that primary (internal) radiation decomposition contributed 0·005 per cent to the observed impurity.

(*d*) The weight of the Whatman No. 3 paper was 18 mgm. cm.$^{-2}$.

(*e*) The fraction of the energy absorbed by the sugar or sugar syrup was calculated either (i) by using the relationship deduced above, although the geometry is some way from the ideal; or (ii) by assuming the compound and the paper to absorb all the radiation in the ratio of their weights.

(*f*) Defined as the number of molecules permanently altered or decomposed per 100 eV. absorbed by the sugar or sugar syrup.

secondary radiation effects, probably as a result of the moisture content of the paper. However, under optimum conditions, the overall magnitude of decomposition is markedly reduced by storage under such conditions and so, since it is easy to recover the sugar quantitatively, this method is now used to facilitate the supply of carbohydrates with high specific radioactivities from the Radiochemical Centre.

An example of the varied decomposition by secondary radiation effects was recently observed by us. [^{14}C]-dextran sulphate containing *c.* 20 glucose units per molecule was prepared by sulphation of an appropriate [^{14}C]-dextran hydrolysate. It had the relatively low specific radioactivity of 22·4 mc. per gm. atom of carbon (*c.* 3 mc./m.mole dextran sulphate). It was stored in the freeze-dried form, but when inspected after three weeks it was charred and became a total loss, whereas inactive dextran sulphate did not decompose during storage for several months. The decomposition of the labelled dextran sulphate was presumably due to a secondary radiation effect arising from the prior liberation of sulphuric acid. The sulphuric acid, being fortified rather than diluted in the process, destroyed the rest of the material.

Macromolecules

We quoted earlier an expression for the magnitude of the primary (external) effect which includes a term for specific radioactivity, s_0, expressed in curies per mole. While it is, of course, true for all compounds, it is particularly important when dealing with macromolecules to appreciate that this term needs to be expressed on a molar, and not on a unit weight, basis. To illustrate this we may consider the case of some glucose, maltose and amylose (containing n glucose units), all prepared from [^{14}C]-carbon dioxide of the same specific radioactivity, and stored under identical conditions, so that the irradiation per unit mass is the same in each case. It is clear that for each destructive interaction between a β-particle and a carbohydrate molecule the impurity produced will contain x, $2x$, and nx atoms of carbon-14 respectively in the three cases. Nevertheless, if we hydrolyse such partially decomposed samples, the resultant glucose will have the same purity in each case, neglecting any effects due to other causes.

How secondary effects will vary with increasing chain-length will depend on what the result of the particular effect is. In some cases the above principle will hold, whereas in others—such as attack on reducing end-groups—it will not.

It is sometimes necessary to consider the contribution of the primary (internal) effect to the production

of radiochemical impurity from a labelled macromolecule. This will depend, of course, on the pattern of labelling. For example, in a polysaccharide prepared directly from [^{14}C]-carbon dioxide, so that the labelling is statistically uniform throughout all its molecules, the number of radioactive atoms in the form of an impurity initially produced per nucleus disintegrating is the multiple of its isotopic abundance expressed as a fraction and $(n - 1)$, where n is the number of atoms of the given element in the molecule. Production of radiochemical impurity due to this cause, will, of course, fall exponentially with time, as nuclei in the resultant impurity themselves disintegrate; but it is important to appreciate that a similar compound, prepared from [^{14}C]-carbon dioxide at twice the specific radioactivity, and then diluted with an equal weight of inactive carrier, will produce a different amount of radiochemical impurity as a result of the primary (internal) effect, even though its pattern and degree of labelling appears superficially to be identical with the compound first mentioned.

The amount of radiochemical impurity arising as a result of this effect can be surprisingly large with macromolecules, even when the isotope has a long half-life. For example, a glucose polymer containing 1,000 glucose units, and prepared from [^{14}C]-carbon dioxide having the same isotopic abundance as that used to prepare the sucrose quoted in Table 1, would give rise to 0·28 per cent of radiochemical impurity in one week as a result of this effect.

Conclusion

We have not attempted to give a comprehensive review of this subject. We have rather aimed at illustrating certain principles and have endeavoured to show that material of very high specific radioactivities can be used, and even stored, under certain conditions, but that a constant awareness of the phenomenon is called for on the part of all users of compounds labelled with radioactive isotopes.

We are indebted to Prof. E. J. Bourne, Dr. J. R. Catch, and Dr. J. S. Glover for helpful discussions.

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993. *Self-decomposition of* [^{14}C]*Glucose.*

By E. J. Bourne, D. H. Hutson, and H. Weigel.

D-[^{14}C]Glucose undergoes appreciable self-decomposition when stored *in vacuo* as a freeze-dried sample. Some of the products have been identified by chromatography, electrophoresis, and carrier-dilution analysis. A comparison of these products with the products of the oxidation of D-glucose with Fenton's reagent suggests a similarity of the two reactions, and the participation of hydroxyl radicals in the self-decomposition.

In the application of [^{14}C]-tracer techniques to chemical or biochemical reactions, it is normally important that the [^{14}C]-compounds employed should be chemically pure, and it is frequently assumed that such compounds are virtually stable during storage before their use. In 1953, Tolbert *et al.*[1] and Lemmon [2] reported that considerable radiation decomposition had occurred to [^{14}C]-labelled amino-acids, amino-alcohols, purine derivatives, calcium glycollate, cholesterol, thyroxine, and succinic acid during storage. The products of self-decomposition of [^{14}C]methanol were examined by Scraba *et al.*,[3] who identified some of them as methane, hydrogen, ethylene glycol, glycerol, and erythritol. Wagner and Guinn [4] have studied the self-decomposition of [^{14}C]methyl iodide. From the magnitudes of the decompositions of these compounds it appears that various groups of [^{14}C]-labelled compounds are differently affected by radiation self-decomposition.

Bayly and Weigel [5] reported the self-decomposition of [^{14}C]carbohydrates, stored under various conditions, and noted that this decomposition could be due to one or more of four effects, *i.e.*, primary (internal) radiation, primary (external) radiation, secondary radiation effect, and chemical decomposition. They concluded that the self-decomposition of [^{14}C]sucrose when stored as a freeze-dried sample *in vacuo* at room temperature was due mainly to the primary (external) radiation effect, and that the secondary radiation effect was prevalent in the decomposition of D-[^{14}C]glucose stored under the same conditions. A further illustration of the importance of chemical structure in determining the susceptibility of a [^{14}C]-compound to self-decomposition is the rapid destruction of [^{14}C]-dextran sulphate (*ca.* 100% in 3 weeks) which was attributed to a secondary radiation effect involving the liberation of sulphuric acid.

The products of self-decomposition are of interest as their presence can lead to erroneous interpretations of tracer experiments, and a knowledge of their nature can give a guidance to the purification of labelled compounds before use. We now report the analysis of the products of self-decomposition of freeze-dried D-[^{14}C]glucose and evidence regarding the reaction mechanism involved.

A sample of D-[^{14}C]glucose (*ca.* 6 mg., generally labelled), with a specific radioactivity of *ca.* 14·44 mc per mmole, which had been purified by paper chromatography and crystallisation, was stored in the freeze-dried state in a vacuum-sealed tube in the dark. Analysis after 26 months revealed that appreciable decomposition had occurred. Radiochromatograms with a butanol solvent showed the presence of at least 11 new components (Table 2) and the disappearance of 14·5% of the glucose. The $R_{glucose}$ values of the products suggested that acids and neutral compounds with the same or a smaller number of carbon atoms than glucose had been produced, together with polymeric material. These included compounds such as gluconic, ketogluconic, arabonic, and smaller acids, and arabinose, erythrose, and glycerose.

Electrophoresis of the self-decomposition product in phosphate buffer (pH 7·2) revealed the presence of a large quantity of acidic compounds (8·2%). Although at least three fractions, in addition to neutral compounds, could be distinguished, considerable streaking prevented their quantitative estimation. Similarly, electrophoresis in borate buffer [6] did

not yield sufficient separation for the fractions to be determined quantitatively, although at least 9 components were evident.

A high resolution of the products was achieved by two-dimensional paper chromatography–paper electrophoresis, which revealed the presence of 37 components (Fig. and Table 3). The fractions were numbered according to their $R_{glucose}$ values as shown in Table 2; the letters refer to their sequence in electrophoresis. Fractions 5-C, 7-B, 9-B, and 12-A had $R_{glucose}$ and $M_{glucose}$ values identical with those of glucose (86·4%), arabinose (0·44%), erythrose (0·59%), and glycerose (0·12%). Other fractions corresponded to aldohexonic acids, aldopentonic acids, their keto-derivatives, and the lactones of these acids.

In order to make a more accurate determination of some of the products of the self-decomposition, the mixture was analysed for specific compounds by the carrier-dilution technique. With those compounds which establish equilibria in aqueous solution, such as α- and β-sugars, acids and lactones, it was necessary to allow sufficient time for the carrier to equilibrate with the [^{14}C]-product of the self-decomposition (cf. 2-keto-D-gluconic acid results). Neglect of this step could have given a determination of only one of the components of the equilibrium. The results are shown in Table 1. It will be seen that

TABLE 1. *Products of self-decomposition of* D-[^{14}C]*glucose and oxidation of* D-*glucose by Fenton's reagent.*

Compound	Yield (%) on self-decompn. for 26 months	Yield (%) on oxidn. by Fenton's reagent	Compound	Yield (%) on self-decompn. for 26 months	Yield (%) on oxidn. by Fenton's reagent
D-Glucose	79·95	40·12	D-Glucurone	<0·10	
D-Arabinose	0·43	0·49	D-Arabonic acid ...	0·07	1·11
D-Erythrose	*		Oxalic acid	<0·0005	1·02
Glycerose	*		D-Glucosone		12·6 *
D-Gluconic acid	0·62	11·68	Formaldehyde		0·02
2-Keto-D-gluconic acid	0·38	2·32	Carbon dioxide		0·49

* Identified by chromatography. † Total osones calculated as D-glucosone.

these measurements indicated that the [^{14}C]glucose sample had decomposed to the extent of 20% in 26 months, giving *inter alia* D-arabinose (0·43%), D-gluconic acid (0·62%), and 2-keto-D-gluconic acid (0·38%). No attempt was made to determine all the many products because the pattern was already evident and because of the small amount (6 mg.) of the stored parent compound at our disposal.

The $G(-M)$ values of freeze-dried samples of [^{14}C]sucrose and D-[^{14}C]glucose, when stored in vacuum-sealed tubes at room temperature, have been found to be 4 and 53 respectively.[5]

Sucrose can, with efficient freeze-drying techniques, be obtained anhydrous. A freeze-dried sample of D-glucose, prepared in the same way as the radioactive sample, was shown, by the presence of a broad absorption band at 1640 cm.$^{-1}$ in its infrared spectrum, to contain an appreciable quantity of non-bonded water. It is therefore reasonable to assume that the freeze-dried D-[^{14}C]glucose sample also contained non-bonded water. The interaction of the β-particles from ^{14}C with the water could thus produce hydroxyl radicals, which on reaction with D-[^{14}C]glucose would enhance the degree of decomposition.

To test the validity of this theory a comparison was made with the oxidation of D-glucose with Fenton's reagent,[7] which is known [8] to generate hydroxyl radicals in solution, according to the process $H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH\cdot$. A mixture of the products formed by Fenton's reagent and by self-decomposition was analysed by paper chromatography. A radiochromatogram showed seven components, six of which had $R_{glucose}$ values identical with six of the eight coloured spots which appeared when the chromatogram was sprayed with alkaline silver nitrate [9] and which arose from the chemical oxidation (Table 4).

A quantitative determination of some of the products from the chemical oxidation was made by treating freshly purified D-[^{14}C]glucose with Fenton's reagent (1·014 mol. of peroxide) and applying carrier-dilution techniques for D-glucose, D-arabinose, D-gluconic acid, 2-keto-D-gluconic acid, D-glucurone, D-arabonic acid, and oxalic acid. D-Glucosone,[7,10] formaldehyde,[11] and carbon dioxide were also determined. The results are shown in Table 1. The production of D-glucosone was confirmed when paper chromatography in phenol–water (4 : 1; to give a clear separation from glucose) revealed a component with R_F 0·25, identical with that previously reported.[12] A radiochromatogram of the products of the self-decomposition of D-[^{14}C]glucose did not reveal D-glucosone.

Immobile components were present on paper chromatograms of the products from both the self-irradiation and the chemical oxidation of D-glucose. That from the former was treated with sulphuric acid and then with barium carbonate, but no radioactivity was found in the filtrate. It is suggested that this material was a non-glycosidic acidic polymer, which was either precipitated by the sulphuric acid or formed an insoluble barium salt; this is supported by its streaking towards the anode during electrophoresis. It is possible that it arose by synthesis of carbon–carbon bonds from radicals, since it is known [3] that [^{14}C]methanol gives ethylene glycol, glycerol, and erythritol. The immobile product from the chemical oxidation of D-glucose was precipitated from aqueous solution by acetone and contained 17·9% of iron. It may have been similar to an iron complex found by Küchlin,[13] and was not necessarily related to the material formed by self-irradiation.

Clearly, there is a marked similarity between the products arising from self-decomposition of D-[^{14}C]glucose and those formed when glucose is oxidised by Fenton's reagent. The only significant difference was the absence of D-glucosone from the products of irradiation. This may have been due to the fact that the irradiation decomposition proceeded in the absence of air, whereas no precautions were taken to exclude air during the chemical oxidation. Moreover, glucosone is a very reactive compound and may well have undergone considerable chemical change during the long storage period, but not during the rapid chemical oxidation.

The similarity between the products of the chemical and radiation-induced reactions suggests that they arise by similar routes, presumably involving the participation of hydroxyl radicals. Abstraction of hydrogen by these radicals would yield polymers, keto-groups, and carboxyl groups, thus producing gluconic acid and keto-gluconic acids. In conjunction with C–C bond fission, lower aldoses and their acids and keto-derivatives would result. This is also supported by the work of Phillips *et al.*[14] and Grant *et al.*,[15] who studied the action of ionising radiation on aqueous solutions of D-glucose in the presence of oxygen and *in vacuo*, respectively. The similarity between some of their products and those produced by self-decomposition suggests a similarity of the reaction mechanisms.

Experimental

Material.—D-[^{14}C]Glucose, generally labelled, was obtained from the Radiochemical Centre, Amersham. It had been prepared by photosynthesis and had been purified by chromatography and crystallisation, after which no impurities could be detected.

Determination of Radioactivity.—Radioactivity was determined after conversion of the compound into carbon dioxide, and thence into barium carbonate.[16] The amount used was sufficient to give a thickness greater than 20 mg. per cm.^{2}. The β-emission was measured by using a Geiger–Müller end-window tube with an EKCO scaler (type N 529A) and for times 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. All figures quoted are corrected for background and paralysis time. 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.

Chromatography.—(i) *Solvents.* The solvents used in paper chromatography were: (*a*) butanol–ethanol–water (4 : 1 : 5) (organic phase); (*b*) acetone–water (4 : 1); (*c*) phenol–water (4 : 1).

(ii) *Radiochromatograms.* Radiochromatograms were obtained by exposure of the paper

chromatograms to Ilford *X*-ray films (Industrial G) for an appropriate length of time, or by scanning the paper chromatograms with a Geiger–Müller end-window counter.

Chromatography and Electrophoresis of Self-decomposition Products from D-[^{14}C]*Glucose.*—D-[^{14}C]Glucose (481 μc contained in *ca.* 6 mg.; spec. radioactivity *ca.* 2400 mc per g.-atom of carbon) was stored in the freeze-dried state in a vacuum-sealed tube in the dark for 26 months. A radiochromatogram in solvent (*a*) revealed the presence of 11 components in addition to glucose, as shown in Table 2. Fractions No. 2, 3, 5, 6, 8, 9, and 11 had $R_{glucose}$ values similar to those of reference samples of D-gluconic acid and 2-keto-D-gluconic acid, D-arabonic acid, D-glucose and D-glucosone, D-arabinose, D-gluconolactone, D-arabonolactone and D-erythrose, and glycerose, respectively.

TABLE 2. *Radiochromatogram of* D-[^{14}C]*glucose stored for* 26 *months.*

Fraction no.	1	2	3	4A	4B*	5	6	7	8	9	10	11	12	13 †
$R_{glucose}$	0	0·1	0·2	0·3		1·0	1·3	1·5	2·1	2·6	2·9	3·1	3·5	
Radioactivity (% of total)	2·9	2·6	1·6	1·2	1·1	85·5	1·4	1·3	0·7	1·0	0·3	0·2	0·1	0·1

* Trail between fractions 4A and 5. † Trail between fraction 12 and bottom of chromatogram.

Electrophoresis (15 v per cm.) of the self-decomposition product in 0·2M-phosphate buffer (pH 7·2) and estimation of the distribution of the radioactivity showed that 8·2% of the material moved towards the anode and was thus acidic. In addition to the neutral material (91·8%), three fractions having $M_{gluconic\ acid}$ values of 1·00, 1·13, and 2·43 could be distinguished, but the considerable streaking did not allow their independent quantitative determination.

Paper electrophoresis in 0·2M-borate buffer [6] (pH 10), with subsequent exposure to *X*-ray film, revealed nine components with M_G values of 0·12, 0·25, 0·37, 0·60, 0·70, 0·80, 1·00, 1·15, and 1·87. Streaking prevented their quantitative determination.

Two-dimensional paper chromatography [solvent (*a*)]–paper electrophoresis (0·2M-borate buffer, pH 10), with subsequent exposure to *X*-ray film and determination of the distribution of the radioactivity, revealed the presence of 37 components (Fig. and Table 3).

TABLE 3. *Self-decomposition products from* D-[^{14}C]*glucose after* 26 *months' storage.*

Fraction	1-A	1-B	2,3-A	2,3-B	2,3-C	2,3-D	2,3-E	Trailing of 4-A	4-A
Radioactivity *	0·64	0·11	2·14	1·46	0·07	0·04	0·02	0·44	0·89
Fraction	4-B	4-C	5-A	5-B	5-C	5-D	6-A	6-B	7-A	7-B
Radioactivity *	0·01	0·03	0·30	0·30	86·4	0·03	0·09	0·47	0·06	0·44
Fraction	7-C	7-D	8-A	8-B	8-C	8-D	8-E	9-A	9-B	9-C
Ratioactivity *	3·19	0·04	0·04	0·06	0·32	0·70	0·08	0·11	0·59	0·25
Fraction	9-D	10-A	11-A	11-B	11-C	11-D	12-A	12-B	12-C	
Radioactivity *	0·11	0·06	0·05	0·05	0·15	0·04	0·12	0·08	0·02	

* *I.e.*, radioactivity as % of total.

Fractions 2,3-A, 2,3-B, 4-A, 5-C, 7-B, 8-D, 9-B, 9-D, and 12-A had $R_{glucose}$ and $M_{glucose}$ values similar to those of aldohexonic acids and their keto-derivatives, aldopentonic acids and their keto-derivatives, D-glucuronic acid, D-glucose, D-arabinose, lactones of fraction 2,3-A, D-erythrose, D-arabonolactone, and D-glycerose, respectively.

Paper chromatography of fraction 5 (Table 2) in solvent (*c*) revealed only one component with R_F value identical with that of D-glucose. D-Glucosone was thus not present.

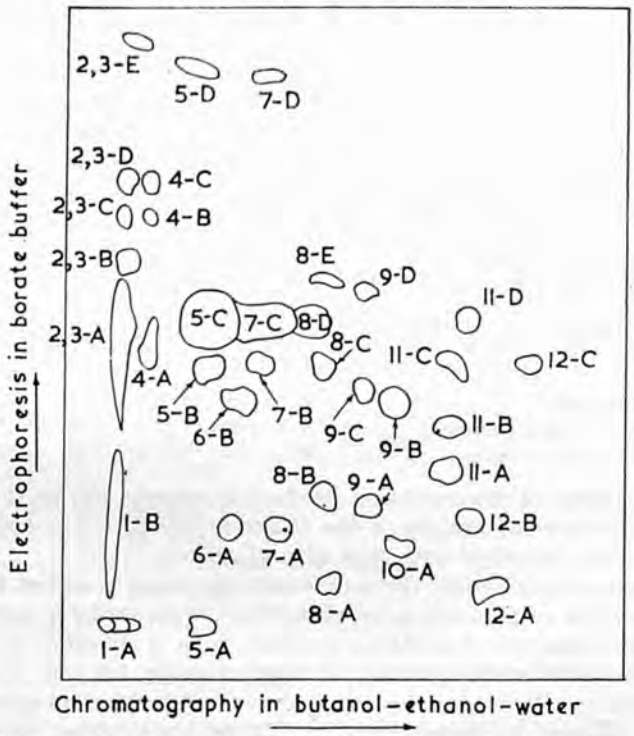
Carrier-dilution Analysis of Self-decomposition Products from D-[^{14}C]*Glucose.*—(i) α-D-Glucose (3·998 g.) was dissolved in an aliquot part of a solution of the self-decomposition product (20·83 μc) in water (15 ml.). The pH of the solution was adjusted with ammonia solution to 7·5. The solution was stored until the optical rotation had reached equilibrium value, and then freeze-dried. The solid was crystallised by dissolving it in boiling 96% methanol (15 ml.) and adding propan-2-ol (10 ml.), and was recrystallised until three consecutive samples possessed constant specific radioactivity (125·07 μc per g.-atom of carbon; 79·95%; m. p. 147°).

(ii) D-Glucono-δ-lactone (1·019 g.) was dissolved in an aliquot part of a solution of the self-decomposition product (9·98 μc) in water (10 ml.). The solution was allowed to equilibrate for 12 hr., as it was found by measurement of the optical rotation that maximum concentration of free acid was obtained after 7 hr. The solution was neutralised with aqueous potassium hydroxide. Potassium D-gluconate was obtained by evaporation to *ca.* 5 ml., addition of ethanol (15 ml.) to incipient cloudiness, and crystallisation at 0°. The material was recrystal-

lised from aqueous ethanol until three consecutive samples possessed constant specific radioactivity (1·81 μc per g.-atom of carbon; 0·62%; m. p. 176°).

(iii) 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^+], 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., then treated with De-Acidite F.F. (carbonate form) (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 neutralised with aqueous potassium hydroxide. Potassium 2-keto-D-gluconate (2·98 μc per g.-atom of carbon; 0·38%; m. p. 152°) was obtained as described for potassium D-gluconate.

Self-decomposition products of D-[^{14}C]*glucose.*



(iv) Potassium 2-keto-D-gluconate (0·471 g.) was dissolved in an aliquot part of the self-decomposition product (19·04 μc) in water (25 ml.), and the solution stored for 1 hr. Potassium 2-keto-D-gluconate (1·43 μc per g.-atom of carbon; 0·09%; m. p. 152°) was obtained as described for potassium D-gluconate.

(v) 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 set aside for 20 hr., evaporated to a syrup, and seeded with a trace of D-glucurone. The crystalline product did not show constant specific radioactivity after 11 recrystallisations (0·45 μc per g.-atom of carbon; maximum 0·10%).

(vi) A solution of D-arabonic acid in water (10 ml.), obtained by treatment of potassium D-arabonate (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 equilibrate for 24 hr. D-Arabonolactone was separated by paper chromatography in solvent (*a*). Potassium D-arabonate was obtained after neutralisation with aqueous potassium hydroxide and crystallisation from aqueous ethanol. It was recrystallised from aqueous ethanol until three consecutive samples possessed constant specific radioactivity (0·45 μc per g.-atom of carbon; 0·07%; m. p. 219°, decomp.).

(vii) 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 6 recrystallisations its specific radioactivity (0·002 μc per g.-atom of carbon; maximum 0·0005%; m. p. 99°) was below the limit of accurate determination.

(viii) D-Arabinose (0·544 g.) was dissolved in an aliquot part of the self-decomposition

product (19·16 μc) in water (10 ml.) and isolated by chromatography on Whatman paper No. 3 in solvent (*a*). The eluate was freeze-dried, and the solid crystallised by dissolving it in boiling methanol (4 ml.) and adding propan-2-ol (10 ml.). It was recrystallised until three consecutive samples possessed constant specific radioactivity (4·58 μc per g.-atom of carbon; 0·43%; m. p. 159°).

(ix) D-Mannitol (1·974 g.) was dissolved in an aliquot part of the self-decomposition product (4·75 μc) in boiling 90% methanol (40 ml.), and crystallised by cooling. Six recrystallisations yielded non-radioactive D-mannitol (m. p. 166°).

Examination of Polymeric Component of the Self-decomposition Products from D-[^{14}C]*Glucose.*—This component, remaining immobile during paper chromatography (Fraction 1, Table 2), was eluted with boiling water. Sulphuric acid was added to portions of the eluate to obtain 0·05N- and 0·5N-sulphuric acid, severally. The solutions were kept at 100° for 2·5 hr., neutralised with barium carbonate, and concentrated. No radioactivity could be detected in the concentrates.

Analysis of Products of Oxidation of D-*Glucose by Fenton's Reagent.*—(i) *Chromatography.* Hydrogen peroxide (20-vol.) (10 × 0·32 ml.) was added to a solution of ferrous sulphate heptahydrate (30 mg.) and D-glucose (1 g.) in water (10 ml.). After each addition, time was allowed for the deep yellow colour to disappear or fade to a light yellow. Paper chromatography [solvent (*b*)] of the solution in admixture with the self-decomposition products from D-[^{14}C]-glucose, exposure to *X*-ray film, and spraying with acetone–silver nitrate–alcoholic sodium hydroxide revealed 8 coloured spots, 6 of which had $R_{glucose}$ values similar to those of 6 of the 7 components present in the self-decomposition product, as shown in Table 4.

TABLE 4. *Comparison of self-decomposition products of* D-[^{14}C]*glucose with products from* D-*glucose and Fenton's reagent.*

	Fraction	1	2	3	4	5	6	7	8
$R_{glucose}$ in solvent (*b*)	Fenton's reagent	0	0·60	0·77	1·00	1·20	1·37	1·57	1·70
	Self-decompn.	0	0·70	0·78	1·00	1·22	1·37	1·56	—

Paper chromatography of the oxidation product in solvent (*a*), elution of the components with $R_{glucose}$ 1·00, and chromatography of the eluate in solvent (*c*) revealed the presence of a component with R_F 0·25, identical with that of D-glucosone.

In the five following experiments, the same conditions were used but the weight of salt and the volume of the portion of peroxide were varied on a scale noted in parentheses.

(ii) *Carrier-dilution analysis of oxidation products from* D-[^{14}C]*glucose.* The reagents (one-tenth scale) were used with freshly purified D-[^{14}C]glucose (*ca.* 100 mg., 1165 or 1229 μc per g.-atom of carbon) in water (1 ml.), as described above. 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 carrier-dilution analysis of the self-decomposition product. Potassium 2-keto-D-gluconate, potassium D-arabonate, and D-arabinose were separated by paper chromatography on Whatman paper No. 3 in solvent (*a*) and then purified as described for the carrier-dilution analysis of the self-decomposition product. The details of the analysis are shown in Table 5.

TABLE 5. *Carrier-dilution analysis of products from pure* D-[^{14}C]*glucose and Fenton's reagent.*

Carrier compound	W_G (mg.)	S_o (μc per g.-atom of C)	W_c (g.)	S_i (μc per g.-atom of C)	Yield (%)
D-Glucose	100	1229	3·988	12·24	40·12
K D-gluconate	100	1229	0·980	17·25	11·68 *
K 2-keto-D-gluconate	98·7	1165	0·503	6·29	2·32 *
D-Arabinose	96·9	1165	0·500	1·10	0·49
K 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 oxidised. S_o = Specific radioactivity of D-[^{14}C]glucose. W_c = Weight of carrier added. S_i = Specific radioactivity of isolated sample. * Calc. for free acid.

(iii) *Osones.* The reagents (original scale) were used with D-glucose (1 g.) in water (10 ml.). Acids were removed by treatment with barium carbonate (2 g.). Acetone (50 ml.) was added,

and the precipitate centrifuged off. The acetone was distilled off, and the solution concentrated to *ca.* 4 ml. Addition of phenylhydrazine (2 g.) in glacial acetic acid (1·5 ml.) and water (15 ml.) produced an immediate precipitate. This was filtered off after 10 min. and dried *in vacuo* over P_2O_5 (0·25 g.; 12·6%, calculated as D-glucosone). The recrystallised D-glucosazone had m. p. 204°, $[\alpha]_D^{27}$ −77°.[17] D-Glucose, treated with phenylhydrazine for 10 min. at room temperature, did not give a precipitate.

(iv) *Formaldehyde.* The reagents (10-fold scale) were used with D-glucose (10 g.) in water (100 ml.). The mixture was steam-distilled, and formaldehyde determined with chromotropic acid (2·3 mg.; 0·02%).

(v) *Carbon dioxide.* The reagents [scale as in (i)] were used with D-glucose (971 mg.) in carbon dioxide-free water (10 ml.). The solution was warmed to 40° for 30 min. The carbon dioxide evolved was isolated as barium carbonate (32 mg., 0·49%).

(vi) *Iron complex of oxidation product.* The reagents (20-fold scale) were used with D-glucose (20 g.) in water (200 ml.). Addition of acetone (1·2 l.) produced a precipitate (40 mg.) (Found: Fe, 17·9%)..

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INFRARED SPECTRA OF CARBOHYDRATES. PART VI. AVOIDANCE OF SPECTRAL CHANGES WITH POTASSIUM BROMIDE FILMS

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In Part V of this series[1] an account was given of attempts to apply the method of Stimson and O'Donnell[2] and Schiedt and Reinwein[3] to the determination of infrared absorption spectra of carbohydrates; in this method the sample is ground with potassium bromide and then pressed to form a film. Cases were reported[1] in which the spectra changed progressively as the films were stored, which made the method suspect for routine work in the carbohydrate field (and indeed in other fields) until the reason for the changes could be ascertained. Meanwhile, Farmer[4] has shown that spectral changes occur with phenols, carboxylic acids and simple alcohols in alkali halide films and has suggested that in these cases the phenomenon is due to adsorption of the samples on the alkali halide particles. Evidence is now presented that the spectral changes observed with films

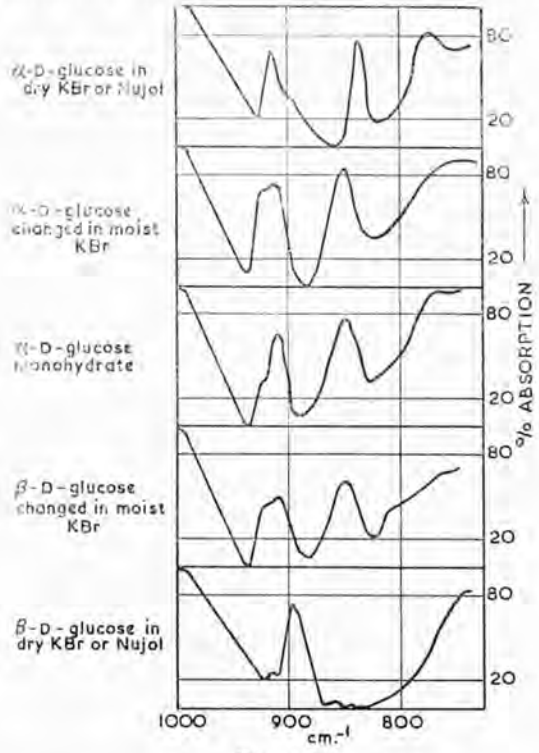

FIG. 1

containing carbohydrates are caused by traces of water in the potassium bromide used; the rate of change is dependent on the water concentration.

A vibration mill[5] was used in order to obtain a uniform and finely ground dispersal of the sample in the potassium bromide. The methods for preparing and storing the films and for determining the infrared absorption spectra were essentially the same as those described previously.[1] As before, the proportion of carbohydrate in each film was *ca.* 1%; this meant that, in the case of a hexose for example, as little as 0·1% of water in the potassium bromide represented an equimolar quantity with respect to the sugar.

In conformity with earlier results,[1] it was found that the spectral modification of α-D-glucose in a potassium bromide film was nearing completion in about 7 days when the components had been dried for several days at 60° C *in vacuo* over phosphoric anhydride before being mixed. However, when the potassium bromide used had not been specially dried (moisture content, 0·3–0·5%), the same type and degree of change were observed immediately after preparation of the film. By contrast, films prepared from potassium bromide which had been dried at 650° C for four hours showed no spectral changes when stored. Thus water is essential for the transformation. Potassium bromide is not essential; this was evident when a sample of α-D-glucose which had been exposed to water vapour at room-temperature gave the modified spectrum when examined by the "Nujol" mull technique, as also did a syrupy αβ-D-glucose mixture.[6]

Similarly β-D-glucose afforded different spectra when films were prepared from dry and moist potassium bromide. In the latter case, the spectrum exhibited absorption peaks at the same frequencies as those observed when α-D-glucose was examined under the same conditions (see Fig. I). It was apparent, therefore, that the α- and β-anomers were giving rise to the same product in the presence of moisture. Since this product absorbed strongly at *ca.* 850 cm.$^{-1}$, it was believed to possess the α-configuration for which absorption at 847 ± 6 cm.$^{-1}$ is to be expected.[7] There was a strong possibility that it was in fact the monohydrate of α-D-glucose. This was confirmed by examination of a film of the monohydrate in dry potassium bromide (see Fig. I).

The infrared spectra of a number of other sugars have been examined using films prepared from potassium bromide which had been previously dried at 650° C. for four hours; in no case was a spectral modification observed during storage. This drying procedure is clearly desirable whenever the compound under examination is prone to hydrate formation, whether it is a carbohydrate or not. It should be noted, however, that Stimson and O'Donnell[2] drew attention to the possibility of bromine being liberated from the salt at high temperatures and this may interfere in certain cases.

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7.

Some Infrared Studies on the Use of Deuterium in the Carbohydrate Group

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Previous studies[1, 2] of the infrared spectra of a large number of glucopyranose, mannopyranose, galactopyranose, arabopyranose and xylopyranose derivatives revealed that absorption peaks in the region 960 to 730 cm^{-1} could be correlated with certain stereochemical features in the molecules. In particular Type 2*a* absorption shown by all α anomers of the first three above-mentioned sugars at *ca.* 825 to 844 cm^{-1} was tentatively attributed to the deformation of the equatorial $C_{(1)}$–H while the corresponding Type 2*b* absorption shown by β anomers of the same three sugars at *ca.* 890 cm^{-1} was attributed to the deformation of the axial $C_{(1)}$–H. In the arabinose series the presence or absence of absorption at 830 to 855 cm^{-1} again seemed a good method of differentiating between α and β forms; in this case, however, it was the β compounds which showed absorption in this region. The xylopyranose series was exceptional inasmuch as neither the α nor the β anomers showed absorption at 830 to 855 cm^{-1}. To determine whether or not Types 2*a* and 2*b* absorption were due to equatorial and axial $C_{(1)}$–H deformation frequencies, 1-deutero derivatives of D-glucose, D-galactose, D-mannose, D-arabinose and D-xylose were synthesised. The result of a comparison of their infrared spectra with those of the normal sugars is the subject of the present communication.

The same general method (*cf.* Topper and Stetten[3]) was used for the synthesis of all the 1-deutero sugars. This involved initial exhaustive replacement of all –OH groups in a sugar lactone with –OD groups. The *oxygen*-deuterated lactone was then reduced with sodium amalgam[4] in deuterium oxide under slightly acidic conditions. The reducing aldose sugar so obtained carried a deuterium atom directly linked to $C_{(1)}$. All the –OD groups were then exchanged back to –OH groups by leaving in water (H_2O). Such a process gave 1-deutero sugars with a high degree (>99%) of isotopic purity. This detection limit was confirmed by measurement of the infrared spectra of synthetic mixtures of $C_{(1)}$–H and $C_{(1)}$–D sugars. In the case of a mixture of α-D-xyloses (98% $C_{(1)}$–D; 2% $C_{(1)}$–H), peaks at 935 and 760 cm^{-1} due to the $C_{(1)}$–H compound were clearly visible. To check that all the deuterium was attached to $C_{(1)}$, 1-deutero-D-arabinose was oxidised to D-arabonic acid. The D-arabonic acid obtained had an infrared spectrum which showed the complete absence of any absorption due to C–D stretching and which was identical with that of a sample of D-arabonic acid obtained by oxidation of normal D-arabinose.

EXPERIMENTAL

In all the syntheses described, heavy water of the same isotopic purity (99.78% D_2O) and sodium amalgam[4] of the same composition (2.44% sodium) was used.

Preparation of 1-Deutero-D-Arabinose

D-Arabono-γ-lactone (0.42 g, 2.8 mM) was dissolved in heavy water (5 g, 0.25 M) in a dry flask, which was stoppered, sealed and kept in a desiccator overnight. The solid obtained by freeze-drying was redissolved in heavy water (5 g) and kept overnight in an open flask in a desiccator containing phosphorus pentoxide. The solution was freeze-dried and the syrup obtained heated at 60° for 5 hr *in vacuo* without removing the flask from the freeze-drier. Heavy water (5 g) was then added together with one drop of concentrated sulphuric acid and a crystal of bromocresol purple indicator. Sodium amalgam (8.25 g, = 8.7 mM sodium) was added in several portions (during 1.25 hr) with vigorous stirring. The pH was maintained below 6 to 7 by addition of further concentrated sulphuric acid. The solution was decanted from the mercury, neutralised with sodium hydroxide and freeze-dried. The solid was extracted with dry methanol, the extract concentrated and the syrup obtained dissolved in water (H_2O, 100 ml) and left overnight. This exchange process was repeated twice. The final solution was freeze-dried with cellulose powder and placed on top of a cellulose column (*l*, 6.5 cm; diam., 3.5 cm)

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which was washed with the organic phase of an *n*-butanol–ethanol–water (4:1:5) mixture. The fraction containing 1-deutero D-arabinose (identified by paper chromatography) was concentrated to a syrup and crystallised from dry methanol. The 1-deutero-β-D-arabopyranose obtained (0.15 g) had mp 156.4° and showed $[\alpha]_D^{+21.5} - 153.5°$ (3.75 min)→ −106.3° equil. (*c*, 1.206 in H_2O). Admixture with β-D-arabopyranose, mp 158–158.5°, $[\alpha]_D^{20} - 179°$ (1.5 min)→ −107.2° equil. (*c*, 1.520 in H_2O), caused no depression in melting point.

Conversion of 1-Deutero-D-Arabinose to D-Arabonic Acid

1-Deutero β-D-arabopyranose (0.0771 g) was dissolved in a water (0.14 ml)–methanol (1.13 ml) mixture and added to a solution of iodine (0.268 g) in methanol (3.6 ml). A solution of sodium hydroxide (4%; 4.2 ml) in methanol was added dropwise over 15 min when the solution became colourless. The sodium arabonate (0.09 g; 93%) was filtered, washed with methanol and ether and dried *in vacuo*. Treatment with warm glacial acetic acid (1.2 ml) gave D-arabonic acid (0.057 g; 67%) having mp and mixed mp 120–120.5°.

Preparation of 1-Deutero-D-Xylose

D-Xylose (3 g) was dissolved in water (300 ml), and bromine (1.2 ml) added. The two-phase system was kept in the dark at room temperature for 9 days. Paper chromatographic analysis of an aliquot showed that no xylose remained. Excess bromine was removed by a stream of air, and hydrogen bromide by neutralisation with silver carbonate. Passage of the solution down a column of Amberlite IR–120[H^+] and concentration *in vacuo* gave syrupy xylonolactone. Conversion to 1-deutero D-xylose was effected under similar conditions to those used above for 1-deutero D-arabinose. The preliminary exchange was carried out in heavy water (10 g; 15 g) and the reduction effected in heavy water (25 g) with sodium amalgam (53.5 g). The 1-deutero-α-D-xylopyranose (1.005 g), isolated after column fractionation and recrystallisation from methanol, had mp 146.5–147° and showed $[\alpha]_D^{20} + 85.7°$ (1.5 min) → +19.96° equil. (*c*, 2.342 in H_2O). Admixture with α-D-xylopyranose, mp 147.5–148° and $[\alpha]_D^{17} + 82°$ (1.7 min) → +18.7° equil. (*c*, 4.946 in H_2O), caused no depression in mp.

Synthetic mixtures of 1-deutero α-D-xylopyranose containing 2.12%, 4.07% and 10.2% α-D-xylopyranose respectively were crystallised and their spectra measured.

Preparation of 1-Deutero-D-Mannose

Bromine (1.6 ml) was added to an ice-cold solution of D-mannose (4.8 g) and barium benzoate (16 g) in water (400 ml). The two-phase system was kept in the dark at room temperature for 60 hr until the solution no longer reduced Fehling's solution. Excess bromine was removed as above. Barium ions were removed by addition of sulphuric acid and the solution decolourised with charcoal. Dissolved benzoic acid was removed by chloroform extraction (4 × 50 ml). The aqueous solution was concentrated in the presence of Amberlite IR–120[H^+] resin, the resin filtered off and the crystals, which separated on further concentration, recrystallised from dry methanol–ethanol and then water. The D-mannono-γ-lactone (1.7 g) so obtained had mp 149–151° and $[\alpha]_D^{20} + 46.7°$ (*c*, 1.14 in H_2O). Part (0.99 g) of the D-mannono-γ-lactone was exchanged twice with heavy water (10 g) and reduced in heavy water (10 g) with sodium amalgam (20 g) as described above. After column separation and crystallisation from methanol, the 1-deutero-α-D-mannopyranose had mp 124–126° (mixed mp with α-D-mannopyranose, 124–126°) and showed $[\alpha]_D^{22} + 25.2°$ (2 min) → +13° (14 hr) (*c*, 1.742 in H_2O). Authentic α-D-mannopyranose had mp 130–134° and showed $[\alpha]_D^{22} + 25°$ (2 min) → +14.1° equil. (*c*, 2.406 in H_2O). 1-Deutero-β-D-mannopyranose, mp 123–124° and $[\alpha]_D^{24} - 14.4°$ (1.5 min) → +12.8° equil. (*c*, 1.154 in H_2O), was obtained by seeding a syrup with β-D-mannopyranose and crystallising from methanol. Authentic β-D-mannopyranose had mp 123–124° and showed $[\alpha]_D^{20} - 125°$ (1 min) → +15.8° equil. (*c*, 2.73 in H_2O). Mixed mp 123–125°.

Preparation of 1-Deutero-D-Glucose

Potassium D-gluconate (10 g) in water (25 ml) was passed down a column of Amberlite IR–120 [H^+] (100 ml) and the eluate concentrated to a syrup, which was then heated at 60° *in vacuo* for 5 hr. Part (0.5 g) of the syrupy D-glucono-δ-lactone so obtained was exchanged twice with heavy water (5 g) and reduced in heavy water (5 ml) with sodium amalgam (8.2 g) as described for 1-deutero-D-arabinose. After column fractionation and several crystallisations from dry methanol, 1-deutero-α-D-glucopyranose was obtained (0.082 g) having mp 145–146° and $[\alpha]_D^{20} + 105.6°$ (2 min) → +50.2° (19.5 hr) (*c*, 1.676 in H_2O). No depression in mp was observed on admixture with α-D-glucopyranose, mp 145–145.5° and $[\alpha]_D^{22} + 108°$ (2 min) → +53° equil. (*c*, 3.22 in H_2O), crystallised from methanol. The β anomers of both the 1-deutero derivative and the normal sugar were obtained by the same procedure. The sugar was dissolved in dry pyridine, filtered, kept at 0° for several days and scratched to induce crystallisation. After removal of pyridine by drying at 100° *in vacuo* 1-deutero-β-D-glucopyranose had $[\alpha]_D^{22} + 24.6°$ (1.5 min) → +47° equil. (*c*, 0.406 in H_2O) and β-D-glucopyranose had $[\alpha]_D^{17} + 20.6°$ (1.25 min) → +51.5° equil. (*c*, 1.842 in H_2O).

Preparation of 1-Deutero-D-Galactose

D-Galactono-γ-lactone (1.5 g) was exchanged twice with heavy water (15 g) and reduced in heavy water (15 g) with sodium amalgam (24.85 g) as described for

Table 1. Infrared Spectra of Normal and Deuterium Labelled Sugars (650–1500 cm^{-1})

	α-D-*Glucopyranose*		β-D-*Glucopyranose*	
	$C_{(1)}$–*H*	$C_{(1)}$–*D*	$C_{(1)}$–*H*	$C_{(1)}$–*D*
1	1462 s	1462 s	1480 m	1466 m
2	1450 s	1435 m	1455 s	1450 s
3	1430 s	1420 s	1445 vw	1440 w
4	1425 m	1412 s	1420 m	1420 m
5	1412 m	1400 m	1415 s	1413 s
6	1385 s	1375 s	1375 s	1363 s
7	1375 s[a]		1363 s[a]	
8	1342 s	1350 s	1355 w	1350 s
9	1335 m	1330 s	1345 vw	1332 m
10	1298 m	1292 s	1312 s[b]	
11	1284 m[b]		1275 s	1270 s
12	1265 w	1267 s	1260 m	1255 w
13	1227 s	1232 s	1227 s	1240 s
14	1206 s	1209 m	1205 s	1208 s
15	1150 vs	1167 vs	1155 vs	1178 vs
16	1120 s	1135 s	1130 m	1130 m
17	1113 vs	1120 s	1112 vs	1115 s
18	1106 s	1106 s	1080 vs	1085 vs
		1095 s[a]		1072 s[a]
19	1080 s	1065 vs	1070 m	1050 m
20	1054 vs	1043 vs	1035 vs	1032 vs
21	1025 vs	1024 vs	1025 vs	1020 vs
22	998 vs	997 vs	1015 vs	1010 s
		965 s[b]		972 s[b] / 965 s
23	917 s	876 s	914 m	900 s
			902 s	875 w
24	840 s	810 s	859 vw	835 vw
25	776 s	762 s	735 m	730 m
26	725 m	720 m	705 m	700 m

	α-D-*Mannopyranose*		β-D-*Mannopyranose*	
	$C_{(1)}$–*H*	$C_{(1)}$–*D*	$C_{(1)}$–*H*	$C_{(1)}$–*D*
	1456 m	1456 m	1485 m	1480 w
	1440 w	1435 w	1460 m	1452 m
	1425 s	1422 s	1432 s	1430 w
	1400 m	1395 m	1425 s	1420 s
	1386 s	1380 s	1412 s	1405 m
	1372 s[a]		1375 s	1373 m
	1358 m	1358 m	1367 m[a]	
	1335 w	1330 m	1350 m	1348 w
	1320 vw	1318 m	1336 m	1339 m
	1285 w	1305 m	1324 s[b]	
	1275 s[b]		1310 s	1318 m
	1255 s	1288 s	1282 m	1300 w
	1240 m	1255 s	1265 s	1278 s
	1225 w	1226 w	1240 s	1242 s
	1210 s	1212 s	1215 m	1235 w
	1200 w	1200 w	1170 s	1216 s
	1170 w	1170 w	1125 w	1130 w
	1130 s	1155 m	1120 s	1125 s
	1115 w	1130 m	1113 m	1120 w
	1110 s	1110 s		1106 s[a]
	1100 m	1090 vs	1087 vs	1090 vs
	1070 vs	1080 s	1073 vs	1080 vs
	1045 m	1065 s	1060 vs	1066 vs
	1040 vs	1045 vs	1045 vs	1055 vs
	1020 s	1025 s	1036 s	1033 s
		1000 s[a]	1010 s	985 w
	973 s	973 m		
	960 s	966 m		969 s[b]
	940 vw	945 vw		930 m[c]
	915 m	930 s	936 s	911 s
		912 m[b]	900 s	885 m

	α-D-*Mannopyranose*		β-D-*Mannopyranose*	
	$C_{(1)}$–*H*	$C_{(1)}$–*D*	$C_{(1)}$–*H*	$C_{(1)}$–*D*
	885 s	864 s / 854 s	862 s	856 m
	845 m	830 w	856 s	828 s
	830 s	818 s		790 m[c]
	810 s	790 s	772 s	758 s
	775 vw	765 vw	731 s	725 s
	710 m	705 m	685 s	675 s
	680 m	670 m		

	α-D-*Xylopyranose*		β-D-*Arabinopyranose*	
	$C_{(1)}$–*H*	$C_{(1)}$–*D*	$C_{(1)}$–*H*	$C_{(1)}$–*D*
	1480 s	1478 s	1472 m	1476 m
	1465 w	1460 s	1450 vw	1450 m
	1455 s	1450 m	1425 m	1425 m
	1445 w	1425 s	1402 w	1397 w
	1425 vw	1405 w	1375 s	1375 s
	1398 m	1375 s	1357 s[a]	
	1375 s	1360 s	1329 m	1332 s
	1360 w	1340 m	1318 s[b]	
	1340 s[a]		1260 s	1266 s
	1315 m	1322 s	1247 w	1255 w
	1305 s[b]		1234 s	1227 s
	1255 vw	1275 w	1135 vs	1162 vs
	1250 w?	1260 m		1115 s[a]
	1240 m	1240 s	1103 s	1108 m
	1205 m	1218 m	1093 s	1088 vs
	1195 s	1200 s	1065 s	1076 s
	1150 s	1188 s	1053 vs	1047 vs
	1130 vs	1125 vs		1035 s[b]
	1110 w	1110 w	1001 vs	985 s
	1080 m	1095 w	998 s	974 s
	1070 m	1082 m	943 m	920 s
		1065 sa	925 vw	893 vw
	1055 s	1050 s	893 s	864 s
	1035 vs	1033 vs	865 vw	858 vw
	1020 s	1022 s	843 s	824 s
		975 s[a]	785 s	777 s
	935 s	900 s / 881 s	710 m	699 m
	905 s	865 s	675 s	672 s / 667 s
	762 s	742 s		
	675 w	675 w		

	α-D-*Galactopyranose*			
	$C_{(1)}$–*H*	$C_{(1)}$–*D*	$C_{(1)}$–*H*	$C_{(1)}$–*D*
	1498 s	1498 s		1132 s[a]
	1458 s	1458 s	1105 s	1110 s
	1450 m	1442 s	1080 s	1090 s
	1427 s	1424 s	1068 vs	1080 vs
	1405 m	1405 m	1055 vs	1064 vs
	1394 w	1394 w	1046 s	1050 s
	1380 vw	1380 vw		1036 m[b]
	1363 s[a]		998 m	995 s
	1340 w	1340 w	976 s	976 s
	1330 s	1322 s	958 s	933 s
	1315 m[b]		890 vw	873 w
	1300 s	1290 s	885 vw	864 s
	1285 m	1282 m	840 s	822 s
	1252 s	1255 s	795 s	788 s
	1242 s	1240 s	765 s	760 s
	1153 vs	1194 vs	705 m	695 m
	1140 m	1148 w	655 m	648 m

[a, b] Frequencies so marked are considered as due largely to $C_{(1)}$–H and $C_{(1)}$–D vibrations, the "a" referring to the higher value in each case.
[c] Probably impurity due to α anomer.

) Frequencies so paired are believed to arise from Fermi resonance with weak summation bands and to have, therefore, only one visible counterpart in the companion spectra.

1-deutero-D-arabinose. The product, after several crystallisations from dry methanol and then glacial acetic acid, was 1-deutero-α-D-galactopyranose (0.98 g), mp 165–166° and $[\alpha]_D^{17}+145°$ (2 min) → +81° equil. (*c*, 0.78 in H_2O). Commercial D-galactose was freed from a lactose impurity and was crystallised from dry methanol as α-D-galactopyranose, mp 166–167° and $[\alpha]_D^{16}+140.9°$ (3.75 min) → +80.3° equil. (*c*, 1.83 in H_2O). Mixed mp of the 1-deutero derivative and normal sugar showed no depression.

Infrared Spectra

The spectra of the sugars prepared above were measured in a Perkin–Elmer 21 double-beam spectrometer having a sodium chloride prism. The samples were dispersed in pressed potassium chloride films; the potassium chloride was dried for several hours at 600° and the sugars at 100° *in vacuo* before being pressed into disks. Table 1 shows the frequencies (cm^{-1}) of the absorption bands together with indications of their relative intensities after subtracting an approximate allowance for overlapping. The intensities were roughly related to the molar extinction coefficients as follows: $\epsilon > 300$, vvs; 100–300, vs; 30–100, s; 10–30, m; 3–10, w; 1–3, vw; <1, vvw. Since the area of the disk was 1.33 cm^2,

$$\epsilon = 1.33 \frac{\text{Mol. wt. of sugar}}{\text{Wt. of sugar (mg)}} \log_{10} \frac{I_0}{I}.$$

DISCUSSION

Before discussing in detail the spectral changes resulting from the introduction of the deuterium, it is desirable to examine the type of behaviour expected. In the approximation of perfect isolated characteristic C–H vibrations, the C–H stretching motion and the two perpendicular bending motions could be factorised from the secular equation for both the proteo (normal) and the deutero compounds. Each frequency would be reduced by a factor closely approaching $2^{\frac{1}{2}}$ and the intensity would be halved. This approximation is reasonably valid for the C–H and C–D stretching motions but is less satisfactory for the bending motions as indicated in Table 2.

Under the characteristic frequency approximation, all frequencies but the three C–H/C–D pairs would remain unchanged in intensity and value. If the approximation is relaxed, then for the sugar molecules which have no symmetry all frequencies are lowered as a result of the increase of mass. Hence the *n*th fundamental frequency of the deutero compound must lie below the *n*th frequency of its proteo analogue.

A guide to the intensities may be obtained from the following discussion. The vibrational modes of the proteo molecule may be numbered in order of descending fundamental frequencies and most of these mode forms will appear, but slightly modified, in the fundamental modes of the deutero analogue and with very similar intensities. The counterparts may have a different number in the frequency series. Omitting the C–H and C–D stretching frequencies which are adequately covered by the stricter approximation above, there are four cases to consider. (i) The *n*th normal mode of the proteo molecule approximates to the *n*th mode of the deutero molecule. The deutero frequency must be the lower and will have comparable intensity. This case applies in two regions, namely, above the highest $C_{(1)}$–H deformation and below the lowest $C_{(1)}$–D. (ii) The *n*th mode of the proteo molecule approximates to the $(n-1)$th of the deutero molecule. Again the two intensities are comparable but there is no requirement that the deutero frequency should be lower. This case applies for frequencies lying between the two $C_{(1)}$–H deformations and for those between the two $C_{(1)}$–D deformations. (iii) The *n*th mode of the proteo molecule approximates to the $(n-2)$th mode of the deutero molecule. Again intensities are similar but the frequency of the deutero molecule must lie higher. This is because the natural frequency for the mode without admixture of C–H or C–D deformation is depressed by the C–H admixture, since C–H deformations lie at a higher frequency, and raised by C–D deformations which lie at lower frequencies. Case (iii) applies to frequencies lying between the lower $C_{(1)}$–H deformation and the higher $C_{(1)}$–D deformation. (iv) The *n*th mode, is a C–H deformation and approximates to the $(n+k)$th mode of the deutero molecule. The frequency is reduced by about $2^{\frac{1}{2}}$ and the intensity by about $\frac{1}{2}$. Larger than average frequency shifts may occur in the $n \pm 1$ frequencies of the proteo molecule and the $n+k \pm 1$ frequencies of the deutero molecule and these may be accompanied by exchange of intensity between neighbouring frequencies. This last feature results from admixing the modes and may make exact identification uncertain or even meaningless.

Table 2. Stretching and Bending Frequencies of $C_{(1)}$–H and $C_{(1)}$–D

Pyranose sugar	*Stretching frequencies*			*Deformation frequencies*						*Product ratio (650–1500 cm^{-1})*
				Higher			*Lower*			
	$C_{(1)}$-H	$C_{(1)}$-D	*Ratio*	$C_{(1)}$-H	$C_{(1)}$-D	*Ratio*	$C_{(1)}$-H	$C_{(1)}$-D	*Ratio*	
α-D-Glucose	*c.* 2910 s	2180 m	*c.* 1.34	1375 s	1095 s	1.26	1284 m	965 s	1.33	1.914
β-D-Glucose	*c.* 2920 s	2140 m	*c.* 1.36	1363 s	1072	1.27	1312 s	972 s	1.35	1.943
								965 s	1.36	
α-D-Mannose	*c.* 2900 s	2200 m	*c.* 1.32	1372 s	1000 s	1.37	1275 s	912 m	1.40	1.885
β-D-Mannose	*c.* 2860 s	2155 m	*c.* 1.33	1367 m	1106 s	1.24	1324 s	969 s	1.37	1.769
α-D-Galactose	*c.* 2920 s	2195 m	*c.* 1.33	1363 s	1132 s	1.20	1315 m	1036 m	1.27	1.653
β-D-Arabinose	*c.* 2900 s	2180 m	*c.* 1.33	1357 s	1115 s	1.22	1318 s	1035 s	1.27	1.818
α-D-Xylose	*c.* 2870 s	2210 m	*c.* 1.30	1340 s	1065 s	1.26	1305 s	975 s	1.34	1.968

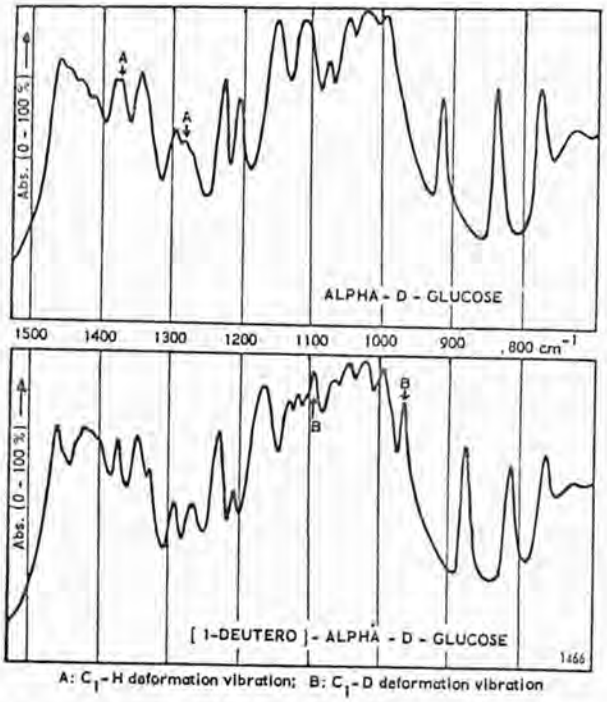

Figure 1. Infrared spectra of α-D-glucose and 1-deutero-α-D-glucose. A. $C_{(1)}$–H deformation vibration. B. $C_{(1)}$–D deformation vibration

The way in which these rules apply to the sugars can be seen by considering α-D-glucopyranose and 1-deutero-α-D-glucopyranose (Table 1). Twenty-six frequencies in the range 1500–700 cm^{-1} may be distinguished for the proteo compound and frequencies $n = 1$ to 6 fall in case (i), and for each there is a slightly lower frequency in the deutero compound. Frequency 7 is fairly clearly a $C_{(1)}$–H deformation and Fig. 1 shows that intensity has been lost in this range. Frequency 8 is in case (ii) with a higher frequency in the deutero body, whereas 9 and 10 have lower counterparts. Frequency 11 at 1284 cm^{-1} has been chosen as the second $C_{(1)}$–H deformation though this is less certain than the choice of the 1375 cm^{-1} band. Frequencies 12 to 15 all have clear counterparts in the deutero molecule at higher frequencies, as is proper for case (iii) modes. The strong band at 1113 cm^{-1} in the proteo compound is broad and clearly multiple and not inconsistent in shape with three unresolved frequencies. The deutero body shows four clear frequencies at 1135, 1120, 1106 and 1095 cm^{-1} and these appear to include a $C_{(1)}$–D deformation. If it is necessary to choose one as predominantly a $C_{(1)}$–D deformation, that at 1095 cm^{-1} fits this interpretation best, giving a frequency ratio of 1.255 with its counterpart at 1375 cm^{-1}. The fact that this ratio is less than 1.414 and the appearance of neighbouring bands which differ from those in the proteo compound suggests that considerable mixing of vibrational modes has occurred. Modes 19 to 22 refer to case (ii) and in all cases a drop in frequency is to be observed. The strong band at 965 cm^{-1} in the deutero compound has no clear counterpart and is assigned to the second $C_{(1)}$–D vibration. It has gained intensity through mixing with other modes and if related to that at 1284 cm^{-1} the frequency ratio is 1.33. Modes 23 to 26 are again under case (i), but the falls in frequency for the first three are surprisingly large, namely 41, 30 and 14 cm^{-1}. These falls again indicate appreciable mixing. The total frequency product ratio for these 26 pairs of frequencies is 1.914 compared with the Redlich–Teller product rule value of 2.00, which shows that the frequencies below 700 cm^{-1} must be almost unchanged in value.

As indicated above, the $C_{(1)}$–D stretching frequencies (some of which appear double) are easily identified and are rather more than $2^{-\frac{1}{2}}$ times the centre of the C–H stretching regions as shown in Table 2. Although measured with a sodium chloride prism and not therefore of great accuracy, it is significant that the stretching frequency of the axial $C_{(1)}$–D in β-D-glucose and β-D-mannose is about 40 cm^{-1} lower than the corresponding equatorial $C_{(1)}$–D in α-D-glucose and α-D-mannose. This corresponds to findings in the steroids.[5] In the hexoses the higher $C_{(1)}$–H bending frequencies lie within a very narrow range, i.e. 1363–1375 cm^{-1}. In those cases where both sets of anomers were available the lower $C_{(1)}$–H bending frequency was higher (1312, 1324) for the β anomer than the α anomer (1284, 1275). What is evident from Table 2 is that Type 2*a* and Type 2*b* absorption cannot be due to $C_{(1)}$–H deformations as previously suggested.

In all the sugars in Table 1, a strong peak at *ca.* 1150 ± 20 cm^{-1} in the normal sugar shows a large upward shift (17–48 cm^{-1}) in the corresponding deutero anomer. This peak may correspond to the very strong frequency found at 1097 cm^{-1} in tetrahydropyran and allocated by Burket and Badger[6] to a ring stretching vibration. The implied correspondence of the very weak double peak (890, 885) shown by α-D-galactopyranose with those of 873 w and 864 m respectively in the spectra of the corresponding deutero anomer appears justified on examination of the spectra of derivatives of α-D-galactopyranose which show absorption (Type 2*c*)[2] near here of very variable intensity.

The results shown in Table 1, especially if considered with the spectra themselves, indicate that the C–H deformations are relatively characteristic and easily distinguished by the change of spectra on deuteration. The two bands chosen disappear and only minor rearrangements of the other frequencies in their vicinity occurs. In contrast their appearance as C–D deformations is less plain and in most cases several possible candidates present themselves. All differ by 200 or more wave numbers from bands of corresponding intensity in the proteo compounds. Although a selection has been made on the balance of the evidence it is clear that the C–D deformations are hardly to be distinguished as characteristic vibrations. Instead all vibrations in the relevant range partake of appreciable C–D deformation character. This less distinguishable nature is to be expected since in the pure vibration the carbon atom must move through twice the amplitude of the corresponding C–H case in order to preserve the

centre of gravity unchanged. Such large carbon motions will involve mixing of other modes.

To what can the Type 2*a* and Type 2*b* absorption be attributed? Since they are not $C_{(1)}$–H deformations and a wide diversity of groups [$(O\text{–}Gluc)_n$, OAc, OMe, OEt, Br, etc.] can be introduced at position 1 without increasing appreciably the small over-all frequency standard deviation of Type 2*a* and Type 2*b* absorption, it is concluded that a vibration of the whole grouping at $C_{(1)}$ is responsible for Type 2*a* and Type 2*b* absorption. The frequency exhibited is dependent on the stereochemical configuration at $C_{(1)}$ (i.e., α or β).

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8.

Interaction of Sucrose Stearate with Starch

It is generally agreed that changes in the starch component of flour are responsible for the staling of bread[1]. Following a report by Bohn[2] that the sucrose stearates are effective as anti-staling agents, a study was made of the interaction of starch with a commercial sample of sucrose stearate, kindly supplied by F. D. Snell, Inc.

Addition of a sucrose stearate solution to a 1 per cent solution of 'AnalaR' starch in 0·1 per cent sodium chloride resulted in the precipitation of some starch. The maximum amount of precipitate (about 20 per cent of the weight of starch) was obtained with concentrations of sucrose stearate above 0·06 per cent.

Extension of the study to undegraded starches (Table 1) showed that the amount of precipitate depended upon the type of starch. Fractionation of the potato starch into amylose and amylopectin[3] followed by precipitation with excess sucrose stearate showed that most of the amylose but little of the amylopectin was precipitated (see Table 2). Treatment with excess sucrose stearate of an artificial mixture of amylose and amylopectin in the ratio in which they occur in starch gave a figure intermediate between that of the whole starch and that which would be expected on the basis of the separate amylose and amylopectin precipitations. These results indicate that the amylopectin is precipitated more efficiently in the presence of amylose than in its absence.

Controlled acid hydrolysis of potato starch showed that a high molecular weight was important in determining the amount of precipitate formed with sucrose stearate and it is likely that the low yield of precipitate from 'AnalaR' starch could be attributed to this factor.

A study of the change in diameter of wheat starch granules on heating in aqueous suspension in the presence and absence of sucrose stearate showed that the rate of swelling was reduced in the presence of sucrose stearate. From an examination of the change in turbidity on heating wheat starch suspensions (0·1 per cent), it appeared that in the presence of 0·01 per cent sucrose stearate the gelatinization temperature is raised about 10 deg.

Table 1. PRECIPITATION OF STARCHES BY SUCROSE MONOSTEARATE

Type of starch	Polysaccharide concentration (per cent)	Sucrose monostearate concentration (per cent)	Precipitate (per cent of polysaccharide)
'AnalaR' starch	1·0	0·1	21·6
Potato starch	1·0	0·1	70·9
Wheat starch	1·0	0·1	92·4
Waxy maize starch	1·0	0·1	13·0

Table 2. PRECIPITATION OF STARCH FRACTIONS BY SUCROSE MONOSTEARATE

Type of starch fraction	Polysaccharide concentration (per cent)	Sucrose monostearate concentration (per cent)	Precipitate (per cent of polysaccharide)
Potato starch	0·4	0·04	72·7
Potato amylose	0·4	0·04	79·9
Potato amylopectin	0·4	0·04	11·1
20 per cent amylose, 80 per cent amylopectin	0·4	0·04	43·9

As sucrose stearate is firmly bound by starch, it seemed likely that it would interfere with the starch/iodine reaction. This was shown to be so, and that iodine and sucrose stearate compete for the amylose fraction. There was no evidence for any marked interaction between sucrose stearate and amylopectin by this technique. It is possible that the stearate chain of the sucrose stearate molecule occupies a position down the centre of an amylose helix, in the same way that iodine does[1].

A complete account of this work will be published elsewhere.

We are grateful to the Sugar Research Foundation for financing these investigations.

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9.

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Interaction of Anti-Staling Agents with Starch

FOLLOWING an observation that sucrose stearate, a compound claimed to have anti-staling activity, precipitated starch from solution (to be published), the study was extended to other substances known to have anti-staling properties.

As most known anti-staling agents have surface-active properties, two surfactants were included in the programme—a sulphonated hydrocarbon (anionic) and cetyl trimethyl ammonium iodide (cationic). For purposes of comparison, *n*-butanol and thymol (amylose precipitants) were also included.

The following compounds, claimed to have anti-staling activity, were tested: sucrose monostearate, sucrose distearate, polyoxyethylene monostearate, glyceryl monostearate (commerical), glyceryl monostearate (pure, Myverol 18-06) and stearoyl tartrate.

Solutions of the test agents were added to solutions of wheat starch to give final concentrations: starch 0·5 per cent, sodium chloride 0·05 per cent, test agent 0·005–0·075 per cent (except in the case of stearoyl tartrate where the maximum concentration was 0·03 per cent, due to its low solubility). The amount of precipitate was determined turbidimetrically.

Butanol, thymol and the two ionic surfactants had virtually no precipitating effect in this concentration range. Among the anti-staling agents only stearoyl tartrate showed little precipitating power. The most effective precipitants were sucrose monostearate, glyceryl monostearate (pure) and polyoxyethylene monostearate. Glyceryl monostearate (commercial) was slightly less effective and sucrose distearate much less effective.

These results show that five out of six substances with anti-staling activity give a precipitate with starch. Whether or not this reaction is a pre-requisite for all anti-staling agents is not certain, but in any event this reaction must change the characteristics of flour products.

Ofelt *et al.*[1] reported that glyceryl monostearate decreased the crumb firmness of bread (an anti-staling characteristic) and that glyceryl distearate had no such effect, nor did it act synergistically with the monostearate. Our results show that glyceryl monostearate (pure) is a more effective precipitant for starch than the commercial material, but only slightly so. However the commercial glyceryl monostearate employed contained about 33 per cent monostearate

with the remainder largely distearate. If there were a strict parallel between the baking and precipitation tests, it would be expected that there would be a greater difference between the two samples of glyceryl monostearate in the precipitation tests. Ofelt *et al.*[2] also found that the crumb softening effect decreased in the order polyoxyethylene monostearate, glyceryl monostearate, sucrose monostearate but the precipitation tests showed little difference between the three compounds. In addition, Axford and his colleagues[3] have found no direct correlation between the amount of precipitate which we have observed with starch and the effectiveness of an anti-staling agent in bread. Thus one is led to the conclusion that complex formation between known anti-staling agents and starch must occur in flour products and that it may well explain the action of these agents as bread improvers; if this is so, then the effectiveness of such an agent in bread must be determined not only by the amount of complex formed, but also by the properties of that complex, such as its permeability to moisture.

We are grateful to the Sugar Research Foundation for financing this investigation and to Dr. D. W. E. Axford, of the British Baking Industries Research Association, for helpful discussion.

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INTERACTION OF STARCH WITH SUCROSE STEARATES AND OTHER ANTISTALING AGENTS

By E. J. BOURNE, A. I. TIFFIN and H. WEIGEL

The reaction of sucrose monostearate (SMS) with starches and starch fractions is similar to that of various substances with 'antistaling' activity with starch. SMS forms an insoluble complex with starches and competitively inhibits the reaction of iodine with amylose. Wheat starch granules adsorb SMS and it is suggested that the formation of an insoluble complex on the surface of the granules may play a part in the 'antistaling' activity of SMS.

Introduction

It has been reported[1] that sucrose stearates are of value as antistaling agents or crumb softeners. Since the staling of bread almost certainly involves changes in the starch constituent,[2] it was decided that a chemical study should be made of the effect of sucrose stearates and other 'antistaling agents' on starch itself and on its components.

Experimental

(i) *Materials*

The '*sucrose monostearate*' (SMS) used initially was a commercial sample supplied by the Sugar Research Foundation, which contained a considerable proportion of the distearate. A small number of experiments was carried out with another sample of the 'monoester' (SMS pure) analysis of which, supplied by the Herstein Laboratories Inc., indicated that it contained 78·5% monostearate and 17·3% distearate. Pure samples of SMS were not available because of the facility with which the ester undergoes transesterification.

The *sucrose distearate* (SDS) was a commercial sample which was recrystallised from cold acetone to remove a small percentage of free sucrose.

AnalaR Starch (British Drug Houses Ltd.) was used as supplied.

Undegraded potato starch was prepared by blending peeled potatoes in water and filtering the aqueous suspension through butter muslin. The filtration was repeated three times and followed by decantation and washing of the surface of the sedimented starch granules until no discoloration remained. The starch was washed three times with acetone and dried in air at room temperature.

Potato amylose and amylopectin were prepared from this potato starch by a modification of the technique of Greenwood & Robertson.[3] Oxygen-free nitrogen was bubbled through a suspension of 0·5% potato starch in 0·1% NaCl. While the mixture was being heated to boiling, a pre-determined amount of NaOH was added so that the final dispersion would be neutral. With nitrogen still passing, the temperature was kept at 100° for 30 min., then the solution was cooled to ~40° and centrifuged at 2500 *g*. The amylopectin (sediment) was washed twice with 0·1% NaCl.

The supernatants were combined, excess of n-butanol was added, the mixture heated to 70° and shaken overnight at room temperature. The amylose–n-butanol complex was removed by centrifugation and washed twice with n-butanol-saturated water. The complex was pasted with water until solution was complete and the n-butanol distilled off under reduced pressure. The volume of the solution was adjusted to make the amylose concentration approximately 0·15% (assuming that 20% of starch is amylose), and thymol was added to give a saturated solution. This mixture was heated to 70°, shaken, centrifuged and washed as before. The amylose–thymol complex was pasted with water and the thymol removed by distillation under reduced pressure. The amylose was lyophilised and dried at 60° over P_2O_5. [Care is necessary in this stage that the amylose is dried completely as quickly as possible as, if drying is not complete, the amylose becomes insoluble.] The blue value[4] of the amylose was 1·43.

The amylopectin sediment was suspended in 0·05N-NaOH and the mixture heated to boiling under nitrogen until solution was complete (~30 min.); the solution was neutralised while hot, and treated with excess n-butanol. After being shaken overnight, a small amount of amylose–n-butanol complex was removed by centrifugation. The supernatant was dialysed against

running tap water for 3 days and then against distilled water for 1 day, with frequent changes of water, before lyophilisation. The blue value of the amylopectin was 0·18.

Undegraded wheat starch was prepared from a sample of Manitoba wheat flour by a modification of the technique of Bechtel & Meisner.[5] Wheat flour (400 g.) and water (300 ml.) were thoroughly kneaded by hand at room temperature until the whole was a cohesive glutinous mass. This was kneaded with excess of water (~400 ml.), when much of the starch separated in aqueous suspension from which loose particles of gluten were removed by filtering through butter muslin. The dough was again extracted with water (~400 ml.). The combined starch suspensions were washed several times by decantation, washed three times with acetone and dried in air at room temperature.

Waxy maize starch was supplied by the American Maize-Products Co. It had probably been slightly degraded during extraction since dilute sulphurous acid had been used in the milling process.

In all experiments on starches and their components allowance was made for their moisture contents.

(ii) *Precipitation experiments*

Starch solutions (other than AnalaR starch) were prepared in an inert atmosphere to reduce as far as possible degradation due to oxidation.

(a) *Starch and components soluble in water.*—The starch plus 10% of its weight of NaCl was made into a slurry with a little cold water and added to boiling water which was being stirred vigorously and through which oxygen-free nitrogen was being passed.

(b) *Starch and its components insoluble in water.*—The starch was made into a slurry with a little cold water and added to boiling dilute NaOH solution which was being stirred vigorously and through which was being passed a stream of oxygen-free nitrogen. The concentration of NaOH was such that after neutralisation with HCl, the concentration of NaCl was 10% of that of the starch.

The starch solution so formed, i.e. from (*a*) or (*b*), was centrifuged at ~2500 *g* for 15 min. to remove any residual insoluble material. To the solution was added a known volume of a solution of the ' antistaling ' agent. Final concentrations were in the range : starch, 0·4–1·0% ; NaCl, 0·04–0·1% ; ' antistaling ' agent, 0·005–0·1%.

For spectrophotometric determinations using AnalaR starch, the turbidity was measured immediately, using a Spekker Photoelectric Absorptiometer with neutral filters, as on keeping or on heating, flocculation was liable to occur. For other starches and starch components, the mixture was heated to 100°, kept overnight at 4° and, after shaking, the turbidity measured as above.

For gravimetric determinations, the mixture was heated to 100°, kept at 4° for 18 h. and then centrifuged at ~2500 *g*. The precipitate and supernatant were separated and lyophilised and then dried over P_2O_5 before being weighed.

(iii) *Blue value determination*

Blue values of starches were determined using the technique of Bourne *et al.*[4] on solutions prepared as above. The blank in the absorptiometer was distilled water.

(iv) *Reaction of iodine with starch/SMS mixtures*

(a) *Rate of reaction of starch with SMS.*—A 1% solution of AnalaR starch was prepared by boiling in 0·1% NaCl. The test solution was prepared at 25° by adding 0·1% SMS solution (100 ml.) to the starch solution (100 ml.), and the control solution was similar except for the SMS. Samples (10 ml.) were withdrawn immediately and at intervals from the test solution and pipetted into 400 ml. of water containing 5 ml. of 0·2% iodine, 2% KI and one drop of conc. HCl, and the volumes made up to 500 ml. The visible absorption spectrum of each solution was measured immediately and compared with that obtained from the control solution (see Fig. 3).

(b) *Effect of SMS on the reaction of amylose with iodine.*—Amylose was prepared from AnalaR starch by precipitation from 1% starch solution with n-butanol.[6]

From solutions of 0·1% amylose in 0·1% NaCl, 0·1% SMS in water, and 0·2% iodine in 2% KI, the following three mixtures were prepared, the reagents being added in the order indicated :

1. amylose solution (5 ml.), water (400 ml.), conc. HCl (1 drop), I/KI solution (5 ml.) ;
2. amylose solution (5 ml.), water (400 ml.), conc. HCl (1 drop), SMS solution (0·5 ml.), I/KI solution (5 ml.) ;
3. [amylose solution (5 ml.) + SMS solution (0·5 ml.)] heated to 100° and cooled, water (400 ml.), conc. HCl (1 drop), I/KI solution (5 ml.).

The mixtures were made up to 500 ml., their absorption spectra measured immediately and again after 48 hours (Fig. 4).

(v) *Hydrolysis*

An approximately 1·5% solution of starch was prepared in 0·01N-NaOH as described in Experimental (ii) (b). With the solution at 100° and the nitrogen flow maintained, the solution was made 0·01N in HCl. Samples were withdrawn at intervals and neutralised. Part of each sample was precipitated with half its volume of 0·3% SMS as described in Experimental (ii) ; part was kept for viscosity measurement as described in Experimental (vi) (see Fig. 6).

(vi) *Viscosity measurement*

Viscosities were measured at 25° in a standard relative U-shaped viscometer. The limiting viscosity number was determined[3] for each of the samples in (v).

(vii) *Microscopical techniques*

(a) *Swelling of starch granules.*—A micro-melting point apparatus (Messrs. A. Gallenkamp & Co. Ltd.) was used to determine the rate of swelling of the granules of a 1% suspension of wheat starch in 0·1% NaCl in the presence and absence of 0·1% SMS. Measurements were made on granules, the initial diameters of which were in the range 14–30μ. The rate of heating was approximately 1°/min.

(b) *Sectioning and staining of starch granules.*—1% wheat starch suspensions in 15% gelatin were prepared at 60° with and without 0·5% SMS. The solutions were heated at 75–80° for 5 min. and cooled to 50° before 15μ sections were cut on a freezing microtome. The sections were stained with very dilute I/KI solution and examined under a microscope (×1000).

(viii) *Macroscopic techniques*

(a) *Swelling of starch granules.*—An 0·2% suspension of wheat starch in 0·02% NaCl was shaken and divided into two parts, each of 100 ml. To one was added 100 ml. of water and to the other 100 ml. of 0·02% SMS. The solutions, in three-necked flasks (fitted with stirrer and thermometer), were placed in a water bath and the temperature slowly raised to ~95°. Samples were withdrawn at intervals, the temperature noted and the turbidity measured against water. The rate of rise of temperature was approximately constant at 1½°/min. (see Fig. 7).

(b) *Uptake of SMS by starch granules.*—Three mixtures were prepared : (P) 1% suspension of wheat starch in water ; (Q) 0·025% solution of SMS ; (R) 1% suspension of wheat starch in 0·025% SMS. The mixtures were kept for 2 h. at 25° and samples of P and R were withdrawn.

Suspensions P and R were heated to 50° in 1 min. and held at this temperature for 1 min. while samples were withdrawn. The temperature was raised to 75° in 1 min. and further samples were withdrawn. All samples were cooled and centrifuged. To part (5 ml.) of the supernatants from samples P were added solution Q (5 ml.). These solutions (S) were set aside overnight at 4° and then centrifuged.

Anthrone sugar determinations were carried out on the nine supernatants and on solution Q, using two volumes from supernatants S, and only one volume from the other samples. The appropriate blank (see Table III) was used in this colorimetric estimation.

Results

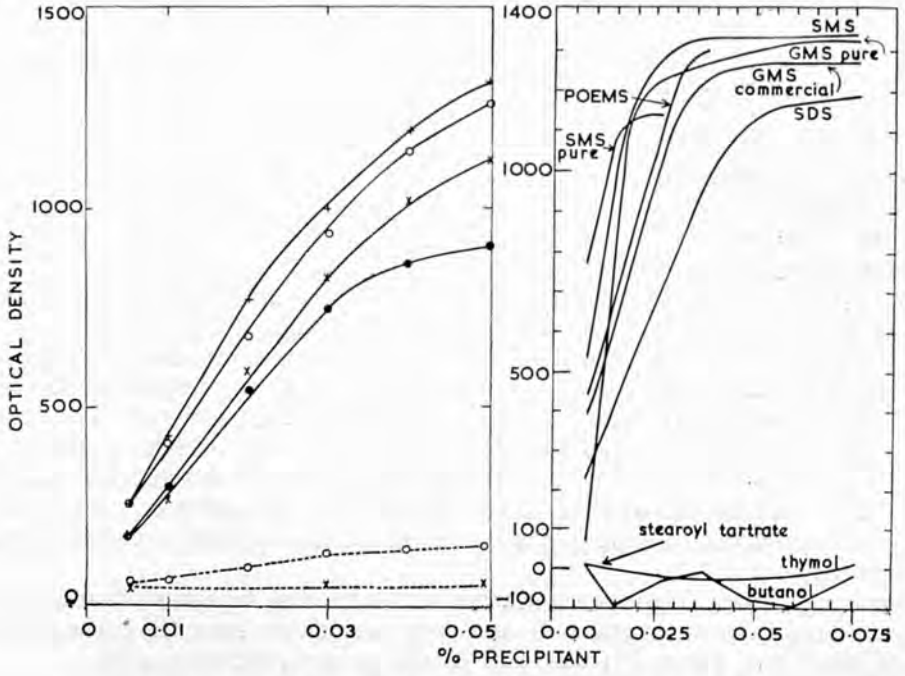
In the initial series of experiments, AnalaR starch was used, as it is readily soluble in water and solutions of reproducible characteristics are easily available. An immediate precipitate was

obtained on mixing solutions of SMS and this starch (final concentrations starch 1·0%, NaCl 0·1%). Turbidimetric estimation showed that the amount of precipitate was proportional to the amount of SMS added, up to a certain level, above which there was little increase in precipitate. Other substances claimed to have ' anti-staling ' activity were found to behave in a similar manner. These compounds are non-ionic and exhibit some degree of surface activity. To determine whether the phenomenon of starch precipitation was a general characteristic of surfactants, ' Teepol '—a higher hydrocarbon sulphonate—and cetyltrimethylammonium iodide (anionic and cationic respectively) were tested. As may be seen from Fig. 1, the ionic surfactants gave a negligible amount of precipitate.

A series of experiments under similar conditions with undegraded wheat starch (final concentrations starch 0·5%, NaCl 0·05%) and a more comprehensive group of precipitants, showed substantially the same picture (see Fig. 2). As the concentration was increased, solutions containing pure SMS and polyoxyethylene monostearate (POEMS) showed a tendency to flocculate, and hence a considerable drop in turbidity was observed. This is not shown in the graph. It is of interest to note that stearoyl tartrate, which is in commercial use as an ' antistaling ' agent, is virtually without effect, although, by reason of its low solubility, concentrations above 0·03% could not be achieved. Weighing the precipitates produced by 0·025% of these compounds showed that the turbidimetric estimation was a reasonably reliable indication of the amount of precipitate, a straight line relationship being found between optical density and log (% precipitation) for stearoyl tartrate, SDS, GMS, SMS and POEMS. At low concentrations both butanol and thymol appear to increase the solubility of starch.

When AnalaR starch (50 g.) was treated with SMS (2 g.) (final volume : 5 l.) and the resultant precipitate and supernatant lyophilised, Soxhlet extraction of the solids with acetone resulted in the recovery of only 1·36 g. of SMS (assuming that all recovered acetone-soluble material was SMS), indicating that the SMS is in some way bound to the starch.

Confirmation of this came from a study of the interaction of iodine with starch/SMS mixtures. When a mixture of starch and SMS was prepared, addition of iodine to samples at intervals, resulted in a series of solutions, the absorption spectra of which showed that a gradual



Precipitation of (FIG. 1) *AnalaR starch*, (FIG. 2) *wheat starch*

× —— × SMS (sucrose monostearate)
+ —— + POEMS (polyoxyethylene monostearate)
○ ---- ○ Cetyltrimethylammonium iodide
○ —— ○ SDS (sucrose distearate)
● – – ● Polyoxyethylene sorbitan
× ----- × Teepol
GMS (glyceryl monostearate)

change occurred in the starch (Fig. 3). This change corresponded to a gradual reduction in the amount of amylose component available for complex formation with the iodine.

Addition of iodine to amylose solutions, before and after the addition of SMS, showed that SMS prevented the access of iodine to the amylose helix, but that on keeping the mixture for some time the iodine replaced the SMS to some extent, with a resultant increase in the blue amylose/iodine complex (Fig. 4). Thus SMS and iodine are in competition for the same sites

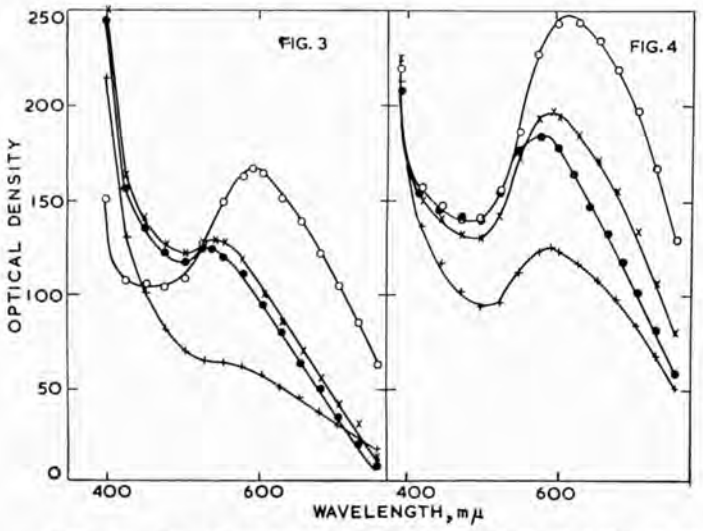


FIG. 3.—*Rate of reaction of iodine with starch/SMS*
○ control ● 8 min. ○ amylose + amylose + SMS (heated)
× 0 min. + 48 h. × amylose + SMS ● amylose + SMS after 48 h.

FIG. 4.—*Effect of SMS on reaction of iodine with amylose*

on the amylose molecule, presumably inside the helix. A similar experiment, carried out with amylopectin prepared from AnalaR starch, showed that SMS had little effect on the uptake of iodine by amylopectin.

Gravimetric determination of the precipitate produced from AnalaR starch (final concentrations: starch 1·0%, NaCl 0·1%) and SMS showed that a maximum of about 20% of the starch could be precipitated (Fig. 5); this point was reached when the SMS/starch ratio reached approx. 7% by weight. From this figure and from the experiments on the interaction of iodine with SMS/starch, it seemed probable at this stage that the precipitation was actually a fractionation of the starch—the amylose being precipitated. As the SMS cannot be extracted fully from the precipitate and no convenient method of estimating the amount of SMS in the precipitate could be found, the amount of precipitate is expressed as a percentage of the original weight of polysaccharide.

However, on testing undegraded starches, it was found that up to about 90% of wheat starch and about 70% of potato starch could be precipitated by SMS. In addition, it was found that with waxy maize starch, which is composed principally of the branched polyglucose, amylopectin, 13% was precipitated (see Table I).

Precipitation of purified potato amylose and amylopectin with excess SMS gave a precipitate of almost 80% of the amylose and 11% of the amylopectin (cf. precipitate from waxy maize starch), whereas from an artificial mixture of amylose and amylopectin, in the ratio 1 : 4) in which they occur naturally, only 44% of the original weight of polysaccharide could be precipitated. (The precipitate from amylopectin alone, was soluble in acetone to the extent of 26%; it gave a purple coloration with iodine. Thus there was some polysaccharide precipitated, and the precipitate did not consist solely of the SMS which amounted to 10% of the weight of amylopectin.) On the basis of the separate amylose and amylopectin precipitations, the mixture would be expected to give 24·8% of precipitate. It thus appears that in the presence of amylose, additional amylopectin is precipitated. Undegraded potato starch gave 70% of precipitate,

and it is thus apparent that even more amylopectin is being precipitated here, although this difference may be due in part to degradation of the amylose and/or amylopectin during separation. Wheat starch gives a still larger precipitate, which may be correlated with the finding of Bengough *et al.*[7] that barley starch (another cereal starch) is not readily separated by the technique of Baum & Gilbert[8] (cf. [3]). An association of amylose and amylopectin in the undegraded starches is indicated.

Since AnalaR starch is prepared by mild hydrolysis with HCl, it seemed possible that the difference in the amounts of precipitate obtained with SMS from AnalaR starch and from undegraded potato starch was due to degradation of the former. Potato starch was hydrolysed at 100° under nitrogen in 0·01N-HCl. Samples were withdrawn at intervals, neutralised and used for viscosity measurements, and for determination of the amount of precipitate produced with SMS. The intrinsic viscosity was obtained graphically[3] and its log plotted against the log % precipitated. A similar experiment was carried out with wheat starch and gave similar results. The amount of precipitate was found to be proportional to the square root of the intrinsic viscosity $[\eta]$ down to $[\eta] = 10^{-1}$ and, in the case of wheat starch, below this value of $[\eta]$ there was a sudden fall in the amount of precipitate (see Fig. 6). The hydrolysis of the potato starch was not sufficiently prolonged to determine whether its behaviour was identical with that of the wheat starch in the later stage of the hydrolysis. When the break in the curve for wheat starch occurs, the intrinsic viscosity has dropped by a factor of about four, which corresponds to the cleavage of an average of three bonds per molecule of starch. Clearly molecular weight is a major factor in the precipitation of starch-type polysaccharides with SMS, the larger molecules being precipitated far more effectively than smaller ones.

As the diameter of starch granules shows considerable variation, a simple graphical representation of observations on the rates of swelling of wheat starch granules in presence and absence of SMS is of doubtful validity. A statistical treatment of the results substantiated the simple graphical interpretations and is outlined below.

As change in diameter is being studied and the initial diameters show considerable variation, it is logical to express all values in terms of the ratio of the diameter at a given temperature to the diameter at an arbitrary starting temperature. No change in volume was observed in the range 25–50°, so the arbitrary starting temperature was taken at 45°. The means of the ratios are given in Table II. However, when applying statistical tests to the data, it appeared preferable to use the logarithms of these ratios, since the variability of the logarithms is less from temperature to temperature. Also given in the table are the mean values of the logarithms and the standard error of these means.

The difference between means for water and SMS is significant (at the 5% level) for those temperatures where the ' t ' value has an asterisk. It is seen therefore that the ratio for the water group is significantly greater than that for the SMS group for all temperatures of 60° and over.

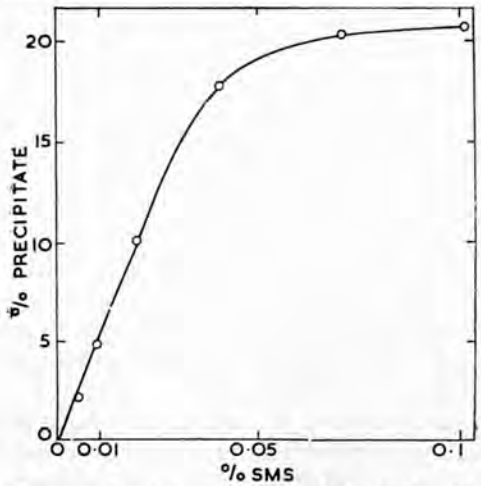


FIG. 5.—*Precipitation of AnalaR starch with SMS*

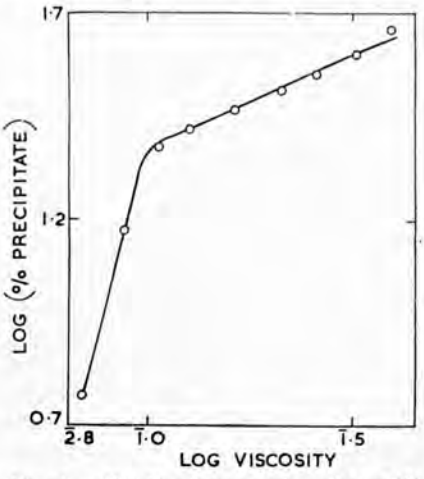


FIG. 6.—*Relation between viscosity and % precipitate*

Table I

Precipitation of starches and starch fractions by SMS

Type of starch or starch fraction	Polysaccharide concn., %	SMS concn., %	Precipitate (% of polysaccharide)
AnalaR starch	1·0	0·1	21·6
Potato starch	1·0	0·1	70·9
Wheat starch	1·0	0·1	92·4
Waxy maize starch	1·0	0·1	13·0
Potato starch	0·4	0·04	72·7
Potato amylose	0·4	0·04	79·9
Potato amylopectin	0·4	0·04	11·1
20% amylose, 80% amylopectin	0·4	0·04	43·9

Table II

Statistical treatment of the increase in diameter of wheat starch granules heated in presence and absence of SMS

Ratios are expressed as $\frac{\text{diameter of granule at } t^\circ}{\text{diameter of granule at } 45^\circ}$

SMS treated							
Temp., ° c (*t*)	45	55	60	65	70	75	80
Mean ratio	1·000	1·026	1·136	1·435	1·600	1·660	1·720
Mean log ratio	0·000	0·0154	0·0572	0·1546	0·2065	0·2252	0·2425
Standard error of the mean log		0·0026	0·0067	0·0119	0·0121	0·0106	0·0090
Water							
Mean ratio	1·000	1·058	1·490	1·608	1·740	1·812	1·902
Mean log ratio	0·000	0·0262	0·0976	0·1918	0·2454	0·2628	0·2848
Standard error of the mean log		0·0050	0·0140	0·0063	0·0098	0·0096	0·0154
Value of ' t ' between means		2·10	2·93*	2·32*	2·24*	2·43*	2·47*

Thus, in presence of SMS, starch granules swell less than in water alone. (Application of a ' t ' test to each temperature does not take into account the increase in diameter of individual granules, but a fuller examination of variance fully confirmed the simpler treatment.)

These results were further substantiated by a study of the change in turbidity on heating wheat starch suspensions in the presence and absence of SMS (Fig. 7). At temperatures above 60°, starch suspensions in the presence of SMS show greater opacity than in the control suspension. It should be pointed out that although an increase in diameter of the granules results in an increase in the turbidity of the suspension, there is a concomitant decrease in the opacity of the granules, so that by this technique only the resultant effect is measured. It will be seen that 0·01% SMS increases the gelatinisation temperature of an 0·1% wheat starch suspension by approximately 10°.

An attempt was made to assess the penetration of SMS into swollen wheat starch granules (such as might occur in bread) by sectioning such granules and staining the sections with a very dilute solution of iodine and potassium iodide. The sections showed, however, an even pale blue colour, and were indistinguishable from controls obtained from starch which had not been treated with SMS.

As another approach to the problem of the uptake of SMS by swollen starch granules, it was thought that it might be possible to detect the adsorption of dissolved SMS by a suspension of starch granules. Although the anthrone determination of the sucrose content of SMS had not proved satisfactory, the results were sufficiently reproducible to enable a relative estimation of the amount of carbohydrate in solution to be made. Thus a wheat starch suspension was made and the amount of polysaccharide in solution determined against a water blank (Table III, A). To this suspension was added SMS and the amount of carbohydrate in solution determined against an SMS blank (Table III, B). Some of the original starch suspension was centrifuged and SMS was added to the supernatant. After further centrifugation the dissolved carbohydrate was again determined against an SMS blank (Table III, C). At any given temperature, if the SMS had been without effect, the set of three figures should have been the same. It may be

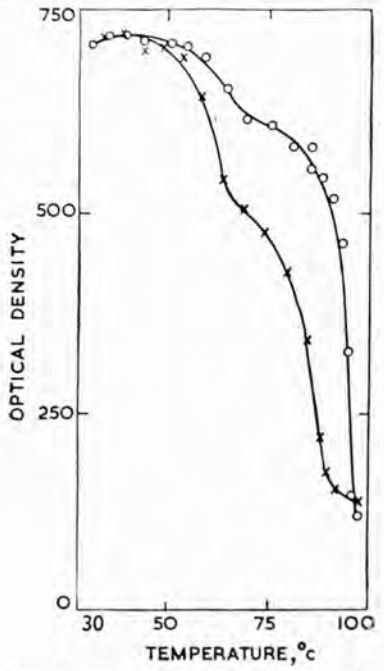


FIG. 7.—*Swelling of starch granules*
× water ○ 0·01% SMS

Table III

Uptake of SMS by wheat starch granules

Material	Temperature, °C	Optical density against appropriate blank
A	25	0·244
B	25	−0·072
C	25	−0·038
A	50	0·382
B	50	0·044
C	50	0·038
A	75	1·330
B	75	0·799
C	75	0·580
D	—	0·565

A = supernatant from wheat starch suspension
B = supernatant from SMS + wheat starch suspension
C = supernatant from wheat starch suspension with SMS added later
D = SMS solution, concentration as in A, B and C

noted (A and B in the Table) that on addition of SMS there is a considerable decrease in the amount of carbohydrate that would be expected in the solution. This decrease is less than would result from the complete precipitation of the SMS (A − D). It may be explained as a partial precipitation or adsorption of the SMS and/or a decrease in the amount of starch going into solution. In C in Table III, the possibility of adsorption of the SMS on starch granules has been ruled out, and the results are close to those in group B at temperatures 25° and 50°. At 75° the difference between B and C is considerable and can only be explained by assuming that the SMS is primarily adsorbed on the surface of the gelatinised starch and some starch is left in the solution, whereas in the absence of the granules, the SMS removes the larger molecules from solution. At the lower temperatures, the difference between B and C is insufficient to allow definite conclusions to be drawn, but the indication is that with increasing temperature up to 75° there is a transition between precipitation and adsorption being of major importance.

From the above results it is clear that SMS, even in very small relative concentration, forms insoluble complexes with solutions of starches; in addition it is adsorbed by swollen starch granules. One is led to the inescapable conclusion that similar reactions must occur in bread which contains SMS. Other commercial 'antistaling' agents have been shown to form insoluble complexes with starches and these too must behave in a similar fashion when used in bread. The interesting question is thus posed whether it is the formation of such complexes which prevents the staling of bread. The staling of bread has been attributed to the gradual passage of water from starch to other components in the bread, and it can be seen that the formation of an insoluble outer coating on the surface of swollen starch granules could restrict this passage of water and hence slow down the staling process. The efficiency of such a coating as a water barrier would depend not only on its weight (i.e. thickness) but also on its permeability. Thus it would be expected that a starch/SDS complex would be less permeable to moisture than a starch/SMS complex because of its probable higher fatty ester content. It follows that the above determinations of the amounts of precipitates obtained from starches with antistaling agents may well provide a guide to the function of these agents in bread, but may not actually reflect the relative antistaling properties of these agents.

Acknowledgments

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469. *Mechanism of the Enzymic Synthesis of a Branched Trisaccharide containing the α-1 : 2-Glucosidic Linkage.*

By E. J. Bourne, J. Hartigan, and H. Weigel.

A trisaccharide produced during the growth of *B. arabinosaceous* on a [^{14}C]sucrose medium containing lactose has been characterised as *O*-β-D-galactopyranosyl-(1 → 4)-*O*-{[^{14}C]-α-D-glucopyranosyl-(1 → 2)}-D-glucose. The distribution of ^{14}C in its three monosaccharide units is in accordance with a mechanism involving the transfer of the glucose residue from sucrose to the reducing moiety of lactose.

It has been shown [1] that certain simple sugars can serve as chain initiators when the dextransucrase of *Leuconostoc mesenteroides* (NRRL B-512) acts on sucrose. Such sugars cause a marked decrease in dextran production, and lead to the synthesis of oligosaccharides. A similar phenomenon was observed with dextransucrase preparations and with growing cultures of *B. arabinosaceous* (Birmingham).[2-5] The oligosaccharides produced in cultures containing sucrose as a substrate, to which isomaltose, maltose, methyl α-D-glucoside, or 3-*O*-methyl-D-glucose had been added as receptors, were formed by the successive transfer of glucosyl units from sucrose molecules to the receptor, with the formation of α-1 : 6-glucosidic linkages. The addition of lactose or cellobiose to a culture led to the synthesis of a trisaccharide in which a glucosyl unit was attached through an α-1 : 2-linkage to the reducing moiety of the disaccharide.[2,4,5] Thus the trisaccharide produced in a lactose–sucrose medium was characterised as *O*-β-D-galactopyranosyl-(1 → 4)-*O*-[α-D-glucopyranosyl-(1 → 2)]-D-glucose, whilst that produced in the presence of cellobiose was analogous, namely, *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-D-glucopyranosyl-(1 → 2)]-D-glucose.

These two trisaccharides are of interest for several reasons. They contain the rare α-1 : 2-glucosidic linkage rather than the α-1 : 6-linkage normally synthesised by dextransucrase. They are believed to be the first "branched" trisaccharides obtained by a controlled enzymic synthesis *in vitro*; their formation involves the use of a carbohydrate primer in which a sugar residue, other than the usual non-reducing end unit, has the correct structure to act as a receptor of a transferred sugar residue. The mechanism of their formation could, in fact, be a general one for the production of branched oligosaccharides and, moreover, could represent the first stage in the synthesis of branched polysaccharides, by a route which does not involve prior formation of the linear polymer.[2] It was important therefore to study the synthesis of the branched trisaccharides in greater detail. Although it seemed likely that each was formed by transfer of a glucosyl unit from sucrose to a preformed lactose (or cellobiose) molecule, as follows:

$$\text{Glu 1}\text{—}2\ \text{Fru} + \text{Gal 1}\text{—}4\ \text{Glu} \longrightarrow \text{Gal 1}\text{—}4\ \text{Glu 2}\text{—}1\ \text{Glu} + \text{Fru} \quad . \quad . \quad (1)$$

there were other possibilities, such as:

$$\text{Gal 1}\text{—}4\ \text{Glu} + \text{Gal 1}\text{—}4\ \text{Glu} \longrightarrow \text{Gal 1}\text{—}4\ \text{Glu 2}\text{—}1\ \text{Glu} + \text{Gal} \quad . \quad . \quad (2)$$

We now report the use of ^{14}C-tracer techniques to elucidate this problem; at the same time confirmatory evidence is presented that the structure previously assigned to the "branched" trisaccharide, derived from lactose, was correct.

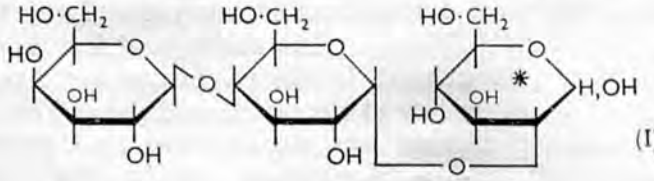
A culture of *B. arabinosaceous* (Birmingham), incubated with lactose and [^{14}C]sucrose, produced a [^{14}C]trisaccharide (A), which was isolated from the culture medium by fractionation on a charcoal–"Celite" column.[6] Chromatograms of the trisaccharide indicated that it was pure in both the chemical and the radiochemical sense. The specific radioactivity of the trisaccharide, determined as barium carbonate by the infinitely thick

disc method,[7] was 523 μc per g.-atom of carbon, corresponding to 9417 μc per mole of trisaccharide. In a direct determination on the trisaccharide by the infinitely thin film method, the corresponding figures were 519 and 9341. Chromatographic analysis of the culture medium during the incubation period revealed [^{14}C]sucrose, [^{14}C]glucose, [^{14}C]-fructose, the [^{14}C]trisaccharide A, and lactose. This indicated that an enzyme system involving an equilibrium between sucrose, glucose, and lactose was not present.

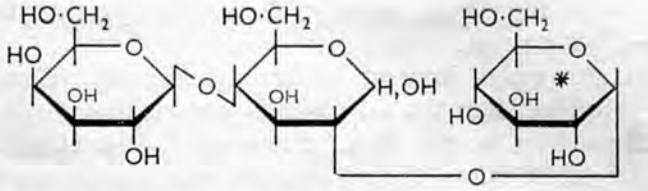
The trisaccharide A had the same mobility on paper chromatograms and on electrophoretograms as the trisaccharide *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose isolated and characterised by Bailey *et al.*[5] With aniline hydrogen phthalate, it gave a yellowish colour characteristic of a 2-*O*-substituted reducing aldose. It was not revealed by alkaline triphenyltetrazolium chloride which detects all reducing glucosaccharides except those with a 2-*O*-substituent.[8] Its low mobility in borate buffer confirmed the conclusion that the reducing unit was linked through position 2.

Further evidence for the structure of trisaccharide A and qualitative evidence for the distribution of ^{14}C in the three monosaccharide units was obtained by total and partial hydrolysis. The hydrolysates were analysed by paper chromatography. The total hydrolysate was shown to contain galactose and [^{14}C]glucose. The partial hydrolysate contained galactose, lactose, [^{14}C]glucose, and [^{14}C]kojibiose.

The presence of one α- and one β-glycosidic link was confirmed by treatment of trisaccharide A with almond β-glucosidase which produced galactose and [^{14}C]kojibiose.



(I)



(II)

The evidence presented so far suggests two possible structures (I and II) for trisaccharide A. The first structure could have resulted from the transfer of a lactosyl unit to position 2 of [^{14}C]glucose, derived from [^{14}C]sucrose; the second could have been formed by transfer of a [^{14}C]glucosyl unit from [^{14}C]sucrose to position 2 of the reducing moiety of lactose [eqn. (1)]. Since the radioactivity of the [^{14}C]sucrose employed was roughly 17,000 μc per mole, the radioactivities of structures (I) and (II) should have been approx. 8500 μc per mole, compared with the values 9341 and 9417 actually obtained for trisaccharide A.

The possibility that trisaccharide A arose from two lactose molecules [eqn. (2)] was eliminated when it was shown that no galactose was liberated and that the lactose was inactive throughout the incubation period. Indeed, even if instantaneous equilibration of the glucose units in the lactose and [^{14}C]sucrose had occurred, the radioactivity of the trisaccharide produced in this way would have been only approx. 2840 μc per mole.

In an attempt to determine quantitatively the distribution of ^{14}C in its monosaccharide units, trisaccharide A was treated with phenylhydrazine, a technique which is known to convert, under suitable conditions, 2-*O*-substituted aldoses into their phenylosazones.[9] Thus compounds (I) or (II) should each have yielded D-[^{14}C]glucosazone and lactosazone. The products were separated on a "Celite" column with a pyridine–benzene–water solvent, in conditions which had been established with artificial mixtures. The specific activity of the "glucosazone" (4203 μc per mole) was much lower than that expected

(*ca.* 9400 μc per mole), whereas the " lactosazone," expected to be inactive, had 1184 μc per mole. If specific cleavage of the 1 : 2-linkage had occurred, these values would require the specific radioactivity of the trisaccharide to be only 5387 μc per mole. Clearly, phenylhydrazine under these conditions was rupturing also a percentage of the 1 : 4-linkages, as was confirmed subsequently by treatment of lactose itself.

Conclusive evidence for the distribution of ^{14}C in the monosaccharide units of trisaccharide A was obtained by reduction with potassium borohydride, followed by hydrolysis and radioactivity assay of the products. Paper chromatography of the total hydrolysate of trisaccharide A alcohol revealed the presence of galactose, sorbitol, and [^{14}C]glucose. These three products were isolated by preparative paper chromatography, and their radioactivities determined by the infinitely thin film method.[10] The β-emission measured was shown to bear a linear relation to the amount of compound applied and was calibrated against a standard [^{14}C]glucose sample. The results are shown in the Table. From the

	Counts per min. per μmole	μc per mole		Counts per min. per μmole	μc per mole
D-Glucose	952	9253	Sorbitol	17	165
D-Galactose	6	58	Trisaccharide A	961	9341

ratio of the emission of the glucose to the sum of the emissions of glucose, galactose, and sorbitol, the glucose fragment was calculated to contain 97·6% of the ^{14}C of trisaccharide A alcohol. An alternative calculation was based on a direct comparison of the radioactivities of the trisaccharide A and the glucose fragment of trisaccharide A alcohol. This indicated that 99·1% of the ^{14}C of trisaccharide A was present in the non-reducing glucose unit. These results are conclusive in favour of the " branched " structure (II) for trisaccharide A, rather than (I), which would have given glucose, galactose, and [^{14}C]sorbitol. Moreover, they confirm the structure assigned earlier [2,4,5] on chemical grounds alone, to a trisaccharide prepared in a similar manner.

Thus it is established that the enzymic synthesis of the " branched " trisaccharide *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose proceeds by transfer, directly or indirectly, of the glucosyl residue from sucrose to position 2 of the reducing unit of lactose. When [^{14}C]sucrose is used, as above, the equation becomes:

$$\overset{*}{\text{Glu}}\ 1\text{——}2\ \overset{*}{\text{Fru}} + \text{Gal}\ 1\text{——}4\ \text{Glu} \longrightarrow \text{Gal}\ 1\text{——}4\ \text{Glu}\ 2\text{——}1\ \overset{*}{\text{Glu}} + \overset{*}{\text{Fru}} \quad . \quad (3)$$

where the asterisk denotes a radioactive sugar residue. It remains to be seen whether the transfer is catalysed by dextransucrase itself, or by a closely related enzyme which accompanies it.

Experimental

Materials.—[^{14}C]Sucrose, generally labelled, was obtained from the Radiochemical Centre, Amersham.

Determination of Radioactivity.—(i) *Apparatus.* Radioactivities were determined by means of a Geiger–Müller end-window counter. The β-emission of a radioactive specimen was measured for a time sufficient to give a standard counting error of better than ±2% except for samples of specific radioactivity lower than 1·6 μc per g.-atom of carbon when measured by the infinitely thick disc method, or 30 μc per g.-atom of carbon when measured by the infinitely thin film method.

(ii) *Infinitely thick disc method.* Radioactivities were determined after conversion of the compound into carbon dioxide, and thence into barium carbonate.[7] The amount used was sufficient to give a thickness of greater than 20 mg. per cm.^{2}. 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.

(iii) *Infinitely thin film method.*[10] Three drops of a 0·01% " Teepol " solution were placed on a polished aluminium disc (diam. 2·5 cm.). The disc was flooded with water, and the " Teepol " solution evaporated to dryness in a stream of warm air and under an infrared lamp.

An aqueous solution of the compound, containing 30—600 μg., was placed on the disc, and the disc flooded with water in order to spread the compound evenly over the surface. The solution was evaporated to dryness as described above. [^{14}C]Sugars were used as substandards.

Chromatography.—(i) *Solvents.* The solvents used in chromatography were (*a*) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase); (*b*) benzene–pyridine–water (5 : 4 : 4) (organic phase); (*c*) acetone–water (4 : 1); (*d*) ethyl acetate–acetic acid–saturated aqueous boric acid (9 : 1 : 1); (*e*) butan-1-ol–pyridine–water–saturated aqueous boric acid (6 : 4 : 2 : 1); (*f*) butan-1-ol–ethanol–water–ammonia (4 : 1 : 4·9 : 0·1) (organic phase).

(ii) *Radiochromatograms.* Radiochromatograms were obtained by scanning the chromatograms with a Geiger–Müller end-window counter or exposure to Ilford *X*-ray films (Industrial G) for an appropriate time.

Oligosaccharide Synthesis in a Lactose–[^{14}C]*Sucrose Medium.*—An aqueous medium (100 ml.) containing yeast extract (1%), disodium ammonium phosphate (0·5%), potassium dihydrogen phosphate (0·1%), hydrated magnesium sulphate (0·05%), lactose (10%), and [^{14}C]sucrose (2%, *ca.* 100 μc) was adjusted to pH 7 with sodium hydroxide and sterilised by filtration. The medium was inoculated with *Betacoccus arabinosaceous* (Birmingham) and incubated at 25° for 4 days. Paper chromatography of the culture medium in solvent (*a*) revealed components with R_F values identical with those of lactose (present throughout the incubation period), sucrose (first 24 hr.), fructose (24 hr. only), glucose, and *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.[5] The sugars were detected by acetone–silver nitrate–alcoholic sodium hydroxide.[11] Radiochromatograms showed ^{14}C-activity corresponding to glucose, fructose, sucrose, and *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.

The culture medium was adjusted to pH 7 and heated at 90° for 10 min. The bacterial cells were removed on a centrifuge (30 min. at 4500 r.p.m.). After the addition of ethanol (100 ml.), centrifuging, and removal of the ethanol, the oligosaccharide mixture was fractionated on a charcoal–"Celite" column[6] (40 × 4 cm.). Water (2·5 l.) eluted the monosaccharides and salts: 5% aqueous ethanol (2·5 l.) removed lactose. Trisaccharide A (351 mg.) was obtained by elution with 10% aqueous ethanol (4 l.).

Characterisation of Trisaccharide A.—(i) Paper chromatography of trisaccharide A in solvent (*a*) and of its benzylamine derivative[12] in solvent (*f*) showed that each moved as a single radioactive component with $R_{glucose}$ 0·11 and 0·37, respectively. Paper ionophoresis[13] in borate buffer (pH 10) again showed a single component with M_G 0·33. It was detected with acetone–silver nitrate–alcoholic sodium hydroxide,[11] and with aniline hydrogen phthalate,[14] but not with alkaline triphenyltetrazolium chloride.[8] In all of these tests the behaviour of trisaccharide A was identical with that of authentic *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.[5]

(ii) *Radioactivity of trisaccharide A.* The specific radioactivity of trisaccharide A, determined by the infinitely thick disc method, was 523 μc per g.-atom of carbon, *i.e.*, 9417 μc per mole of trisaccharide.

(iii) *Hydrolysis.* Trisaccharide A (4·7 mg.) was hydrolysed in 1·5N-sulphuric acid (1 ml.) at 100° for 4 hr. Paper chromatography of the hydrolysate in solvent (*a*) showed components corresponding to glucose and galactose, while radiochromatograms revealed ^{14}C only in the former spot. Paper chromatography of a partial hydrolysate (N-sulphuric acid for 1 hr. at 90°) of trisaccharide A (8·4 mg.) in solvent (*a*) showed components with R_F values identical with those of glucose, galactose, kojibiose, lactose, and trisaccharide A. Radiochromatograms revealed [^{14}C]components corresponding to glucose, kojibiose, and trisaccharide A.

(iv) *Treatment with almond emulsin.* Trisaccharide A (1 mg.) was added to almond β-glycosidase solution[15] (0·1 ml.). Paper chromatography of the digest after 72 hours' incubation at 37° revealed components corresponding to [^{14}C]trisaccharide A (*ca.* 70%), [^{14}C]kojibiose, galactose, and glucose (trace, radioactivity not determined). Under similar conditions lactose was completely hydrolysed and maltose gave a trace of glucose.

(v) *Treatment with phenylhydrazine.* Trisaccharide A (74·7 mg.) in water (1·25 ml.) was treated with phenylhydrazine (165 mg.) in acetic acid (0·165 ml.) at 100° for 2·5 hr. and then left overnight at 0—2° before the mixture of phenylosazones (10 mg.) was isolated. Paper chromatography of the mixture of osazones in solvent (*b*) revealed components corresponding to the phenylosazones of glucose (R_F 0·69) and lactose (R_F 0·38). The osazones were detected by ultraviolet light and by acetone–silver nitrate–alcoholic sodium hydroxide.

The mixture of osazones was fractionated on a " Celite " (40 × 1·7 cm.) column. Dry " Celite " was wetted with benzene-saturated water, and made into a slurry in solvent (*b*), which was also the developing solvent. Paper chromatography of the two isolated fractions, using the same solvent, showed that they moved as single components with R_F values corresponding with those of the phenylosazones of glucose and lactose, respectively. Their specific radioactivities, determined by the infinitely thick disc method, were 4203 μc per mole of monosaccharide and 1184 μc per mole of disaccharide, respectively.

(vi) *Reduction of trisaccharide A.* Trisaccharide A (33·8 mg.) in water (7·3 ml.) was reduced with potassium borohydride (37·5 mg.) at room temperature for 20 hr. The excess of borohydride was destroyed by 3N-sulphuric acid (0·4 ml.), and the volume made up to 15 ml. Part (3 ml.) of the resulting solution was adjusted to pH 7 and passed through a column of Permutit " Biodeminrolit " (15 g.) pretreated with carbon dioxide, and then evaporated to dryness *in vacuo*. Dry methanol (3 × 10 ml.) was added and the whole evaporated to dryness *in vacuo*. The residue was dissolved in water (10 ml.) and freeze-dried. Paper chromatography of the reduced trisaccharide A in solvent (*c*) showed that it moved as a single component with $R_{glucose}$ 0·51. It was detected with acetone–silver nitrate–alcoholic sodium hydroxide [11] and with periodate–permanganate–benzidine,[16] but not with aniline hydrogen phthalate. Radiochromatograms revealed that it moved as a single radioactive compound.

(vii) *Hydrolysis of trisaccharide A alcohol.* Another part (10 ml.) of the solution of the reduced trisaccharide A was adjusted with 3N-sulphuric acid (2·4 ml.) to an acid normality of 0·5N, and heated at 100° for 4 hr., then passed through a column of Permutit " Biodeminrolit " (45 g.), pretreated as above. Paper chromatography of the concentrated solution in solvent (*e*) showed components with R_F values corresponding to glucose, galactose ($R_{glucose}$ 0·85), and sorbitol ($R_{glucose}$ 0·3). Sorbitol did not give a discrete spot, but was well separated from glucose and galactose. Radiochromatograms revealed the presence of ^{14}C in the component corresponding to glucose. Portions of the hydrolysate were fractionated by paper chromatography. A pure glucose fraction was obtained in the above solvent. The eluted aqueous solutions containing glucose and galactose, respectively, were freeze-dried. Dry methanol (3 × 25 ml.) was added to each and the whole evaporated to dryness *in vacuo*. The galactose fraction was further purified in solvent (*a*). A pure sorbitol fraction ($R_{glucose}$ 2·2) was obtained by paper chromatography in solvent (*d*), and was freed from boric acid by the above method.

The concentrations of the aqueous solutions of glucose and galactose were determined by the methods of Somogyi and Nelson.[17] The concentration of sorbitol was determined by oxidation with sodium periodate and determination of the formaldehyde produced with chromotropic acid.[18] The specific radioactivities of glucose, galactose, and sorbitol were determined by the infinitely thin film method, on aliquot portions of the analysed solutions of the compounds. The results obtained are shown in the Table.

Compound	Wt. (μg.)	Counts per min.	Counts per min. per μmole	Average counts per min. per μmole	μc per mole
D-Glucose standard	44·19	284	1158	1156	11,236
	66·63	427	1154		
Trisaccharide A	103·3	194	947		
	221·0	431	984		
	419·0	795	957	961	9,341
	508·0	965	958		
	607·0	1154	959		
D-Glucose	32·83	168	922		9,253
	52·02	276	956	952	
	58·83	319	977		
D-Galactose	32·12	1	6	6	58
D-Glucitol	53·10	5	17	17	165

Phenylhydrazine Treatment of Lactose.—Lactose (68·2 mg.) was dissolved in a solution of acetic acid (1·65 ml.) and water (12·5 ml.). Phenylhydrazine (0·22 g.) was added and the solution was heated at 80° for 2·5 hr. and kept overnight at 0—2°. The solid product obtained was analysed by paper chromatography [solvent (*b*)], which revealed a component with R_F value corresponding to lactose phenylosazone and a trace of a component with R_F value corresponding to a monosaccharide phenylosazone.

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219. *Biosynthesis of α-D-Glucopyranosyl D-Galactofuranoside and Other D-Galactose-containing Saccharides by* Betacoccus arabinosaceous.

By E. J. Bourne, J. Hartigan, and H. Weigel.

B. arabinosaceous grown on a galactose–sucrose medium produces di- and oligo-saccharides. One of the disaccharides has been characterised as α-D-glucopyranosyl D-galactofuranoside. The glucose : galactose ratios of the oligosaccharides suggest that they are formed by successive addition of glucosyl units to a galactose-containing receptor.

It has been shown [1] that in cultures of *Betacoccus arabinosaceous* (Birmingham), containing sucrose as a substrate and isomaltose, maltose, methyl α-D-glucoside, or 3-*O*-methyl-D-glucose as receptor, oligosaccharides are formed by successive transfer of glucosyl units from the sucrose molecules to the receptor, with the formation of α-1,6-glycosidic linkages; with isomaltose and maltose the transfer was to the non-reducing moiety. In a previous paper [2] we established that the synthesis of the " branched " trisaccharide *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose in a lactose–sucrose medium proceeds by transfer of the glucosyl residue from sucrose to position 2 of the reducing unit of lactose. A similar trisaccharide, *O*-β-D-glucopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose is elaborated by the same organism when grown on a cellobiose–sucrose medium.[3] It was thought that the non-reducing moieties, β-D-galactosyl or β-D-glucosyl, in lactose and cellobiose did not provide suitable acceptor sites and thus tended to favour the transfer of the glucosyl residue from sucrose to the reducing moiety (αβ-D-glucose) of the receptor disaccharide. The present investigation, in which D-galactose was used as receptor, was of interest because the sugar was now present as α- and β-D-galactose and, moreover, for the preferred C1-conformation, its 4-hydroxyl group was axial, in contrast to the non-glucosidic hydroxyl groups of glucose.

A culture of *B. arabinosaceous*, grown on a D-galactose–sucrose medium, produced three disaccharides (A, B, and C) and two oligosaccharides (D and E). The sugars were isolated from the culture medium by fractionation on a charcoal–" Celite " column [4] and, when necessary, purified by paper chromatography. We now report the structure of disaccharide A and some properties of the other sugars.

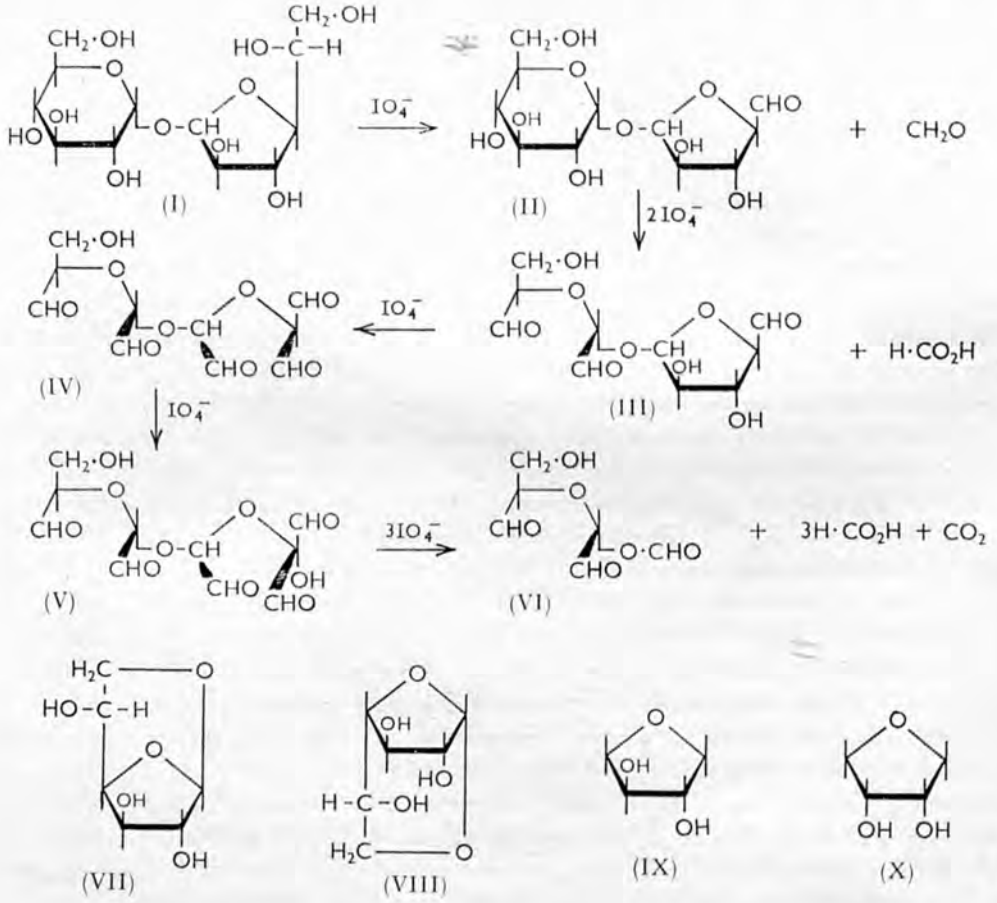
Chromatographically pure disaccharide A could be detected on paper chromatograms with acetone–silver nitrate–alcoholic sodium hydroxide,[5] but not with reagents specific for reducing sugars. Acid-hydrolysis yielded components identical with D-glucose and D-galactose on paper chromatography. Disaccharide A was immobile during electrophoresis in molybdate solution.[6] That it was a disaccharide was shown by the ratio of the constituent monosaccharides (glucose to galactose, 1 : 1·05) together with its rate of elution from a charcoal–" Celite " column and migration during chromatography.

Evidence for the non-reducing nature of disaccharide A was obtained by oxidation with alkaline hypoiodite [7] and Somogyi and Nelson's alkaline copper reagent,[8] and was confirmed when potassium borohydride failed to reduce it (paper chromatography of the product of an attempted reduction revealed a single component identical with disaccharide A; this component did not migrate during electrophoresis in molybdate solution, as would be expected of a disaccharide; [6] acid hydrolysis yielded only glucose and galactose).

Disaccharide A was hydrolysed by acid at a rate intermediate between those of αα-trehalose and sucrose and almost as fast as sucrose. This suggested that the glycosidic link between the D-glucosyl and the D-galactosyl unit was weakened by the presence of a furanosyl residue.

When treated with 5 mol. of periodate disaccharide A consumed in 800 min. 3·7 mol.

of periodate and produced 1·45 mol. of formic acid and 0·83 mol. of formaldehyde. There was immediate consumption of 1 mol. of periodate followed by that of a further 2 mol. during the first 260 min. These values can be rationalised only when a D-glucopyranosyl D-galactofuranoside or a D-glucofuranosyl D-galactopyranoside structure is proposed for disaccharide A. Theoretically, complete oxidation of such a disaccharide (I) would consume 8 mol. of periodate and produce 4 mol. of formic acid, 1 mol. of formaldehyde, and 1 mol. of carbon dioxide (I—VI). The apparent discrepancy between the theoretical and the experimental values can be explained if the immediate oxidation stops with compound (III), whose further oxidation is then rate-determining. The resistance to periodate oxidation might be due to the formation of a cyclic semiacetal in the furanosyl



residue. Molecular models indicate that this is not so. On the other hand, 1,2-glycols derived from five-membered ring systems are not oxidised by periodate when the hydroxyl groups are securely locked in a *trans*-position, as in compounds (VII) [9] and (VIII): [10] L-threitan (IX), which has less securely locked *trans*-hydroxyl groups, reacts with periodate, but more slowly than the *cis*-compound erythritan (X); [11] further, Mitra and Perlin [12] have shown that the glucopyranosyl part of sucrose is more rapidly oxidised by periodate than the fructofuranosyl part. On the basis of these facts we believe that the rate-determining step is the further oxidation of (III), the adjacent hydroxyl groups of which are *trans* to one another.* Thus in the later stages of the oxidation of disaccharide (I) the consumption of $(3 + 5x)$ mol. of periodate should give rise to $(1 + 3x)$ mol. of formic

* *Added*, 21.10.60: Since this paper was submitted, Kjølberg (*Acta Chem. Scand.*, 1960, **14**, 1118) has found that by lowering the temperature to 5° the periodate oxidation of methyl D-galactofuranosides and glucofuranosides can be confined to the 5,6-bond.

acid. The consumption of 3·7 mol. of periodate should, on this theory, produce 1·42 mol. of formic acid and 1·0 mol. of formaldehyde. The experimental results are in good agreement with these values and so, we suggest, prove the pyranosyl furanoside structure of disaccharide A.

That the initial attack of periodate on disaccharide A removed a primary hydroxyl group as formaldehyde was shown when use of 1 mol. of periodate produced 0·58 mol. of formaldehyde. The oxidation product was reduced with potassium borohydride to an acid-labile disaccharide. Paper chromatography of the hydrolysate revealed the presence of components corresponding to D-glucose and L-arabinose. This showed that disaccharide A was a D-glucopyranosyl D-galactofuranoside. D-Galactopyranosyl D-glucofuranoside should have yielded D-galactose and D-xylose.

The configuration of the glycosidic links was investigated by treatment of disaccharide A with hydrolytic enzymes. The disaccharide was hydrolysed by a mixture of yeast α-glucosidase and α-galactosidase, but not by the same mixture when the α-glucosidase was inhibited by the addition of D-glucono-δ-lactone.[13] It was not hydrolysed by almond β-glucosidase. We conclude that the D-glucopyranosyl unit had an α-configuration. There was not sufficient evidence to assign the configuration of the D-galactofuranosyl unit as it is likely that the α-galactosidase was specific for galactopyranosides.

The evidence presented thus characterises disaccharide A as α-D-glucopyranosyl D-galactofuranoside. This is of considerable interest since the synthesis of a furanoside was involved.

Disaccharides B and C could be detected on paper chromatograms with acetone–silver nitrate–alcoholic sodium hydroxide and with reagents specific for reducing sugars. Acid-hydrolysis of each yielded components identical with D-glucose and D-galactose on paper chromatography. They had a glucosyl-galactose structure as reduction with potassium borohydride followed by hydrolysis and paper chromatography revealed glucose and a hexitol.

The chromatographically pure oligosaccharides D and E yielded, when completely hydrolysed, components with R_F values identical with those of glucose and galactose in the ratios of 2 : 1 and 3 : 1, respectively. Partial hydrolyses gave components corresponding to glucose, galactose, and isomaltose on paper chromatograms. These oligosaccharides may thus be formed by successive addition of glucosyl units to a galactose-containing receptor molecule.

It is thus established that αβ-D-galactose serves as receptor of α-D-glucose units transferred from sucrose by *Betacoccus arabinosaceous*, though in view of the relatively low yields, much less efficiently than D-glucose.

Experimental

Chromatography.—The solvents used in chromatography were (*a*) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase); (*b*) butan-1-ol–benzene–pyridine–water (5 : 3 : 2 : 1); (*c*) butan-1-ol–pyridine–water–saturated aqueous boric acid (6 : 4 : 2 : 1).

Oligosaccharide Synthesis in a Galactose–Sucrose Medium.—An aqueous medium (150 ml.) containing yeast extract (1%), sodium ammonium hydrogen phosphate (0·5%), potassium dihydrogen phosphate (0·1%), hydrated magnesium sulphate (0·05%), and sucrose (2%) was adjusted to pH 7 with sodium hydroxide and sterilised, then inoculated with a strongly growing culture of *Betacoccus arabinosaceous* (Birmingham) and incubated at 25° for 60 hr. Galactose (20 g.), which had been purified by charcoal-column chromatography, was added and incubation continued for a further 5·5 days. Sterile 6% sucrose solutions (2·5 ml.) were added to the culture at intervals of 4 hr. throughout the first three days of the second incubation period.

The culture medium was adjusted to pH 7 and invertase (B.D.H. concentrate; 2 ml.) added to remove the residual sucrose. After incubation at room temperature for 1·5 hr. paper chromatography in solvent (*a*) and paper ionophoresis in borate buffer (pH 10)[14] revealed glucose, fructose, galactose, and oligosaccharides other than sucrose. The bacterial cells were

removed by centrifugation (1 hr. at 5000 r.p.m.). After addition of ethanol (200 ml.) to precipitate dextran, the ethanol was removed from the supernatant liquid, and the residual solution was applied to a charcoal–" Celite " column [4] (30 × 8 cm.). The monosaccharides were eluted with water (4 l.), and the disaccharides with 5% aqueous ethanol (6 l.). Higher oligosaccharides were eluted with 10% (6 l.) and 15% aqueous ethanol (10 l.), respectively. Paper chromatography of the disaccharide fraction in solvent (*a*) showed the presence of one component with $R_{glucose}$ 0·46 and two components with $R_{glucose}$ *ca.* 0·38; ionophoresis in borate buffer (pH 10) revealed the presence of three components with M_G 0·54, 0·43, and 0·34, respectively.

Partial fractionation of the mixture of disaccharides was on a charcoal–" Celite " column (41 × 2·5 cm.) impregnated with 0·1M-borate buffer (pH 10), by elution with 0·1M-borate buffer (2·5 l.) and then with borate buffer containing 2·5% of ethanol (3 l.).[4b] Refractionation by paper chromatography in solvent (*a*) gave disaccharide A (47·6 mg.), B (32·7 mg.), and C (40·5 mg.).

Oligosaccharides D and E were obtained as chromatographically pure fractions when the charcoal–" Celite " column was eluted with 10% and 15% aqueous ethanol, respectively.

Properties of Disaccharide A.—(i) On a paper chromatogram irrigated with solvent (*a*) the disaccharide moved as single component with $R_{glucose}$ 0·46. Paper ionophoresis in borate buffer (pH 10) [14] again showed a single component with M_G 0·44. The disaccharide was revealed by acetone–silver nitrate–alcoholic sodium hydroxide [5] as a weak spot that appeared slowly and at the same rate as that due to αα-trehalose. It was not revealed with aniline hydrogen phthalate,[15] *p*-anisidine hydrochloride,[16] or triphenyltetrazolium chloride.[17]

The carbohydrate content, determined by the anthrone method,[18] was 96·1%.

(ii) Disaccharide A (*ca.* 1·5 mg.) was hydrolysed in 0·25N-sulphuric acid (2 ml.) at 100° for 40 min. Paper chromatography of the hydrolysate in solvent (*a*) showed components corresponding to glucose and galactose in equal quantities. The two components were isolated by paper chromatography. Determination of the glucose and galactose [8] in aliquot parts of the eluates revealed that they were present in the ratio of 1 : 1·05.

(iii) Disaccharide A (2·2 mg.), on oxidation with alkaline hypoiodite,[7] consumed iodine equivalent to 0·078 mg. of glucose or galactose (6·7% of the theoretical value for a disaccharide). When treated by the method of Somogyi and Nelson [8] disaccharide A had no reducing power.

(iv) Disaccharide A (2 mg.) was left in 0·05% potassium borohydride solution (0·5 ml.) overnight at room temperature. The solution was de-ionised by Amberlite resin IR-120 (H^+), then evaporated to dryness and distilled with dry methanol (3 × 2 ml.). Paper chromatography of the residue in solvents (*a*) and (*b*) revealed a single component identical with disaccharide A, immobile during electrophoresis in molybdate solution.[6]

This residue was hydrolysed with 1·5N-sulphuric acid at 100° for 4 hr. Paper chromatography of the de-ionised hydrolysate in solvent (*c*) revealed components corresponding to glucose and galactose.

(v) Disaccharide A (0·4 mg.), αα-trehalose (*ca.* 2 mg.), and sucrose (*ca.* 2 mg.) were separately hydrolysed with 0·25N-sulphuric acid (0·25 ml. and 1 ml.) at 100° for 15 min. Paper chromatography of the de-ionised hydrolysates showed that disaccharide A had been almost completely hydrolysed (*ca.* 90%) to glucose and galactose. Sucrose had been completely hydrolysed to glucose and fructose. Only a trace of glucose (<10%) was liberated from αα-trehalose.

(vi) *Periodate oxidation.* Treatment of disaccharide A (10·3 mg.) with 0·015M-sodium metaperiodate (10 ml.) in the dark at room temperature gave the following results. The periodate consumption, expressed in moles per mole of disaccharide A, was 1·2 (10 min.), 2·5 (150 min.), 2·9 (260 min.), 3·3 (480 min.), 3·5 (700 min.), 3·7 (800 min.). After 24 hr. all the periodate had been reduced. After 800 min. 1·45 moles of formic acid and 0·83 mole of formaldehyde were produced per mole of disaccharide A.

On treatment of αα-trehalose under the same conditions, the periodate consumption was 0·7 (10 min.), 3·2 (240 min.), 3·5 (360 min.), 3·6 (540 min.), 3·6 (1380 min.). After 540 min. 1·4 moles of formic acid were produced per mole of αα-trehalose. No formaldehyde was produced.

When the disaccharide A (0·34 mg.), dissolved in water (10 ml.), was treated with 0·001M-sodium metaperiodate (1 ml.) the formaldehyde produced (mole per mole) was 0·36 (7 hr.), 0·58 (23 hr.), 0·58 (48 hr.). The solution was then de-ionised with Permutit " Biodeminrolit "

resin, pretreated with carbon dioxide. Potassium borohydride (50 mg.) in water (1 ml.) was added and the solution left overnight at room temperature, de-ionised by Amberlite resin IR-120 (H^+), and evaporated to dryness. The residue was distilled with dry methanol (3 × 2 ml.), then treated with 0·25N-sulphuric acid (5 ml.) at 100° for 15 min. The de-ionised hydrolysate was analysed by paper chromatography in solvent (*b*) which revealed components corresponding to D-glucose and L-arabinose, but not to galactose and xylose. They were detected with acetone–silver nitrate–alcoholic sodium hydroxide [5] and *p*-anisidine hydrochloride.[16] The latter reagent gave a pink stain, typical of pentoses with the component corresponding to L-arabinose and a yellowish stain with that corresponding to D-glucose.

(vii) A solution of disaccharide A (1%) was sealed in a capillary tube with an equal volume of almond β-glycosidase solution.[19] Paper chromatography of the digest after incubation at 35° for 72 hr. showed that no hydrolysis had occurred. Under the same conditions cellobiose was completely hydrolysed, lactose partially hydrolysed, and maltose was not hydrolysed. A 1% solution of disaccharide A was sealed in a capillary tube with an equal volume of yeast α-glucosidase [20] solution. Paper chromatography of the digest after incubation at 26° for 48 hr. revealed components corresponding to glucose and galactose. Under the same conditions, maltose was completely hydrolysed and melibiose partially hydrolysed. Lactose and cellobiose were not hydrolysed. A 1% solution of disaccharide A was sealed in a capillary tube with an equal volume of yeast α-glycosidase [20] solution, to which D-glucono-δ-lactone (2%) had been added: [13] paper chromatography of the digest after incubation at 26° for 72 hr. revealed absence of hydrolysis. Under the same conditions maltose was not hydrolysed and melibiose was partially hydrolysed.

Investigation of Disaccharides B and C.—(i) On a paper chromatogram irrigated with solvent (*a*) the disaccharides B and C moved with $R_{glucose}$ 0·39 and 0·36, and M_G 0·60 and 0·34, respectively. Disaccharide B was probably contaminated with a trace of leucrose. For detection see p. .

(ii) Materials B (1 mg.) and C (1 mg.) in N-sulphuric acid at 100° for 4 hr. gave glucose and galactose (identified by paper chromatography). Sugar B also gave a trace of fructose.

(iii) Disaccharides B (2 mg.) and C (2 mg.) were separately treated with potassium borohydride (as above). The reduction products were hydrolysed with 1·5N-sulphuric acid at 100° for 4 hr. Paper chromatography of the hydrolysates in solvent (*c*) revealed components corresponding to glucose and a hexitol, but not galactose.

Investigation of Oligosaccharides D and E.—Chromatographically pure oligosaccharides D and E [$R_{glucose}$ 0·16 and 0·18, respectively, in solvent (*a*)] were separately hydrolysed in 1·5N-sulphuric acid at 100° for 4 hr. Paper chromatography then revealed components corresponding to glucose and galactose in the ratios of 2 : 1 and 3 : 1, respectively. Partial hydrolysis of D and E in N-sulphuric acid at 90° gave glucose, galactose, and isomaltose on paper chromatography.

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Substrate Cleavage Point with Glucamylase

THE glycosidic link can, theoretically, be hydrolysed by two alternative routes, proceeding with the fission of the bond between (*a*) the anomeric carbon atom and the glycosidic oxygen or (*b*) the aglycon group and the glycosidic oxygen. For a number of glycosides the mechanism of the acidic and/or enzymatic hydrolysis has been investigated[1–8]. Since it became possible to prepare highly purified glucamylase it was interesting to investigate the cleavage point when this enzyme acts on maltose.

An aqueous extract (8 ml.)[9] of ruptured *Aspergillus niger* '152' cells was fractionated on a column (29 × 1·5 cm) of diethyl-amino-ethyl cellulose (Whatman *DE* 50) by the method of Pazur and Ando[10], using the same citric acid phosphate buffer but a smaller *p*H gradient for elution. Elution was commenced with buffer solution (100 ml.) of *p*H 7·5 and followed successively with the same volume of buffer solutions of *p*H 7·0 and 6·5. Fractions of the eluate (5 ml.) were analysed for transglucosylase and glucamylase activity by incubating portions in digests containing (*a*) amylose and (*b*) maltose. Transglucosylase activity was found in fractions No. 13–16 and glucamylase activity in fractions No. 14–20. Fractions No. 17–20 contained glucamylase activity only. The iodine-absorption value reducing power curve[11] of the pure glucamylase fraction was found to be identical with that of glucamylase purified by acid treatment[9] and remained unaltered after such treatment.

The point of cleavage of the α-1 : 4-glucosidic link when glucamylase acts on maltose was investigated by conducting the hydrolysis in $H_2{}^{18}O$.

The enzymatically produced glucose was isolated as its phenylosazone. Thus, fission of the $C_{(1)}$-O bond, route (*a*), would lead to a glucosazone, all oxygen atoms of which would be isotopically normal, whereas fission of the $C_{(4)}$–O bond, route (*b*), would give a glucosazone, one oxygen atom of which would be isotopically half-enriched.

A digest containing maltose (112 mg) in $H_2{}^{18}O$ (1·5 ml.; oxygen-18 enrichment 4·2 per cent), M-acetate buffer (*p*H 4·0; 0·1 ml.) and glucamylase solution (0·4 ml.) was incubated at 50° C for 16 h, when all the maltose had been hydrolysed to glucose. This was isolated as its phenylosazone with m.p. 204°–205° C

which was not depressed on admixture with authentic glucosazone. A control experiment was carried out in normal water. Samples of glucosazone were pyrolysed by a method similar to that described by Oita and Conway[12]. Mass-spectrometric analysis of the carbon dioxide obtained showed no enrichment in oxygen-18.

The results show that the hydrolysis of maltose by glucamylase proceeds almost entirely by the fission of the bond between the anomeric carbon atom and the glycosidic oxygen. This is in contrast to the acidic hydrolysis of maltose where 78 per cent of the reaction apparently proceeds by this route[8]. On the other hand, with few exceptions such as the hydrolysis of α-D-glucose-1-phosphate by prostatic acid phosphatase[4], enzymatic reactions studied seem to proceed by route (*a*). Thus, the hydrolysis of maltose by glucamylase follows a general pattern for hydrolases and transglycosylases.

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877. *Synthesis of Sucrose Labelled with Carbon-14 in the Fructose Part.*

By E. J. Bourne, J. Peters, and H. Weigel.

A dextransucrase preparation from *Leuconostoc mesenteroides* (Birmingham) syntheses, from sucrose and D-[^{14}C]fructose, sucrose labelled in the fructose part, and other [^{14}C]fructose-containing saccharides.

It is generally accepted that the reaction between sucrose and dextransucrase to give dextran and D-fructose proceeds through several stages, involving association of the enzyme with donor and receptor molecules.[1,2] In the present work an investigation on the effect of added D-[^{14}C]fructose is described.

When dextransucrase preparations from *Leuconostoc mesenteroides* (Birmingham)[3] were incubated with sucrose and D-[^{14}C]fructose, radiochromatograms showed that carbon-14 was incorporated into four components, B, C, D, and E, which had, in solvent (*a*), $R_{fructose}$ values of 0·50, 0·38, 0·22, and 0·11, respectively. Components C, D, and E were produced only in relatively small quantities. This phenomenon was also observed with dextransucrase preparations from *Streptococcus bovis*, kindly supplied by Dr. R. W. Bailey. In the absence of any enzyme preparation, D-[^{14}C]fructose remained the only labelled compound in the mixture.

The following evidence shows that component B was in fact [^{14}C]sucrose: (*a*) in several solvents it migrated as a single component with an R_F value identical with that of sucrose; (*b*) hydrolysis by acid, and by invertase gave glucose and [^{14}C]fructose; (*c*) carrier dilution with sucrose gave crystalline [^{14}C]sucrose, the specific radioactivity of which was not affected by recrystallisation. The fact that acid and enzymic hydrolysis of [^{14}C]sucrose gave [^{14}C]fructose as the only component containing carbon-14 also showed that carbon-14 was present in the fructose portion only.

The specific radioactivity of the [^{14}C]sucrose present at the end of the incubation period (8 hr.) was 60·0 mc/mole by the infinitely-thin film method (Fig. 1), and 60·8 mc/mole by the infinitely-thick disc method. Based on the quantities (and radioactivity) of sucrose and D-[^{14}C]fructose present initially, a complete interchange of D-[^{14}C]fructose between sucrose and free D-fructose should give [^{14}C]sucrose with a specific radioactivity of *ca.* 62·0 mc/mole. The results show that this interchange is, within experimental error, virtually complete after 8 hours' incubation.

This investigation has shown that dextransucrase preparations are capable of transferring D-glucopyranosyl units reversibly from sucrose to the reducing position of D-fructofuranose. If the sucrose synthesising enzyme is in fact dextransucrase then the formation of a D-glucosyl-dextransucrase complex and D-fructose from sucrose is a reversible process. On the other hand, the synthesis of α-1,2- and α-1,6-linkages by our enzyme preparations[3] appears to be irreversible;[4] dextran synthesis is certainly very much slower than the D-fructose interchange.

The component C was, in several solvents, chromatographically identical with leucrose (5-*O*-α-D-glucopyranosyl-D-fructose). No hydrolysis occurred under mild acid conditions suitable for the complete hydrolysis of sucrose, or by treatment with invertase. More drastic conditions, however, gave glucose and a trace of [^{14}C]fructose, by chromatographic evidence. The conditions of this hydrolysis were such that most of the fructose would have been converted into hydroxymethylfurfuraldehyde, which would not have been detected in the chromatographic analysis. It is therefore suggested that component C was, in fact, [^{14}C]leucrose; leucrose is known to be formed in this type of system.[5–7] The apparent ability of dextransucrase preparations to transfer D-glucopyranosyl units from sucrose also to C-5 of D-fructose is in agreement with our findings that six-membered ring

compounds will act as receptors only if they possess two hydrogen atoms and one oxygen atom *cis* related on C-1, C-3, and C-5.[4]

The properties of components D and E suggest that they were produced by successive addition of glucosyl units to sucrose.

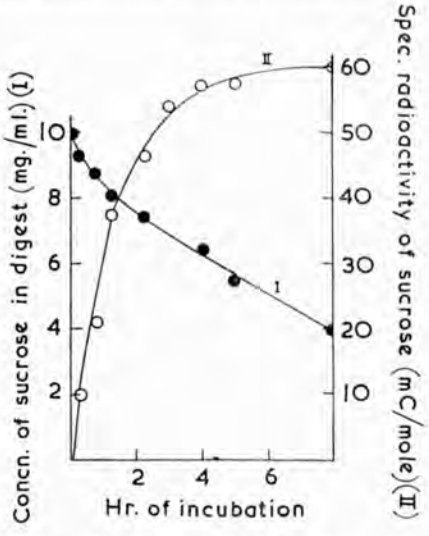
Experimental.

Materials.—D-[^{14}C]Fructose, generally labelled, was obtained from the Radiochemical Centre, Amersham.

Determination of Radioactivity.—The apparatus and the methods used were those described previously.[8]

Chromatography.—(i) *Solvents.* The solvents used in chromatography were (*a*) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase); (*b*) ethyl acetate–acetic acid–water (9 : 1 : 1); (*c*) ethyl acetate–pyridine–water–acetone (10 : 5 : 10 : 2), to which ethyl acetate was added until two layers separated (organic phase).

(ii) *Radiochromatograms.* They were obtained by exposure of chromatograms to Ilford *X*-ray films (Industrial G) for an appropriate time.



Concentration and specific radioactivity of [^{14}C]sucrose during incubation of digest.

Oligosaccharide Synthesis in the Presence of D-[^{14}C]*Fructose.*—A digest containing sucrose (10 mg.), D-[^{14}C]fructose (*ca.* 100 mg.; spec. radioactivity 10·88 mc/g.-atom of carbon, *i.e.*, 65·28 mc/mole of D-fructose), and dextransucrase preparation (2·5 units) in 0·05M-acetate buffer (pH 5·0; 1 ml.) was incubated at 25° for 8 hr. The components of portions (0·05—0·1 ml.), withdrawn at intervals, were fractionated by paper chromatography in solvent (*a*). This revealed, in addition to D-[^{14}C]fructose, A, four components containing carbon-14, B, C, D, and E. The components B and C had R_{fru} values identical with those of sucrose (0·50) and leucrose (0·38), respectively. The R_{fru} values of components D and E were 0·22 and 0·11, respectively.

Specific Radioactivity of Component B at Time Intervals.—Each fraction containing component B, obtained by chromatographic fractionation, was diluted with water to 25 ml. Portions (1 ml.) were analysed for carbohydrate content by the anthrone method.[6] The specific radioactivity of component B in each fraction was determined by the infinitely-thin film method [8] and expressed per mole of disaccharide. The results are shown in the Figure.

Characterisation of Component B.—(i) In each of the three solvents used component B moved as a single radioactive and chemical component with an R_F value identical with that of sucrose.

(ii) *Hydrolysis.* A sample of component B was hydrolysed with 0·25N-sulphuric acid at 100° for 15 min. Another sample of component B was incubated with invertase (B.D.H. concentrate) at 20° for 1 hr. Chromatographic analysis of the hydrolysates revealed in each case components which had R_F values identical with those of glucose and [^{14}C]fructose.

(iii) *Carrier dilution.* Sucrose (255·7 mg.) was dissolved in a portion of the fraction containing component B (0·22 mg.) obtained after incubation of the digest for 8 hr. After being

freeze-dried, the sucrose was crystallised from dry methanol and propan-2-ol. Recrystallisation did not cause a decrease in specific radioactivity (52·26 μc/mole, by infinitely-thick disc method [8]). From this the specific radioactivity of component B at the end of the incubation period was calculated (60·8 mc/mole of disaccharide).

Investigation of Component C.—(i) In each of the three solvents used component C moved as a single radioactive and chemical component with an R_F value identical with that of leucrose.

(ii) Samples of the combined fractions of component C were (*a*) heated with 0·25N-sulphuric acid at 100° for 15 min. and (*b*) incubated with invertase (B.D.H. concentrate) at 20° for 1 hr. Radiochromatograms revealed only a single radioactive component identical with component C. Another sample was heated with 1·5N-sulphuric acid at 100° for 4 hr. Chromatography of the hydrolysate revealed the presence of glucose and a trace of a component containing carbon-14 and having R_F identical to that of fructose.

Investigation of Components D and E.—Samples of components D and E were separately heated with 1·5N-sulphuric acid at 100° for 4 hr. Chromatography revealed in each case components corresponding to glucose and [^{14}C]fructose (trace). Chromatographic analysis of partial hydrolysates (N-sulphuric acid; 90°; 1 hr.) revealed in each case the presence of components which had R_F values identical with those of isomaltose, glucose, and [^{14}C]fructose.

Incubation of Sucrose with D-[^{14}*C*]*Fructose.*—Sucrose (10 mg.) and D-[^{14}C]fructose (100 mg.) dissolved in 0·05M-acetate buffer (pH 5·0; 1 ml.) were kept at 25° for 25 hr. Radiochromatograms of the solution revealed only a single radioactive component with R_F identical to that of fructose.

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Action of a Bacterial Dextranase on Branched Dextrans

SEVERAL mould species[1,2] and strains of Bacteroides[3] have been shown to secrete dextranases which hydrolyse, to different degrees, dextrans of various branched structures. Although glucose, *iso*maltose and *iso*maltotriose[1–3] have been shown to be among the main products of the hydrolysis, no oligosaccharides containing the glucosidic branch linkages have so far been identified in the enzyme hydrolysates. Recently, several rumen strains of an organism, closely resembling *Lactobacillus bifidus*[4], were found to secrete a dextranase which hydrolysed an unbranched *Streptococcus bovis*[5] dextran to a mixture of *iso*malto-triose, -tetraose, -pentaose and -hexaose[6]. The enzyme also hydrolysed a dextran (produced by *Leuconostoc mesenteroides*, Birmingham strain) containing 12–15 per cent of α-1,3-branch links[7] to a mixture of tri- and higher saccharides, which were not examined further. We have now investigated the oligosaccharide mixture produced by the enzyme from this and other branched dextrans.

Solutions were prepared containing dextranase[6] (10 mgm.), purified[7] dextran (30 mgm.), and citrate buffer (0·2 *M*, *p*H 5·5, 5 ml.). A solution of the above composition, containing unbranched *S. bovis* dextran, was used as a control. The solutions were incubated under toluene at 37° C. for 36 hr., after which time there was no further increase in reducing sugars. The final digests were desalted with 'Biodeminrolite', concentrated under reduced pressure to a small volume, and analysed by paper chromatography using the ethyl acetate, pyridine, acetone, water solvent of Malpress and Hytton[8]. Reducing sugars were located with silver nitrate[9]. Intense spots, corresponding to *iso*malto-triose, -tetraose and -pentaose, were present on chromatograms of all the hydrolysed dextrans. In addition, chromatograms of the *Leuconostoc* branched dextran digest showed two minor reducing components moving just in front of *iso*malto-tetraose and -pentaose respectively. These chromatograms also revealed a mass of unresolvable reducing components, $R_G <$ *iso*maltopentaose, which was absent from the control digest. Portions of the two extra oligosaccharides were eluted and hydrolysed in *N* sulphuric acid for 4 hr. at 100° C. The hydrolysates contained a single component which was chromatographically identical to glucose.

Table 1. POSSIBLE OLIGOSACCHARIDES IN ADDITION TO *iso*MALTOTRIOSE— -PENTAOSE DETECTED IN DEXTRANASE–DEXTRAN DIGESTS

Dextran; organism and type of branch link	Fast-moving tetraose	Fast-moving pentaose	Saccharides of $Rg <$ *iso*maltopentaose
(1) *S. bovis*; unbranched (ref. 5)	absent	absent	*iso*malto-hexaose^{+++}, -heptaose^{++}
(2) *L. mesenteroides* (Birmingham); modified, 2 per cent α-1,3- (ref. 9)	trace	trace	*iso*malto-hexaose^{++}, -heptaose^{+}
(3) *L. mesenteroides* (Birmingham); 12–15 per cent α-1,3- (ref. 7)	++	+++	intense unresolved mixture $Rg <$ *iso*maltopentaose
(4) *L. mesenteroides* (N.R.R.L. *B*512); 5 per cent α-1,3- (ref. 10)	trace	trace	possible hexaose^{+++}, heptaose^{+++}, octaose^{++}
(5) *Betabacterium vermiforme*; 5 per cent α-1,3-, 5 per cent α-1,4- (ref. 11)	trace	trace	possible hexaose^{+++}, heptaose^{+++}, octaose^{++}
(6) *Acetobacter capsulatum*; 10 per cent α-1,4- (ref. 12)	absent	absent	possible hexaose^{+++}, heptaose^{+++}, octaose^{++}, nonaose^{+} and unresolved higher saccharides

Gradings allocated on the basis: trace, just visible; +, clearly visible; +++, definite black spot. Under the conditions used *iso*maltotriose spot = +++++++.

The three main components in the digest have been shown, by movement in five solvent systems and reaction with four spray reagents, to be chromatographically identical with *iso*malto-triose, -tetraose and -pentaose, respectively. It seems likely, therefore, that the two extra saccharides contain, in addition to α-1,6-links, the α-1,3-branch links of the original dextran. Their absence from the control digests seems to rule out the possibility that they were formed by an enzymic transferase action on the *iso*maltodextrins produced in the digests. Attempts are now being made to elucidate the structure of these two sugars. As the dextranase has been shown to hydrolyse *iso*malto-heptaose and -octaose, the mass of higher saccharides present in the branched dextran digest probably also contains α-1,3-branch links which prevented their further hydrolysis. Two acid-degraded specimens of the branched *Leuconostoc* dextran, mol. wt. 300,000 and plasma grade, were also treated with dextranase. The same two extra saccharides were detected on chromatograms of the digests at possibly lower concentrations. The three *iso*maltodextrins and the unresolved mixture of $R_G <$ *iso*maltopentaose were also present.

Several other dextrans, the structures of which have been examined by chemical methods, were treated with the dextranase and the hydrolysates examined by paper chromatography. The results,

with those already obtained, are listed in Table 1.

It appears that *L. bifidus* dextranase is unable to cleave branching links in dextrans. This is not surprising as the organism was cultured in a medium containing unbranched *S. bovis* dextran. The mixture of saccharides produced seems to depend not only on the type of branching, but also on the degree of branching; compare dextrans Nos. 3 and 4 in which there appears to be a definite difference in the composition of the saccharide mixture of $R_G <$ *iso*-maltopentaose. Another dextran, produced by *L. mesenteroides* (N.R.R.L., *B*742) and containing 36 per cent of α-1,3- and α-1,4-links[11], was also treated with the enzyme. This dextran was not hydrolysed by the dextranase, a result which is in agreement with the markedly different serological properties reported[13] for a similarly branched dextran produced by another strain (N.R.R.L.-*B*742-*LR*) of this organism. The possibility that dextranases produced by *L. bifidus* and other organisms may be of help in establishing the structures of dextrans is being investigated further.

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Oligosaccharides in Dextran-Producing Cultures of *Streptococcus bovis*

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Species of *Leuconostoc mesenteroides* and *Streptococcus bovis* produce dextran and fructose when grown in media containing sucrose (Hehre, 1951; Tsuchiya *et al.* 1952; Bailey, Barker, Bourne & Stacey, 1957; Dain, Neal & Seely, 1956; Bailey & Oxford, 1958*a*). In addition, reducing disaccharides containing glucose and fructose units are formed. One of these, leucrose (5-*O*-D-glucopyranosyl-D-fructopyranose), has been isolated from cultures of *L. mesenteroides* (NRRL B-512F) and characterized by Stodola, Sharpe & Koepsell (1956), who also mentioned the formation of isomaltulose (Sharpe, Stodola & Koepsell, 1954). Bailey & Bourne (1959) have isolated leucrose from dextran-producing cultures of *S. bovis* and noted that a second disaccharide was produced. The present paper reports a closer examination of the oligosaccharides produced in cultures of *S. bovis*.

EXPERIMENTAL

Organism. Streptococcus bovis (strain I; Bailey & Oxford, 1958*a*, *b*) was used.

Culture medium. The organism was grown in a medium containing yeast extract (1%, w/v), Bacto-tryptose (1·5%, w/v), sucrose (8·5%, w/v) and separately sterilized potassium carbonate (0·004 M), pantothenic acid (20 mg./l.) and biotin (0·01 mg./l.). A 1 l. culture was incubated at 37° for 24 hr., when more sucrose (30 g.) and sodium bicarbonate (1 g.) were added. The pH of the medium was adjusted to 6·5 with N-NaOH and the culture incubated for a further 3 days.

Paper chromatography. Whatman no. 1 filter paper was used. The solvent systems were: (*a*) organic phase of ethyl acetate–water–pyridine (2:2:1, by vol.); (*b*) ethyl acetate–acetic acid–water (9:2:2, by vol.); (*c*) butanol–benzene–pyridine–water (5:1:3:2, by vol.); (*d*) water-saturated butanol.

Paper ionophoresis. Ionophoresis was conducted on Whatman no. 3 MM filter paper in 0·1 M-borate, pH 10 (Foster, 1953), and 0·1 M-molybdate, pH 5·5 (Bourne, Hutson & Weigel, 1959), at 80 v/cm. for about 1 hr.

Selective spray reagents. The spray reagents used were: (*a*) acetone–silver nitrate–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950), for the general detection of carbohydrates; (*b*) urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955), for the detection of ketoses, particularly compounds containing a leucrose unit, which stain a dull brown, strongly fluorescent in u.v. light; (*c*) aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956), found to give a specific green spot with 6-*O*-substituted glucose (e.g. isomaltose); (*d*) triphenyltetrazolium chloride (Feingold, Avigad & Hestrin, 1956), reaction with which was regarded as evidence that reducing glucose units were unsubstituted at the C-2 hydroxyl group.

Reduction of aldoses and ketoses. Aqueous solutions of the sugars (about 1%) were mixed with an equal volume of potassium borohydride solution (1%) and allowed to stand overnight (Bragg & Hough, 1957). The solutions were deionized by treatment with Amberlite resin IR-120 (H^+) followed by evaporation to dryness and repeated distillation with dry methanol. Completion of reduction was checked by ionophoresis in molybdate solution (Bourne *et al.* 1959). The product was always purified by paper chromatography.

Acid hydrolysis. Hydrolysis was effected at 96° in 0·5 N-sulphuric acid for 3 hr. (complete) or 1 hr. (partial). The solutions were deionized by treatment with Amberlite resin IRA-400 (carbonate).

RESULTS

Isolation of oligosaccharides

At the end of the incubation period dextran was removed from the culture fluid (1 l.) by precipitation with acetone (2 vol.). The supernatant liquid was evaporated to 200 ml. and fractionated on a charcoal–Celite column (40 cm. × 7 cm.; Whistler & Durso, 1950). Water eluted fructose and a small amount of glucose. Di- and tri-saccharides were eluted with aqueous 1–6% and 6–10% ethanol respectively. A small quantity of material eluted with aqueous 15% ethanol was not examined further.

Leucrose (m.p. 156°, mixed m.p. 156°) was crystallized from the disaccharide fraction in ethyl acetate (Bailey & Bourne, 1959). The mother liquor was fractionated on a cellulose column (Whatman cellulose powder, standard grade; 60 cm. × 4·5 cm.) with solvent (*c*) as the irrigant. The fraction containing disaccharide A was concentrated, dissolved in water and freeze-dried. From the fraction containing leucrose a disaccharide B was isolated in trace quantity by paper chromatography with solvent (*b*).

Paper chromatography of the trisaccharide fraction (300 mg.) with solvent (*a*) revealed a trace quantity of a trisaccharide A, which was isolated by this method. The main trisaccharide fraction then appeared to be homogeneous in the four chromatography solvents, but 100 mg. was resolved into two components, trisaccharide B (about 50 mg.) and C (about 40 mg.), by paper ionophoresis in borate solution. The yields of oligosaccharides from a 1 l. culture are shown in Table 1.

Structural examination of the oligosaccharides

Disaccharide A. Disaccharide A stained blue with spray (*b*) (ketose-type) and had the characteristics shown in Table 2. Its high rate of mobility during ionophoresis in molybdate solution was indicative of a 6-*O*-substituted fructose (Bourne, Hutson & Weigel, 1960). Acid hydrolysis gave glucose and fructose (paper chromatography), shown by elution and determination with anthrone (Yemm & Willis, 1954) to be present in the ratio 1 : 0·75. Determination of glucose and fructose in an artificial mixture (ratio 1 : 1), treated with acid in the same way as the disaccharide, revealed a glucose/fructose ratio of 1 : 0·81.

Disaccharide A was reduced with potassium borohydride (Bragg & Hough, 1957). Ionophoresis in molybdate solution (pH 5·5) showed that the alcohol migrated as a single component, with

Table 1. *Yields of oligosaccharides from a* 1 *l. culture of* Streptococcus bovis *grown on sucrose* (115 *g.*)

The expected yield of dextran would be about 40 g.

Compound	Yield
Leucrose	1·8 g.
Disaccharide A (isomaltulose)	250 mg.
Disaccharide B (isomaltose)	Trace
Trisaccharide A (isomaltotriulose)	Trace
Trisaccharide B (isomaltotriose)	150 mg.
Trisaccharide C (5-*O*-α-isomaltosyl-D-fructose)	120 mg.

mobility referred to sorbitol (M_S) 0·8 (identical with that of a 5- or 6-*O*-glycosyl-sorbitol or -mannitol), and that on hydrolysis it gave glucose and hexitol but not fructose.

When estimated by the Shaffer & Hartmann (1921) method disaccharide A (4 mg.) had a reducing power equivalent to 1·84 mg. of fructose. This corresponded to 87·7 % of the calculated value for a disaccharide.

Disaccharide A was incubated with almond β-glycosidase at 25° for 24 hr. Paper chromatography showed that no hydrolysis had occurred. Under the same conditions 1-*O*-β-D-glucopyranosyl-D-fructose was completely hydrolysed.

Disaccharide A (50 mg.) was converted into its phenylosazone (30 mg.) by the method of Avigad (1959). The recrystallized phenylosazone had m.p. 171–173°. Admixture with isomaltose phenylosazone, m.p. 173–175°, caused no depression in melting point. The phenylosazone behaved as a pure disaccharide phenylosazone during chromatography in solvents (*a*) and (*d*). Its infrared spectrum was identical with that of isomaltose phenylosazone. Thus disaccharide A was isomaltulose.

Disaccharide B. Disaccharide B could be detected on paper chromatograms and ionophoretograms with spray reagents (*a*), (*c*) and (*d*), and was indistinguishable from isomaltose (Table 2). Spray (*c*) gave a green spot characteristic for a 6-*O*-substituted glucose. Acid hydrolysis gave only glucose (paper chromatography). Thus the trace disaccharide B was almost certainly isomaltose.

Trisaccharide A. Trisaccharide A could be detected on paper chromatograms and ionophoretograms (Table 2) with the spray reagents (*a*) and (*b*). Chromatography and ionophoresis of the complete acid hydrolysate revealed the presence of components identical with glucose and fructose (small amount), and a partial hydrolysate contained also components identical with isomaltose and isomaltulose.

Trisaccharide A alcohol, obtained by reduction of trisaccharide A with potassium borohydride (Bragg & Hough, 1957), had M_S 0·75, identical with that of 6-*O*-isomaltosylsorbitol and different from all but 5- or 6-*O*-biosyl-sorbitols or -mannitols. Complete hydrolysis of the alcohol gave components identical with glucose and a hexitol; a partial hydrolysate contained isomaltose and glucose as the only reducing components. Thus trisaccharide A was most probably isomaltotriulose.

Trisaccharide B. Trisaccharide B could be detected on paper chromatograms and ionophoretograms with spray reagents (*a*), (*c*) (characteristic green for a 6-*O*-substituted glucose) and (*d*). It was indistinguishable from isomaltotriose (Table 2). Complete acid hydrolysis gave only glucose, whereas a partial acid hydrolysis gave isomaltose and glucose. It was not hydrolysed by almond β-glycosidase.

Trisaccharide B was reduced with potassium borohydride (Bragg & Hough, 1957). Ionophoresis in molybdate solution (pH 5·5) showed that the alcohol migrated at the same rate as 6-*O*-isomaltosylsorbitol (M_S 0·75; see above). Complete hydrolysis of the alcohol gave glucose and a hexitol. A partial hydrolysate of the alcohol was shown, by chromatography in solvents (*a*) and (*b*) and ionophoresis in molybdate solution (pH 5·5), to contain two reducing components identical with glucose and isomaltose, and two non-reducing components identical with a hexitol and 6-*O*-glucopyranosyl-sorbitol, as well as the unchanged alcohol.

Table 2. *Properties of some oligosaccharides compared with those isolated from* Streptococcus bovis *cultures*

Solvents: (*a*) organic phase of ethyl acetate–water–pyridine (2:2:1, by vol.); (*b*) ethyl acetate–acetic acid–water (9:2:2, by vol.); (*c*) butanol–benzene–pyridine–water (5:1:3:2, by vol.); (*d*) water-saturated butanol.

	Chromatography R_G				Ionophoresis		
Compound	Solvent (*a*)	Solvent (*b*)	Solvent (*c*)	Solvent (*d*)	M_G (borate)	M_S (molybdate)	Phenylosazone (m.p.)
1-*O*-β-Glucopyranosyl-fructose	0·59	0·41	0·57	0·28	0·74	0·25	—
Sucrose	0·74	0·63	0·81	0·50	0·10	0	—
Turanose	0·75	0·54	0·81	0·51	0·69	0·1	202–204°
Maltulose	0·63	0·44	0·64	0·39	0·63	0·15	202–204
Leucrose	0·55	0·50	0·50	0·32	0·56	0·35	186–188
Isomaltulose	0·64	0·50	0·68	0·33	0·60	0·64	173–175
Isomaltose	0·58	0·27	0·50	0·31	0·70	0	173–175
Isomaltotriose	0·35	0·15	—	0·10	0·58	0	—
Disaccharide A	0·63	0·50	0·69	0·35	0·61	0·65	171–173
Disaccharide B	0·59	0·28	0·50	0·31	0·70	0	—
Trisaccharide A	0·41	0·24	—	0·15	0·50	0·47	—
Trisaccharide B	0·35	0·15	—	0·10	0·57	0	—
Trisaccharide C	0·35	0·15	—	0·10	0·44	0·30	—

Trisaccharide C. Trisaccharide C could be detected on paper chromatograms and ionograms (Table 2) with spray reagent (*b*) as a brown spot which fluoresced in u.v. light (as did leucrose). Acid hydrolysis yielded glucose and a smaller quantity of fructose (paper chromatography). A partial hydrolysate contained glucose, isomaltose and small amounts of leucrose and fructose, as well as the unchanged trisaccharide. Chromatography of an equimolar mixture of isomaltose and leucrose, after the same treatment with acid, gave a similar pattern.

Trisaccharide C was reduced with potassium borohydride (Bragg & Hough, 1957). During ionophoresis in molybdate solution (pH 5·5) the product migrated with M_s 0·75 (see above). Complete acid hydrolysis of the alcohol gave glucose as the only reducing hexose together with a hexitol. Chromatography and ionophoresis (molybdate) of a partial hydrolysate of the alcohol revealed the presence of components identical with glucose, a hexitol, isomaltose, 5- or 6-*O*-glycosylsorbitol and unchanged material.

Trisaccharide C was not hydrolysed by almond β-glycosidase; thus it was most probably O-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 5)-D-fructose (5-*O*-α-isomaltosyl-D-fructose).

Action of partially purified Streptococcus bovis *dextransucrase on leucrose in the presence of sucrose*

Leucrose (50 mg.) and sucrose (50 mg.) were incubated at 37° with dextransucrase (2 mg.) in 0·05M-acetate buffer (pH 5·2, 2 ml.) for 24 hr. The dextransucrase preparation was supplied by Dr R. W. Bailey and prepared according to Bailey (1959). Chromatography of the digest revealed a small amount of a component with R_g 0·35, 0·15 and 0·10 in solvents (*a*), (*b*) and (*d*) respectively. It could be detected with spray reagents (*a*) and (*b*). The latter gave a brown spot which fluoresced strongly in u.v. light. Some of this component was isolated by paper chromatography and subjected to the same analysis as trisaccharide C. In all these tests the compound was identical with trisaccharide C. The enzyme did not produce detectable amounts of this trisaccharide from leucrose or sucrose separately. This confirmed the structure assigned to the trisaccharide.

DISCUSSION

The evidence presented here and by Bailey & Bourne (1959) characterizes three of the oligosaccharides in *S. bovis* cultures as isomaltulose, leucrose and *O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 5)-D-fructose (5-*O*-α-isomaltosyl-D-fructose); other products were almost certainly isomaltose, isomaltotriose and isomaltotriulose. A large quantity of fructose and a smaller amount of glucose were also present; all of the sucrose had been utilized.

It has been shown that certain simple sugars can act as receptors of transferred glucosyl units when dextransucrase preparations from *L. mesenteroides* (NRRL B-512) (Koepsell *et al.* 1953), *L. mesenteroides* (Birmingham) (Bailey *et al.* 1957) and *S. bovis* (Bailey, 1959) act on sucrose. Such sugars lead to the synthesis of oligosaccharides. Isomaltose and isomaltotriose were found, in the *Leuconostoc* system, to be good receptors whereas glucose, fructose and leucrose acted as poor receptors. The present work shows that the same is true for the *S. bovis* dextransucrase system.

Glucosyl transfer of the above type can occur during dextran synthesis and be responsible for by-products. The amount of such by-products will depend not only on the efficiency of the receptor molecules but also on their relative concentrations. Thus transfer to fructose, which is a poor receptor but present in large quantities, results in relatively large amounts of leucrose (mainly) and isomaltulose. Transfer to glucose would result in much less isomaltose. The concentrations of disaccharides could increase until they competed with fructose as receptors, thus leading to 5-*O*-α-isomaltosyl-D-fructose and isomaltotriulose. The production of isomaltotriose is almost certainly via isomaltose. That the latter was found only in very small amount is reasonable as it is a good receptor and would be soon converted into isomaltotriose. It is interesting that fructose appears to act as a receptor in both the pyranose and furanose forms, to give leucrose and isomaltulose respectively, the former being preferred.

After the present work had been completed, Sharpe, Stodola & Koepsell (1960) confirmed the formation of isomaltulose as a by-product during the synthesis of dextran by enzyme preparations of *L. mesenteroides* (NRRL B-512F).

SUMMARY

1. *Streptococcus bovis*, when grown in a medium containing sucrose, has been shown to produce leucrose, isomaltulose, isomaltose, isomaltotriose, 5-*O*-α-isomaltosyl-D-fructose and isomaltotriulose.

2. The mechanism of the formation of these oligosaccharides is discussed.

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The Action of a *Lactobacillus bifidus* Dextranase on a Branched Dextran

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It has been shown that an extracellular dextranase of a rumen strain of *Lactobacillus bifidus*, when incubated with the essentially unbranched dextran of *Streptococcus bovis*, produces isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose and traces of isomaltoheptaose, but no glucose or isomaltose (Bailey & Clarke, 1959). A study of the action of the *L. bifidus* dextranase on various dextrans revealed that the mixture of oligosaccharides produced varied with the type, and degree of branching, of the dextran (Bailey, Hutson & Weigel, 1960). We have now examined in detail the action of this dextranase on the branched dextran elaborated by *Leuconostoc mesenteroides* (*Betacoccus arabinosaceous*, Birmingham strain).

EXPERIMENTAL

Dextranase. The dextranase was isolated from cell-free culture fluid of a rumen strain of *Lb. bifidus* by one of us (R.W.B.) in the Laboratories of the Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand, according to the method described by Bailey & Clarke (1959).

Dextrans. Leuconostoc mesenteroides (Birmingham strain) dextran was synthesized from sucrose. The dextran was from the same batch as was that used for structural studies of the dextran, which was shown to contain, as well as α-1:6-linkages, 12–15% of α-1:3-branch linkages (Barker, Bourne, Bruce, Neely & Stacey, 1954). A virtually unbranched dextran (Bailey, 1959) was isolated from a sucrose-containing culture of *S. bovis* (strain I) (Bailey & Oxford, 1958).

Paper chromatography and ionophoresis. The solvents used for paper chromatography were: (*a*) the upper layer of ethyl acetate–water–pyridine (2:2:1, by vol.) (Jermyn & Isherwood, 1949); (*b*) ethyl acetate–water–pyridine–acetone (Malpress & Hytten, 1958); (*c*) upper layer of butanol–ethanol–water (4:1:5, by vol.); (*d*) ethyl acetate–acetic acid–water (9:2:2, by vol.); (*e*) butanol–benzene–pyridine–water (5:1:3:2, by vol.). Ionophoresis was carried out at about 50 v/cm. in borate solution, pH 10 (Foster, 1953), and in molybdate solution, pH 5·5 (Bourne, Hutson & Weigel, 1959). It will be noticed that some of the M_S (mobility with respect to sorbitol) values reported here differ slightly from those reported earlier. This is due to the application of smaller quantities, thus allowing a more accurate determination of the rates of migration. In all cases comparison was made with known compounds.

The reagents used for the detection of compounds were: (*a*) silver nitrate in acetone–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950); (*b*) aniline hydrogen phthalate (Partridge, 1949); (*c*) *p*-anisidine–HCl (Hough, Jones & Wadman, 1950); (*d*) aniline–diphenylamine–

phosphoric acid (Schwimmer & Bevenue, 1956); (*c*) triphenyltetrazolium chloride (Feingold, Avigad & Hestrin, 1956).

Dextranase digests. Standard digests were prepared from dextran solution (15 mg. in 2 ml. of water), dextranase solution (5 mg. in 1 ml. of water) and 0·2 M-citrate buffer, pH 5·5 (2 ml.). The digests were incubated under a layer of toluene at 37° for 60 hr.

Reducing sugars. Reducing sugars produced were determined at time intervals by the cuprimetric method of Shaffer & Hartmann (1921). The results were calculated in terms of isomaltotriose (Fig. 1). The solutions were desalted with Bio-Deminrolit, pretreated with carbon dioxide and evaporated to dryness *in vacuo*. The residues were dissolved in water (0·2 ml.) for chromatographic analysis.

RESULTS

Oligosaccharides produced by the action of the dextranase on Leuconostoc mesenteroides *dextran*

The rate of liberation of reducing sugars from *L. mesenteroides* dextran by *Lb. bifidus* dextranase is compared with that from *S. bovis* dextran in Fig. 1. The amounts of reducing sugars released indicates the effect of branching on the enzymic hydrolysis. Paper chromatography in solvent (*b*) of the digest containing *L. mesenteroides* dextran revealed, after 30 hr. incubation, the formation of three oligosaccharides, A, B and C, with $R_{\text{isomaltotriose}}$ (R_{IMT}) values of 0·75, 0·50 and 0·35 respectively, in addition to isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose and isomaltoheptaose, also obtained from *S. bovis* dextran. Glucose, isomaltose and oligosaccharides with a degree of polymerization greater than seven glucose units were absent from the digest containing *S. bovis* dextran. Glucose and isomaltose were also absent from the digest containing *L. mesenteroides* dextran, but a large amount of unresolvable material with $R_{\text{IMT}} < 0{\cdot}20$ was produced.

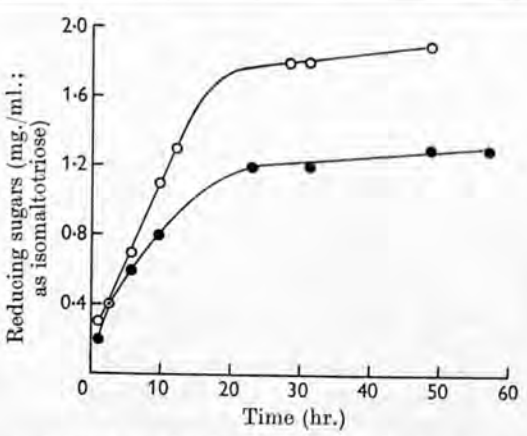

Fig 1. Liberation of reducing sugars in dextran–dextranase digests. Solutions contained dextran (7·5 mg./ml.; 10 ml.), dextranase (5 mg./ml.; 5 ml.) and citrate buffer (0·2 M, pH 5·5; 10 ml.). ○, *S. bovis* dextran; ●, *L. mesenteroides* dextran.

Table 1. *Yields of oligosaccharides produced by the action of* Lb. bifidus *dextranase on* Leuconostoc mesenteroides (*Birmingham strain*) *dextran*

Solutions containing dextran (15 mg./2 ml. of water), dextranase (5 mg./1 ml. of water) and citrate buffer (0·2 M, pH 5·5; 2 ml.) were incubated at 37° for 60 hr. Oligosaccharides were resolved by chromatography in solvent (*b*) and determined by the anthrone method. Grading scheme in visual estimation: + (trace) to + + + + (intense).

	L. mesenteroides dextran (% of recovered material)	*S. bovis* dextran (by visual estimation)
Isomaltotriose	8·8	+ + + +
Isomaltotetraose	9·4	+ + + +
Tetrasaccharide A	3·3	Absent
Isomaltopentaose	11·4	+ + + +
Pentasaccharide B	3·5	Absent
Hexasaccharide fraction	8·6	+ + +
Heptasaccharide fraction	13·1	+
Unresolvable fraction	41·9	Absent

A part of the digest containing *L. mesenteroides* dextran (3·76 mg. of the original dextran; corrected for moisture content) was fractionated by paper chromatography in solvent (*b*). The glucose equivalent of each eluted fraction was determined by the anthrone method (Yemm & Willis, 1954). The results are shown in Table 1. Qualitative results obtained from *S. bovis* dextran are included for comparison.

A digest (500 ml.) containing *L. mesenteroides* dextran (1·90 g.) was incubated under standard conditions. After desalting, the mixture of oligosaccharides was fractionated by paper chromatography in solvent (*b*) on Whatman no. 3 filter paper. Purification of the fractions containing oligosaccharides A and B by the same paper-chromatographic method, followed by desalting and freeze-drying, yielded chromatographically pure oligosaccharide A (30 mg.) and B (30 mg.).

Structural examination of oligosaccharide A. Oligosaccharide A could be detected on paper chromatograms with spray reagents (*a*), (*b*), (*c*) (green spot) and (*d*) (pink spot). During ionophoresis in borate solution (pH 10) it migrated with the same M_G (mobility with respect to glucose) as isomaltotetraose (0·65). When estimated by the Shaffer & Hartmann (1921) method oligosaccharide A (5 mg.) had a reducing power equivalent to 1·15 mg. of glucose. This corresponded to 85·2 % of the calculated value for a tetrasaccharide. It was not possible to determine the ash and moisture contents.

Oligosaccharide A (250 μg.) was heated for 4 hr. at 100° in 1·5 N-HCl (1 ml.) and the solution evaporated to dryness *in vacuo* over solid NaOH to

remove the acid. The hydrolysate contained a single component which was chromatographically identical with glucose. A partial hydrolysate, obtained by heating for 1 hr. at 100° in N-HCl and deionizing with Amberlite IRA-400 (carbonate), was shown by paper chromatography to contain isomaltotriose, isomaltose and glucose.

Oligosaccharide A (12 mg.) was reduced with potassium borohydride (Bragg & Hough, 1957). The solution was deionized by treatment with Amberlite IR-120 (H^+) followed by evaporation and repeated distillation with dry methanol. Oligosaccharide A alcohol was purified and isolated by paper chromatography in solvent (*a*) (R_G 0·32). Ionophoresis in molybdate solution (pH 5·5) (Bourne *et al.* 1959) showed that it migrated with the same M_S as 6-*O*-α-isomaltotriosylsorbitol (0·50).

Oligosaccharide A alcohol was partially hydrolysed by heating for 4 hr. at 100° in 1 % aqueous oxalic acid (5 ml.). The hydrolysate was deionized with Amberlite IRA-400 (carbonate) and fractionated by paper chromatography in solvent (*a*) (see Table 2) into 5 components in addition to unchanged material (about 40 %, visual estimation on chromatograms). Each fraction was subjected to ionophoresis in molybdate solution (pH 5·5), when fractions 3, 4 and 6 were resolved into trisaccharide D and trisaccharide E alcohol, isomaltose and 6-*O*-α-D-glucopyranosylsorbitol, D-glucose and sorbitol respectively. The identity of the products of hydrolysis with the corresponding named compounds was confirmed by ionophoresis in borate solution.

Trisaccharide D, which was immobile during ionophoresis in molybdate solution (pH 5·5), was eluted and, after removal of molybdate with Amberlite IR-120 (H^+) and Amberlite IRA-400 (carbonate), reduced with potassium borohydride (Bragg & Hough, 1957). The solution was deionized as described above. Ionophoresis in molybdate solution (pH 5·5) revealed the presence of trisaccharide F alcohol (M_S 0) and trisaccharide E alcohol (M_S 0·62).

To separate solutions of oligosaccharide A (500 μg.) and isomaltotetraose (500 μg.) in water (1 ml.) was added 0·03 M-sodium periodate (0·5 ml.). After standing for 16 hr. at room temperature the excess of periodate was destroyed with 0·03 M-ethylene glycol (0·5 ml.). Hydrochloric acid was added to give 2 N-HCl solutions. These were heated for 6 hr. at 100° and then evaporated *in vacuo* over solid NaOH. Although paper chromatography in solvent (*a*) of the hydrolysate of oxidized oligosaccharide A revealed an intense spot of glucose, the hydrolysate of oxidized isomaltotetraose was shown to contain only traces of glucose. Measured portions of the hydrolysates (containing 250 μg. of original oligosaccharides) were fractionated by paper chromatography in solvent (*a*). The components corresponding to glucose were eluted with water and determined by the benzidine method (Jones & Pridham, 1954), with glucose as a standard. The yields of glucose from oligosaccharide A and isomaltotetraose corresponded to 27 % and 5 % respectively of the glucose present in a tetrasaccharide containing glucose only.

Structural examination of oligosaccharide B. The methods were as for oligosaccharide A. Oligosaccharide B could be detected on paper chromatograms with spray reagents (*a*), (*b*), (*c*) (green spot) and (*d*) (pink spot). During ionophoresis in borate solution (pH 10) it migrated with the same M_G as isomaltopentaose. Complete hydrolysis gave a single reducing compound which was chromatographically identical with glucose, whereas a partial hydrolysate was shown to contain, by paper chromatography in solvents (*a*) and (*b*), isomaltotetraose, isomaltotriose, isomaltose and glucose. Oligosaccharide B alcohol migrated during ionophoresis in molybdate solution (pH 5·5) with the same M_S as 6-*O*-α-isomaltotetraosylsorbitol (0·44). A partial acid hydrolysate of oligosaccharide B alcohol showed, on chromato-

Table 2. *Fractionation of products from partial hydrolysis of oligosaccharide A alcohol*

Paper chromatography in solvent (*a*)		Ionophoresis in molybdate solution	
Fraction no.	R_G	M_S	Identity
1	0·32	0·50	Oligosaccharide A alcohol
2	0·45	0·60	6-*O*-α-Isomaltosylsorbitol
3	0·55	0	Trisaccharide D
		0·60	Trisaccharide E alcohol
4	0·75	0	Isomaltose
		0·75	6-*O*-α-D-Glucopyranosylsorbitol
5	0·85	0	Nigerose
6	1·0	0	Glucose
		1·0	Sorbitol

graphy in solvents (*a*) and (*d*), in addition to unchanged material (about 50 %, visual estimation), intense spots corresponding to glucose and 6-*O*-α-isomaltotriosylsorbitol. Only very small traces of isomaltose, 6-*O*-α-D-glucopyranosylsorbitol and sorbitol were detectable.

When oligosaccharide B (500 μg.) was oxidized with sodium periodate and hydrolysed in the same way as oligosaccharide A and isomaltotetraose, the yield of glucose corresponded to 24 % of the glucose present in a pentasaccharide containing only glucose.

Action of the dextranase on 6-O-α-*isomaltohexaosyl- and* 6-O-α-*isomaltoheptaosyl-sorbitol*

6-*O*-α-Isomaltohexaosylsorbitol (8 mg.) and 6-*O*-α-isomaltoheptaosylsorbitol (8 mg.), prepared by reduction of isomaltoheptaose and isomaltooctaose respectively with potassium borohydride, were separately dissolved in 0·25 M-citrate buffer (pH 5·5) (1 ml.) and incubated with dextranase (4 and 6 mg. respectively) for 26 hr. at 37°. Paper ionophoresis in molybdate solution of the deionized digest containing 6-*O*-α-isomaltohexaosylsorbitol revealed, in addition to non-migrating material, the presence of two components which migrated at the same rate as 6-*O*-α-isomaltosyl- and 6-*O*-α-isomaltotriosyl-sorbitol respectively. The non-migrating material was eluted from the paper and deionized. Paper chromatography in solvent (*a*) revealed components corresponding to isomaltotriose and isomaltotetraose. 6-*O*-α-Isomaltosylsorbitol, 6-*O*-α-isomaltotriosylsorbitol, 6-*O*-α-isomaltotetraosylsorbitol, isomaltotriose, isomaltotetraose and isomaltopentaose were identified in a similar manner in the digest containing 6-*O*-α-isomaltoheptaosylsorbitol. Visual examination of the paper ionograms and chromatograms suggested that all the products were present in approximately equal quantities.

DISCUSSION

The evidence presented shows that oligosaccharide A is a reducing tetrasaccharide of glucose. The results of the partial acid hydrolysis of tetrasaccharide A and the reduction product showed the presence of an isomaltotriose unit, together with another glucose unit joined by a linkage of a more labile type. One glucose unit was shown to be resistant to oxidation by periodate. This demonstrates the presence of one 1:3-glucosidic linkage in tetrasaccharide A. As the 1:3- as well as the 1:6-glucosidic linkages in dextran are of the α-type it is reasonable to assume that the 1:3-glucosidic linkage in tetrasaccharide A also is an α-1:3-linkage.

There are four possible structures (I–IV) (Fig. 2) for a tetrasaccharide containing an isomaltotriose unit to which a fourth glucose unit is joined by an α-1:3-glucosidic linkage. The results of periodate oxidation eliminate structures (III) and (IV). The migration of tetrasaccharide A alcohol during ionophoresis in molybdate solution conclusively eliminates structures (III) and (IV) because a 3-*O*-substituted sorbitol does not form a complex with molybdate (Bourne *et al.* 1959). This migration and the reaction of tetrasaccharide A with triphenyltetrazolium chloride also eliminate the possibility of substitution at $C_{(4)}$- and $C_{(2)}$-hydroxyl groups respectively of the reducing glucose unit.

The structure of tetrasaccharide A was elucidated further by a partial hydrolysis of the reduction product (V or VI) (Fig. 3). Products which would be expected from both structures, namely 6-*O*-isomaltosylsorbitol (VII), isomaltose (XII), 6-*O*-α-D-glucopyranosylsorbitol (XIII), nigerose (XIV), glucose (XV) and sorbitol (XVI), were identified by paper chromatography and ionophoresis. As would be expected if (VI) were present, two trisaccharide alcohols were obtained (Table 2). One of these (fraction 2) was identical with 6-*O*-isomaltosylsorbitol (VII), the only trisaccharide alcohol expected from (V). The other, trisaccharide E alcohol, must have structure (IX), which is consistent with its M_8.

The possible structures of the reducing trisaccharide D of fraction 3 are (VIII) and (X). Ionophoresis in molybdate solution (pH 5·5) of the reduced trisaccharide D revealed trisaccharide E alcohol (IX) and a non-migrating trisaccharide F alcohol. As 3-*O*-substituted sorbitols do not migrate, trisaccharide F alcohol must have structure (XI), and hence trisaccharide D must have contained both (VIII) and (X).

These results show that tetrasaccharide A is a mixture of 3^3-glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II).

The results obtained with oligosaccharide B provide evidence that it is a reducing pentasaccharide B containing an isomaltotetraose unit to which a fifth glucose unit is joined by a 1:3-

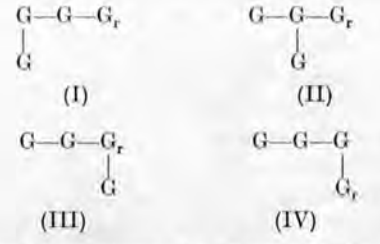

Fig. 2. Possible structures of a tetrasaccharide containing an isomaltotriose unit and a fourth glucosyl unit joined by an α-1:3-link. —, α-1:6-link; |, α-1:3-link; G , reducing glucose unit; G, glucosyl unit.

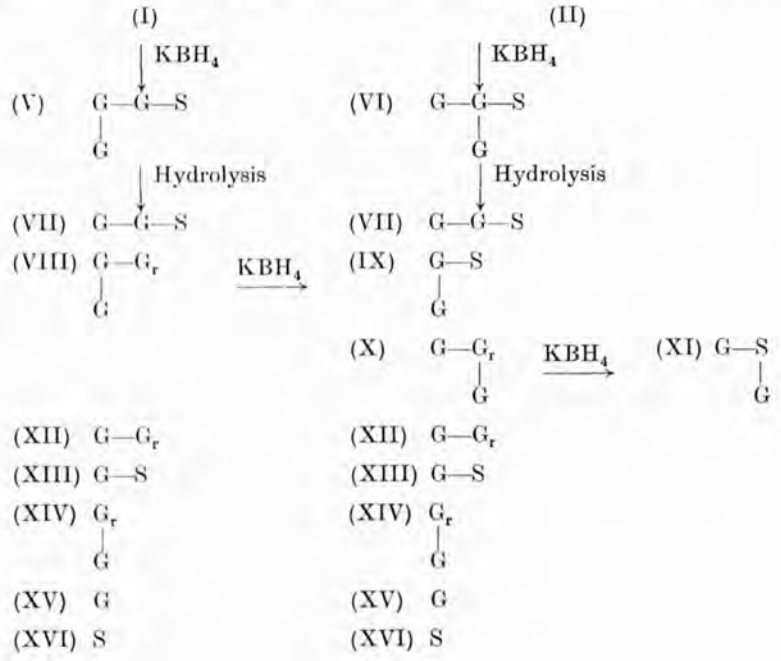

Fig. 3. Products of reduction-hydrolysis of tetrasaccharides (I) and (II). —, α-1:6-link; |, α-1:3-link, G_r, reducing glucose unit; G, glucosyl unit; S, sorbitol.

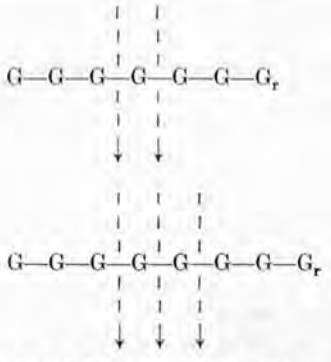

Fig. 4. Action of *Lb. bifidus* dextranase on isomaltodextrins, —, α-1:6-Link; G_r, reducing glucose unit; G, glucosyl unit; - - →, alternative points of hydrolysis.

glucosidic linkage similar to that in (I) or (II). The fact that tetrasaccharide A is a mixture of 3^3-glucosylisomaltotriose and 3^2-glucosylisomaltotriose suggests that pentasaccharide B is also a mixture of pentasaccharides with these types of structures.

Tetrasaccharides (I) and (II) and pentasaccharide B could have arisen by enzymic hydrolysis of *L. mesenteroides* dextran or by chemical or enzymic transglucosylation action on isomaltodextrins. The latter possibility is unlikely as, in this case, they would be expected to have been produced in the control digest containing *S. bovis* dextran. The presence of a 1:3-glucosidic linkage in tetrasaccharides (I) and (II) and pentasaccharide B indicates that the enzyme cannot hydrolyse the 1:3-linkage. We believe that the present work is the first occasion in which oligosaccharides containing the branch linkages of the original dextran have been found.

Bailey & Clarke (1959) established that the action of *Lb. bifidus* dextranase involves random hydrolysis of the dextran chain and that the smallest isomaltodextrin readily hydrolysed is isomaltoheptaose. The action of the enzyme on 6-*O*-α-isomaltohexaosyl- and 6-*O*-α-isomaltoheptaosylsorbitol shows that it has no preference for a particular type of chain end, a result which is in accordance with the properties of an endopolysaccharase. It is probable, therefore, that the dextranase hydrolyses dextran or an isomaltodextrin containing not less than seven glucose units at a point not less than three glucosidic linkages from a chain end, as shown in Fig. 4.

The production of 3^3-glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II) and pentasaccharide B suggests that this type of hydrolysis of dextran by the dextranase is not inhibited by the presence of 1:3-branch linkages. The structure of these products and the absence from the digest of oligosaccharides in which the reducing glucose unit is substituted at $C_{(3)}$ suggests that the α-1:6-link on the reducing side of the branch point of the main dextran chain is resistant to hydrolysis by the dextranase. The branching of *L. mesenteroides* dextran has been reported to occur once in each 6 or 7 anhydroglucose units (Barker *et al.* 1954). It can be calculated that dextrans of regular structures

with branch links on every sixth, seventh and eighth anhydroglucose unit respectively will yield, on hydrolysis with *Lb. bifidus* dextranase, oligosaccharides in unequal proportions. The approximately equal quantities of isomalto-triose, -tetraose and -pentaose produced indicate that the branching of this dextran is random. The presence in the digest of much material containing more than six glucosyl units is further support for a random branching of the dextran molecule.

It is significant that the 'branching' in tetrasaccharide A and pentasaccharide B consists of only one glucosyl unit. 3^3-Glucosylisomaltotriose (I) and 3^2-glucosylisomaltotriose (II) could arise from a part of the dextran molecule where the branching was a single glucosyl unit, or from parts of a randomly branched dextran molecule. Bovey (1959), on the grounds of physical measurements, has suggested that 80% of the branches in *L. mesenteroides* (NRRL-B512) dextran consist of only one glucosyl unit.

SUMMARY

1. *Lactobacillus bifidus* dextranase has been shown to hydrolyse *Leuconostoc mesenteroides* (Birmingham strain) dextran to a complex mixture of oligosaccharides.

2. In addition to isomalto-triose, -tetraose, -pentaose, -hexaose and unresolvable material of degree of polymerization greater than 6, a tetrasaccharide (A) and a pentasaccharide (B) were isolated.

3. Tetrasaccharide (A) was shown to be a mixture of 3^3-glucosylisomaltotriose and 3^2-glucosylisomaltotriose. Pentasaccharide (B) contained one glucose unit joined through a 1:3-linkage to a glucose unit, other than the reducing one, of isomaltotetraose and is probably a mixture of isomers.

4. The dextranase has been shown to hydrolyse 6-*O*-α-isomaltohexaosyl- and 6-*O*-α-isomaltoheptaosyl-sorbitol at two and three alternative glucosidic linkages respectively.

5. The implications of the results, as far as dextranase action and dextran structure are concerned, have been discussed.

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Studies on Dextrans and Dextranases

2. THE ACTION OF MOULD DEXTRANASES ON MODIFIED ISOMALTODEXTRINS AND THE EFFECT OF ANOMALOUS LINKAGES ON DEXTRAN HYDROLYSIS*

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Studies on the degree and types of branching of various dextrans have been carried out by a variety of chemical methods, e.g. methylation, periodate oxidation and cuprammonium complex formation (Neely, 1960). There has been, however, no systematic attempt to study the structures of dextrans by enzymic methods such as those used by Manners (1959) and Bines & Whelan (1960) for glycogens and starches. Dextranases have been obtained from a variety of sources. Exodextranases have been obtained mostly from animal sources, e.g. various types of mammalian tissue, the intestine of *Helix pomatia* and hog intestine. Mould and bacterial sources usually yield endodextranases. The field has been fully reviewed up to 1959 by Fischer & Stein (1960).

Bailey, Hutson & Weigel (1961) reported a detailed study of the action of *Lactobacillus bifidus* dextranase on the branched dextran of *Leuconostoc mesenteroides* (Birmingham strain). We have now examined the mechanism of action of two mould dextranases.

The *Penicillium* dextranases have been chosen for further study because, when the moulds are grown in media containing dextran, culture filtrates containing very high endodextranase activities are readily obtainable. Inducible production of dextranase by certain of these moulds when grown on *L. mesenteroides* (NRRL B-512) dextran was first noted by Tsuchiya, Jeanes, Bricker & Wilham (1952). The resulting extracellular dextranases were shown to cleave dextran randomly to glucose, isomaltose and isomaltotriose. Highly branched dextrans were not readily hydrolysed by these preparations, but no detailed studies were reported.

The availability of essentially unbranched dextran from *Streptococcus bovis* (Bailey, 1959) presented the possibility of inducing the production of a mould dextranase virtually uncontaminated by other carbohydrases, as the substrate contained only α-1→6-linkages. This has in fact been realized and the production, properties and action of two mould dextranases on oligosaccharides and branched dextrans are now reported.

EXPERIMENTAL

Organisms. Streptococcus bovis (strain I), used for the production of virtually unbranched dextran, was obtained from the National Institute for Research in Dairying, Shinfield, Reading. *Penicillium lilacinum* [strains I.M.I. 27830 (NRRL 895) and I.M.I. 79197 (NRRL 896)] and *Penicillium funiculosum* [strains I.M.I. 79195 (NRRL 1132) and I.M.I. 40235 (NRRL 1768)] were obtained from the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey.

Dextrans. Most dextrans were from our Departmental Collection. Others were isolated from cultures on sucrose of *Leuconostoc mesenteroides*, the particular strains of which were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. *S. bovis* dextran, used as substrate for the moulds, was isolated from a 16 l. culture as described by Bailey & Oxford (1958).

Dextranase production. The moulds were inoculated into a

* Part 1: Bailey, Hutson & Weigel (1961).

sterile medium containing *S. bovis* dextran (1%, w/v) and Marmite Co. yeast extract (1%, w/v). The cultures were shaken at 28° for 4 days. Each mould was taken through five subcultures of the same composition. The washed mycelia were then introduced into six 200 ml. batches of media in Erlenmeyer flasks and shaken at 28° for 6 days. Solid material was removed by centrifuging at 3000*g*. The culture fluids were then made 5 mM with respect to sodium citrate, pH 6·0, and dialysed against four changes of 5 mM-sodium citrate, pH 6·0, at 0°. The fluids were then centrifuged at 6000*g* and freeze-dried to brown powders. Yields were about 5 g./1·2 l. of culture fluid.

Dextranase activities. The method used was similar to that of Tsuchiya *et al.* (1952) except that digests were carried out in 0·1 M-sodium citrate buffer and 1 mg. of enzyme preparation was used instead of 1 ml. of culture filtrate. Reducing powers were determined by the method of Shaffer & Hartmann (1921), with isomaltose as a standard. One unit of enzyme is defined as the amount that will produce 1 m-mole of isomaltose monohydrate from *S. bovis* dextran in 1 hr. at 40° under the conditions described above.

Dextran–dextranase digests. Digests were prepared from dextran solutions (100 mg. in 10 ml. of water), 0·2 M-sodium citrate buffer, pH 5·0 (20 ml.), and dextranase (various amounts in 10 ml. of water), and incubated at 37° under toluene.

Variations of dextranase activities with digest conditions were measured at the above concentrations, varying the pH of the buffer and the incubation temperature. Liberation of reducing sugar was measured by the method of Shaffer & Hartmann (1921).

Oligosaccharides. Isomaltodextrins, i.e. oligosaccharides of the isomaltose series, were isolated from a partial hydrolysate of *S. bovis* dextran according to the method of Turvey & Whelan (1957). Isomaltodextrinols, i.e. oligosaccharides of the isomaltose series in which the reducing glucose unit is converted into sorbitol, were made by reduction of the corresponding isomaltodextrins with potassium borohydride (potassium tetrahydroborate) (Bragg & Hough, 1957). 6-*O*-α-Isomaltotriosylfructose (isomaltotetraulose) was made by the epimerization of isomaltotetraose according to the method of Avigad (1959). Other oligosaccharides were from our Departmental Collection, except methyl α-isomaltotrioside for which we are indebted to Dr A. Jeanes. The purity of the carbohydrates was checked by paper chromatography.

Oligosaccharide–dextranase digests. Oligosaccharide (about 10 mg.) in 0·2 M-citrate buffer, pH 5·0 (1 ml.), was incubated with dextranase (2 mg. in 1 ml. of water) at 37° for 16 hr. Digests were de-ionized with Amberlite resin IR-120 (H^+ form) followed by Amberlite resin IR-45 (OH^- form), concentrated in a vacuum desiccator and analysed by paper chromatography and ionophoresis in molybdate solution.

Paper chromatography. The upper layer of an ethyl acetate–pyridine–water mixture (2:1:2, by vol.) was used as a solvent. Compounds were detected with acetone–silver nitrate–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950). Urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955) was used for the detection of ketoses. Aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956) was used for the detection of, and distinction between, oligosaccharides with a maltose- (blue spot) and an isomaltose-type reducing group (green spot).

Paper ionophoresis. Ionophoresis was conducted in 0·1 M-molybdate, pH 5·5 (Bourne, Hutson & Weigel, 1959).

RESULTS

Production of dextranase by strains of Penicillium lilacinum *and* Penicillium funiculosum. Two strains of *P. lilacinum* [I.M.I. 27830 (NRRL 895) and I.M.I. 79197 (NRRL 896)] and of *P. funiculosum* [I.M.I. 79195 (NRRL 1132) and I.M.I. 40235 (NRRL 1768)] grew readily in media containing sucrose, the culture fluid exhibiting a very weak dextranase activity. After subculture into media containing *S. bovis* dextran, in which the moulds also grew readily, high dextranase activity was exhibited by the filtrates of the culture fluids. The activity increased to a maximum after incubation for 5 days and did not change in a further four subcultures. However, it dropped to its original very low level when the moulds were again grown in media containing sucrose.

Four freeze-dried dextranase preparations were obtained and their activities (see the Experimental section) determined. The results are shown in Table 1.

Action of dextranases on Streptococcus bovis *dextran.* Digests were incubated for 20 hr., de-ionized and analysed by paper chromatography. Components with R_G values identical with those of glucose, isomaltose and isomaltotriose only were detected. Jeanes, Wilham, Jones, Tsuchiya & Rist (1953) characterized these compounds and found them to be the main products when *P. funiculosum* (NRRL 1132, i.e. I.M.I. 79195) dextranase hydrolysed *L. mesenteroides* (NRRL B-512) dextran. The liberation of reducing sugar with time in a standard digest was followed and is shown in Fig. 1. No significant increase of reducing sugars was observed after about 3 hr., except with *P. funiculosum* (I.M.I. 40235) dextranase.

Effect of pH *and temperature on dextranase activity.* Standard digests, containing *S. bovis* dextran, were incubated for 0·5 hr., except those of

Table 1. *Activities of dextranase preparations*

Experimental details are given in the text.

Dextranase	Organism	Activity (units/mg.)
A	*P. lilacinum* (I.M.I. 79197; NRRL 896)	2·0*
B	*P. funiculosum* (I.M.I. 79195; NRRL 1132)	1·1
C	*P. lilacinum* (I.M.I. 27830; NRRL 895)	1·8
D	*P. funiculosum* (I.M.I. 40235; NRRL 1768)	0·2

* The activity of the culture fluid from which the dextranase preparation was isolated was at least 8·3 units/ml. (based on the volume and weight-yield). The activity of the culture fluid, when the organism was grown in a medium containing sucrose, was 0·90 unit/ml.

P. funiculosum (I.M.I. 40235), which were incubated for 3 hr., at various temperatures and pH values. The hydrolysis was then stopped by boiling for 1 min. and the reducing powers of the solutions were measured. The *P. lilacinum* dextranases possessed maximum activity at pH 4·5–5·5 and 45–50°, and *P. funiculosum* dextranases at pH 4·3–5·0 and 45–50°. The results are in agreement with those of Tsuchiya *et al.* (1952). The dextranases of the two strains of each mould did not seem to differ appreciably from each other, and therefore the dextranase of one strain of each mould was selected for further study. These were dextranase A of *P. lilacinum* (I.M.I. 70197) and dextranase B of *P. funiculosum* (I.M.I. 79195).

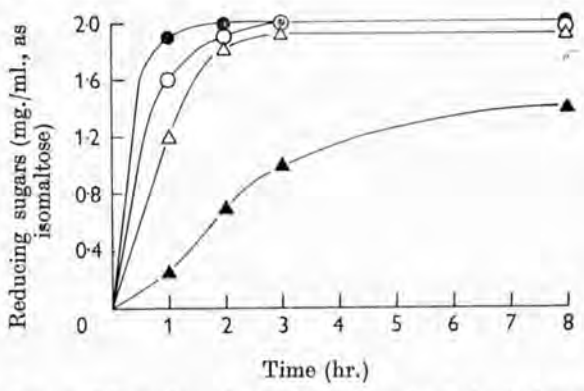

Fig. 1. Liberation of reducing sugars in *S. bovis* dextran–dextranase digests. Identical solutions containing *S. bovis* dextran (about 10 mg./ml.; 10 ml.); 0·2 M-citrate buffer, pH 5·0 (20 ml.), and dextranase (10 mg./ml.; 10 ml.) were incubated at 37°. ○, *P. lilacinum* (I.M.I. 27830) dextranase; ●, *P. lilacinum* (I.M.I. 79197) dextranase; △, *P. funiculosum* (I.M.I. 79195) dextranase; ▲, *P. funiculosum* (I.M.I. 40235) dextranase.

Table 2. *Relative molar yields of glucose, isomaltose and isomaltotriose from isomaltodextrins and dextran on hydrolysis with dextranases A and B*

Substrates were digested with enzymes under standard conditions, and the products separated by paper chromatography and determined with anthrone. Experimental details are given in the text.

Substrate	Products: Glucose	Products: Isomaltose	Products: Isomaltotriose
Dextranase A on:			
Isomaltotetraose	16	100	18
Isomaltopentaose	27	100	79
Isomaltohexaose	23	100	47
S. bovis dextran	20	100	59
Dextranase B on:			
Isomaltotetraose	18	100	10
Isomaltopentaose	23	100	41
Isomaltohexaose	33	100	46
S. bovis dextran	38	100	46

Action of the dextranases on isomaltodextrins. The pattern of hydrolysis of isomaltodextrins was studied by incubation with the dextranases under standard conditions. The products (glucose, isomaltose and isomaltotriose) were fractionated by paper chromatography (Lederer & Lederer, 1957) and their relative molar yields determined with anthrone (Yemm & Willis, 1954). The results are shown in Table 2, where they are compared with those obtained from *S. bovis* dextran. The only significant difference between the activities of dextranases A and B seemed to be that the latter produced slightly more glucose and less isomaltotriose from isomaltodextrins than the former. Two digests were prepared containing isomaltotriose (10 mg.) in 0·1 M-citrate buffer, pH 5·0 (2 ml.), and dextranase (10 mg.), and incubated at 37° for 3 days. De-ionization and analysis by chromatography revealed that slow hydrolysis to isomaltose and glucose had occurred. This hydrolysis had proceeded further with dextranase B than with dextranase A. Elution of sugars from chromatograms and determination with anthrone revealed that, under those conditions, dextranase A hydrolysed about 15% of the isomaltotriose, and dextranase B about 30%.

Action of the dextranases on isomaltodextrinols. Isomaltodextrinols were digested under standard conditions; the products were fractionated by paper chromatography and subjected to ionophoresis in molybdate solution, when reducing and non-reducing sugars were quickly separated (Bourne, Hutson & Weigel, 1961). The results are shown in Table 3. Visual estimation of the chromatograms and ionophoretograms showed that approximately equal quantities of the alcohols and reducing sugars of a particular degree of polymerization were formed. Dextranases A and B appeared to hydrolyse the substrates in the same manner.

Table 3. *Products of the action of dextranases A and B on isomaltodextrinols*

Incubations were carried out under standard conditions for 16 hr., and the products separated by chromatography and ionophoresis in molybdate solution. Experimental details are given in the text.

Isomaltodextrinol	Products
Isomaltitol	Unchanged isomaltitol
Isomaltotri-itol	Unchanged isomaltotri-itol and traces of isomaltitol, isomaltose, glucose and sorbitol
Isomaltotetraitol	Isomaltose, isomaltitol and traces of glucose and sorbitol
Isomaltopentaitol	Isomaltotriose, isomaltotri-itol, isomaltose, isomaltitol and traces of glucose and sorbitol
Isomaltohexaitol	Isomaltotriose, isomaltotri-itol, isomaltose, isomaltitol and traces of glucose and sorbitol

Action of the dextranases on other modified isomaltodextrins. Four oligosaccharides were incubated with dextranases under standard conditions. The products were identified by chromatography with the silver nitrate and aniline–diphenylamine reagents. Ionophoresis in molybdate solution, a specific test for 6-*O*-substituted fructoses (Bourne, Hutson & Weigel, 1960), and the urea–phosphoric acid spray were used to detect isomaltulose in the hydrolysate of isomaltotetraulose. It was not evident, from chromatographic analyses, that the pentasaccharide was cleaved preferentially to a particular mixture of di- and tri-saccharide. The results are shown in Table 4.

Action of the dextranases on branched dextrans. Dextranases A and B were separately incubated under standard conditions with a number of dextrans of various types and degrees of branching. All the dextrans (except *S. bovis* dextran as control) were from various strains of *L. mesenteroides*. The reducing sugar liberated was measured as isomaltose at intervals over 10 hr. No significant increase of reducing sugar was observed after incubation for about 3 hr. The average values obtained after incubation for a further 7 hr. are shown in Table 5.

Table 4. *Products of the action of dextranases A and B on isomaltodextrins containing anomalous linkages and moieties*

Experimental details are given in the text.

Modified 'isomaltodextrin'	Products of dextranase action*
6-*O*-α-Isomaltotriosyl-fructose	Isomaltose, isomaltulose
4-*O*-α-Isomaltotriosylglucose	Isomaltose, maltose
4-*O*-α-Isomaltotetraosyl-glucose	Isomaltotriose, panose, isomaltose, maltose
Methyl α-isomaltotrioside	Isomaltose, methyl α-glucoside†

* Traces of glucose were present in the products; however, only the main hydrolysis products, as revealed by the aniline–diphenylamine spray, are shown above.

† Slow reaction with silver nitrate reagent.

DISCUSSION

Production of extracellular dextranases by strains of *P. lilacinum* and *P. funiculosum* is induced when the moulds are grown in media containing the virtually unbranched *S. bovis* dextran. Their general properties do not seem to differ from the preparations elaborated in media containing the branched dextran of *L. mesenteroides* (NRRL B-512) (Tsuchiya *et al.* 1952).

The products of the action of the dextranases on *S. bovis* dextran are glucose, isomaltose and isomaltotriose, isomaltose being the main product. This suggests that isomaltotetraose is the smallest readily hydrolysed isomaltodextrin. This has been shown to be the case, the molecule being hydrolysed principally at the central glycosidic linkage to give isomaltose.

The composition of the hydrolysate of isomaltopentaitol shows that the two non-terminal glycosidic linkages are hydrolysed at similar rates and much faster than the terminal linkages. Likewise the non-terminal glycosidic linkages of the isomaltohexaitol are readily hydrolysed. It can be assumed that the same is true for isomaltopentaose and isomaltohexaose.

When the reducing glucose unit of isomaltotetraose was replaced by sorbitol (6-*O*-α-isomaltotriosylsorbitol), fructose (6-*O*-α-isomaltotriosylfructose), an α-1→4-linked glucose unit (4-*O*-α-isomaltotriosylglucose) or a methyl group (methyl α-isomaltotrioside), hydrolysis still occurred at approximately the same rate and there was no change in the pattern of the hydrolysis. This shows that the dextranases have no preference for a

Table 5. *Liberation of reducing sugars from various dextrans by dextranases A and B*

Incubations were carried out under standard conditions for 10 hr. Experimental details are given in the text.

Origin of dextran	Percentage of linkage present				Percentage of reducing sugar liberated by dextranases (as isomaltose)	
	1→6	1→4	1→3	1→2	Dextranase A	Dextranase B
*S. bovis**	100	—	—	—	96·6	96·6
L. mesenteroides (Birmingham; NRRL B-1375) modified†	98	—	2	—	86·4	—
L. mesenteroides (NRRL B-512)‡	95	—	5	—	87·9	87·5
L. mesenteroides (NRRL B-1383)‡	84	16	—	—	46·3	—
L. mesenteroides (Birmingham; NRRL B-1375)‡	81	6	13	—	44·4	49·2
L. mesenteroides (NCIB 2706)‡	79	3	18	—	22·7	22·7
L. mesenteroides (NRRL B-742)‡	67	21	12	—	12·5	17·4
L. mesenteroides (NRRL B-1399)‡§	65	6	—	29	36·6	—

* Bailey (1959).

† Barker, Bourne, James, Neely & Stacey (1955).

‡ Jeanes *et al.* (1954).

§ Scott, Hellman & Senti (1957).

particular type of chain-end, a result which is in accordance with the properties of endopolysaccharases. It thus seems that the essential requirements for ready hydrolysis to occur are realized in methyl α-isomaltotrioside, where the terminal glucosidic linkage at the 'reducing end' is merely an α-glucosidic linkage. It is therefore probable that the dextranases hydrolyse readily virtually unbranched dextran, isomaltodextrins or modified isomaltodextrins containing not less than three glucosidic linkages, the hydrolysis being principally at glucosidic linkages other than the terminal ones, as shown in Fig. 2.

Small amounts of glucose were found in all cases when dextran or isomaltodextrins were acted on by the dextranases. Similarly, traces of glucose and sorbitol were found when isomaltodextrinols were hydrolysed. As expected from these results, isomaltotriose was hydrolysed only very slowly. Whereas Walker & Whelan (1960) found that starch and maltotetraose represent the two extremes of molecular size on which salivary α-amylase acts rapidly, the extreme molecular sizes for the dextranases are represented by dextran and methyl α-isomaltotrioside. The marked difference in rate of hydrolysis between methyl α-isomaltotrioside and isomaltotriose might be due to such properties as mutarotation of reducing sugars, the electronic structures of the substituents on the terminal glucosidic oxygen, or both.

Anomalous linkages in natural dextrans usually occur as branch linkages and it is likely that they will affect the pattern of enzymic hydrolysis. Table 5 shows that the final amount of reducing sugar liberated is markedly lowered with increasing degree of branching. This indicates that the products from a branched dextran are of greater average molecular size than those from an unbranched dextran. It is likely that these limit dextrins contain the anomalous linkages and will be formed for two reasons: (i) the dextranases are incapable of hydrolysing the anomalous linkages and (ii) the anomalous linkage renders a neighbouring region in the dextran molecule incapable of being hydrolysed by the dextranases. Analysis of the limit dextrins containing the anomalous linkages would aid both structural studies of dextrans and investigations of the mechanism of dextranase action.

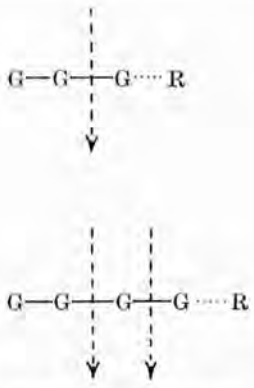

Fig. 2. Action of dextranases on dextran, isomaltodextrins and modified isomaltodextrins. ——, α-1→6-link; · · ·, α-link; G, glucosyl unit; R, glucose, fructose, sorbitol or methyl group; - - - →, principal points of hydrolysis.

SUMMARY

1. Extracellular dextranases are adaptively produced by strains of *Penicillium lilacinum* and *P. funiculosum* grown in media containing the virtually unbranched *Streptococcus bovis* dextran.

2. The products of the action of the dextranases on *S. bovis* dextran are glucose, isomaltose and isomaltotriose, isomaltose being the main product.

3. The dextranases readily hydrolyse virtually unbranched dextran, isomaltodextrins or modified isomaltodextrins containing not less than three glucosidic linkages, the hydrolysis being principally at glucosidic linkages other than the terminal ones.

4. The number of reducing groups liberated from various dextrans by the dextranases is markedly lowered with increasing degree of branching of the dextran molecule.

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Studies on Dextrans and Dextranases

3. STRUCTURES OF OLIGOSACCHARIDES FROM *LEUCONOSTOC MESENTEROIDES* (BIRMINGHAM) DEXTRAN*

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It has been shown that the adaptively produced dextranases of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) and of *P. funiculosum* (I.M.I. 79195; NRRL 1132) hydrolyse the virtually unbranched dextran of *Streptococcus bovis* to give mainly isomaltose and that the degree of hydrolysis of dextrans possessing anomalous linkages depends on the percentage of such linkages in the dextrans (Bourne, Hutson & Weigel, 1962). This suggested that the dextranases could not hydrolyse the anomalous linkages of branched dextrans. Isolation of the oligosaccharides which cannot be hydrolysed by the dextranases should aid the structural analysis of dextrans and also elucidate further the mechanism of dextranase action. An analysis of the structures of 'branched' oligosaccharides produced by the action of the dextranase of *Lactobacillus bifidus* (Bailey & Clarke, 1959) on

* Part 2: Bourne, Hutson & Weigel (1962).

Leuconostoc mesenteroides (Birmingham) dextran has already been made (Bailey, Hutson & Weigel, 1961). The smallest 'branched' oligosaccharides produced were 3^3-glucosylisomaltotriose and 3^2-glucosylisomaltotriose. We have now examined the oligosaccharides produced by the action of the dextranases from the two moulds on the same dextran.

EXPERIMENTAL

Dextranases. The dextranase preparations of *P. lilacinum* (I.M.I. 79197; NRRL 896) (dextranase A) and *P. funiculosum* (I.M.I. 79195; NRRL 1132) (dextranase B) used in the present investigation were as described by Bourne *et al.* (1962).

Dextran. L. mesenteroides (Birmingham) dextran was from the same batch as was that used for structural studies and was shown to contain 12–15% of α-(1→3)-linkages (Barker, Bourne, Bruce, Neely & Stacey, 1954).

Dextranase digests. Digests contained dextran (2%, w/v) and dextranase preparation (0·2%, w/v) in 0·1 M-sodium citrate, pH 5·0. The digests were incubated under toluene for 16 hr. at 37°. The final number of reducing groups liberated was the same when the ratio of dextran to dextranase preparation was 10:1, 4:1 or 2:1 (by weight).

Paper chromatography and ionophoresis. The solvents used for paper chromatography were: (*a*) the upper layer of ethyl acetate–water–pyridine (2:2:1, by vol.) (Jermyn & Isherwood, 1949); (*b*) ethyl acetate–water–pyridine–acetone (Malpress & Hytten, 1958). Ionophoresis was carried out at about 50 v/cm. in molybdate solution, pH 5·5 (Bourne, Hutson & Weigel, 1961), and in borate solution, pH 10 (Foster, 1953). Some of the M_S (mobility with respect to sorbitol) values reported here differ slightly from those reported previously. This is due to the application of smaller quantities, thus allowing a more accurate determination of the rates of migration. In all cases comparison was made with known compounds.

The reagents used for the detection of compounds were: (*a*) silver nitrate in acetone–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950); (*b*) aniline hydrogen phthalate (Partridge, 1949); (*c*) *p*-anisidine–HCl (Hough, Jones & Wadman, 1950); (*d*) aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956); (*e*) triphenyltetrazolium chloride (Feingold, Avigad & Hestrin, 1956).

Borohydride reduction. Aqueous solutions of the sugars (about 1%, w/v) were mixed with an equal volume of potassium borohydride (tetrahydroborate) solution (1%, w/v) and allowed to stand overnight. The solutions were then de-ionized by treatment with Amberlite IR-120 (H^+ form) followed by repeated distillation with anhydrous methanol. The sugar alcohols were always purified by paper chromatography.

Degree of polymerization of oligosaccharides. The degree of polymerization of the oligosaccharides was determined by the following methods: (*a*) measurement of R_M values, $\log[1/(R_G - 1)]$, where R_G is the distance travelled compared with glucose; (*b*) comparison of M_S values (Bourne *et al.* 1961) of the reduced oligosaccharides with those of the reduced oligosaccharides of the isomaltose series; (*c*) determination of reducing power relative to glucose (Shaffer & Hartmann, 1921), with corrections for carbohydrate content; the carbohydrate content was determined by acid hydrolysis and measurement of the reducing sugar produced (Shaffer & Hartmann, 1921), by applying the correction of Pirt & Whelan (1951) for the degradation of glucose by acid; (*d*) comparison of anthrone values before and after reduction with potassium borohydride (Peat, Whelan & Roberts, 1956).

RESULTS

Oligosaccharides produced by the action of dextranases A and B on Leuconostoc mesenteroides *(Birmingham) dextran*

Digests containing *L. mesenteroides* (Birmingham) dextran were separately incubated with dextranases A and B. Paper chromatography in solvent (*a*) of the digest with dextranase A revealed the presence of components with R_G values identical with those of glucose, isomaltose, isomaltotriose and oligosaccharides which had slightly higher R_G values than isomalto-pentaose, -hexaose, -heptaose and -octaose. The digest with dextranase B contained, in addition to the above, a component which had a slightly higher R_G than that of isomaltotetraose. Unresolvable material with R_G smaller than that of isomalto-octaose was present in both digests. As glucose, isomaltose and isomaltotriose are the only products when the dextran-

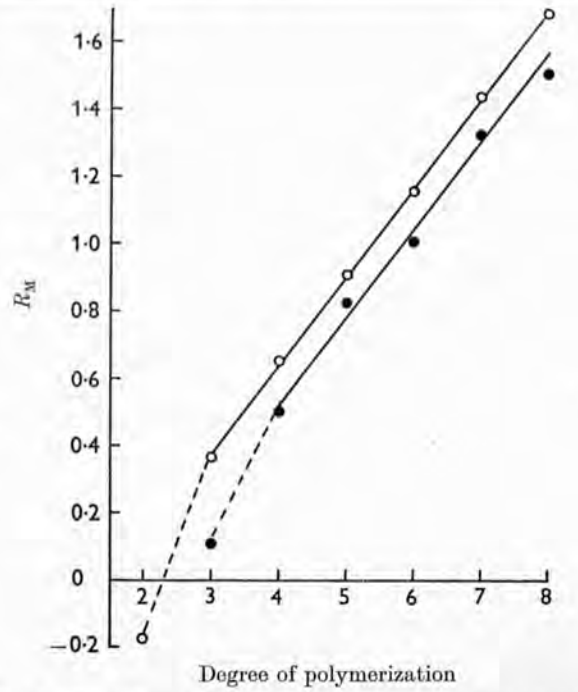

Fig. 1. R_M values (see the text) in ethyl acetate–water–pyridine (2:2:1, by vol.). ○, Oligosaccharides of the isomaltose series; ●, 'branched' oligosaccharides produced by the action of dextranases A and or B on *L. mesenteroides* (Birmingham) dextran. 3^1- (or 3^2-)Glucosylisomaltose (Bailey *et al.* 1961) has been included for comparison.

Table 1. *Ionophoresis of reduced oligosaccharides of the isomaltose series and reduced 'branched' oligosaccharides produced by dextranases A and B*

Experimental details are given in the text. M_S, mobility with respect to sorbitol in molybdate solution (Bourne *et al.* 1961).

Degree of polymerization of reduced oligosaccharides of isomaltose series	M_S	Reduced 'branched' oligosaccharides produced by dextranases A and B	M_S	
			A	B
2	0·78	—	—	—
3	0·62	—	—	—
4	0·53	Tetrasaccharide alcohol	—	0·52
5	0·44	Pentasaccharide alcohol	0·45	0·44
6	0·39	Hexasaccharide alcohol	0·39	0·39
7	0·35	Heptasaccharide alcohol	0·35	0·35
8	0·33	Octasaccharide alcohol	0·33	0·33

ases act on the virtually unbranched dextran of *S. bovis* (Bourne *et al.* 1962), the additional oligosaccharides produced from *L. mesenteroides* (Birmingham) dextran probably contain its α-(1→3)-linkages.

In Fig. 1 the R_M values of these oligosaccharides are plotted against their expected degree of polymerization and compared with those of the oligosaccharides of the isomaltose series. A linear relationship was observed in both series, as was noted for the latter by Turvey & Whelan (1957). 3^1- (or 3^2-)Glucosylisomaltose and isomaltose do not obey this relationship. This indicates that in the solvent used the tetrasaccharide must be regarded as the lowest member of the former polymeric series whereas that of the latter is isomaltotriose.

The oligosaccharides containing one glucose unit joined through a (1→3)-linkage to a glucose unit of isomaltotriose or isomaltotetraose have slightly higher R_G values than those of isomaltotetraose and isomaltopentaose respectively. As the two plots of R_M against degree of polymerization in Fig. 1 are parallel it is reasonable to assume that the oligosaccharides produced by the dextranases are members of a homologous series of 'branched' oligosaccharides of which a 3-glucosylisomaltotriose is the lowest member.

The 'branched' oligosaccharides were eluted from paper chromatograms, reduced with potassium borohydride and subjected to ionophoresis in molybdate solution. The M_S values (Table 1) were identical with those of the reduced oligosaccharides of the isomaltose series of the same expected degree of polymerization.

The products of the action of the dextranases on the dextran were eluted quantitatively from paper chromatograms and their relative concentrations determined by the anthrone method (Yemm & Willis, 1954). The results are shown in Table 2, the assignation of degree of polymerization being based on chromatography and ionophoresis.

Table 2. *Relative weight yields of oligosaccharides produced by the action of dextranase A and B respectively on* Leuconostoc mesenteroides *(Birmingham) dextran*

Experimental details are given in the text. The relative weight yield is expressed relative to isomaltose (= 100).

Compound	Relative weight yield	
	With dextranase A	With dextranase B
Glucose	21	17
Isomaltose	100	100
Isomaltotriose	71	56
Tetrasaccharide	—	8
Pentasaccharide	9	6
Hexasaccharide	21	16
Heptasaccharide	29	22
Octasaccharide and unresolvable material	107	58

Structural examination of oligosaccharides produced by dextranase A

The oligosaccharides (series A) produced by the action of dextranase A on *L. mesenteroides* (Birmingham) dextran (10 g.) were fractionated on a charcoal–Celite column (Whistler & Durso, 1950) and further purified by paper chromatography. The yields, carbohydrate contents, reducing powers, optical rotations and periodate consumptions of the isolated oligosaccharides are shown in Table 3. The properties of the oligosaccharides are consistent with their molecular sizes already assigned.

Pentasaccharide A. Pentasaccharide A could be detected on paper chromatograms with spray reagents (*a*), (*b*), (*c*), (*d*) (green spot, specific for 6-*O*-substituted glucose) and (*e*). During ionophoresis in borate solution, pH 10, it migrated with the same M_G (mobility with respect to glucose) as isomaltopentaose (0·60).

Pentasaccharide A (1 mg.) was heated for 4 hr. at 100° in 1·5 N-hydrochloric acid (2 ml.). Chromatography of the de-ionized hydrolysate showed a single component identical with glucose. A partial hydrolysate, obtained by heating for 4 hr. at 100° in aq. 1 % (w/v) oxalic acid was shown by paper chromatography in solvents (*a*) and (*b*) to contain glucose and isomaltotetraose as the main products. Isomaltose and isomaltotriose were present as traces. After elution from paper chromatograms the isomaltotetraose was further identified by incubation with dextranase A, which resulted in rapid hydrolysis with isomaltose as the main product (Bourne *et al.* 1962).

Pentasaccharide A alcohol (Table 1) was partially hydrolysed by heating for 4 hr. at 100° in aq. 1 % oxalic acid. The de-ionized hydrolysate was fractionated by paper chromatography in solvent (*a*) into six fractions in addition to unchanged material (Table 4). Each fraction was subjected to ionophoresis in molybdate solution, pH 5·5, when fractions 3, 4, 6 and 7 were each resolved into two components. The non-migrating fraction 3 (i) was, after further reduction with potassium borohydride, mobile during ionophoresis in molybdate solution. Fraction 5 was likewise reduced. Ionophoresis in molybdate solution resolved this reduction product into two components, fractions 5 (i) and 5 (ii).

Hexasaccharide A. Hexasaccharide A could be detected with the same spray reagents as pentasaccharide A. Paper chromatography of a complete hydrolysate showed a single component identical with glucose, whereas a partial hydrolysate showed glucose and isomaltopentaose as the main products.

Hexasaccharide A alcohol (Table 1) was partially hydrolysed and the products were fractionated by paper chromatography. In addition to isomaltopentaitol and its expected hydrolysis products, components were found which had the same R_G values as those of the partial hydrolysate of pentasaccharide A alcohol shown in Table 4. The fractions which corresponded to fractions 1, 3 and 5 (R_G values 0·17, 0·29 and 0·44 respectively) were subjected to ionophoresis in molybdate solution. Those corresponding to fractions 1 and 3 were each resolved into an immobile (corresponding to 'branched' pentasaccharide and fraction 3*a* respectively) and a mobile (M_S 0·45 and 0·50 re-

Table 3. *Some properties of oligosaccharides produced by dextranase A*

Experimental details are given in the text. The calculated periodate consumption is for oligosaccharides containing one (1→3)-'branch'-link/molecule.

	Yield (mg.)	$[\alpha]_D$ in water	Carbohydrate content (%)	Reducing power (% of theoretical)	Periodate consumption (mol.prop.)	
					Found	Calc.
Pentasaccharide A	100	178°	98·0	90·1	9·3	9
Hexasaccharide A	340	186	94·2	105·6	11·1	11
Heptasaccharide A	570	190	95·4	97·7	13·2	13
Octasaccharide A	560	194	96·6	95·6	14·3	15

Table 4. *Fractionation of products from partial hydrolysis of pentasaccharide A alcohol*

Experimental details are given in the text.

Paper chromatography in solvent (*a*)		Ionophoresis in molybdate solution			
		Before further reduction		After further reduction	
Fraction no.	R_G	M_S	Identity	M_S	Identity
1	0·17	0·45	Pentasaccharide A alcohol	—	—
2	0·21	0·50	(VIII)	—	—
3	0·29	(i) 0	(IX) or (XI)	0·50	(X) or (XII)
		(ii) 0·50	(X)	—	—
4	0·34	(i) 0	(XV)	—	—
		(ii) 0·65	(XIV)	—	—
5	0·44	0	(XVI) and (XVIII)	(i) 0	(XXIV)
				(ii) 0·65	(XVII)
6	0·60	(i) 0	(XX)	—	—
		(ii) 0·78	(XIX)	—	—
7	1·0	(i) 0	(XXII)	—	—
		(ii) 1·0	(XXIII)	—	—

spectively) component. That with R_G 0·44 (fraction 5) remained immobile during ionophoresis.

Heptasaccharide A. Heptasaccharide A could be detected with the same spray reagents as pentasaccharide A. Complete hyrolysis was shown, by paper chromatography, to give only glucose. The partial hydrolysate contained glucose and isomaltohexaose as the main products.

Heptasaccharide A alcohol (Table 1) was partially hydrolysed and the hydrolysate fractionated as described for pentasaccharide A alcohol. In addition to isomaltohexaitol and its expected hydrolysis products, components were found that had the same R_G values as those of the partial hydrolysate of pentasaccharide A alcohol shown in Table 4. Ionophoresis in molybdate solution resolved the fraction corresponding to fraction 1 (R_G 0·17) into an immobile ('branched' pentasaccharide) and a mobile component with M_S 0·45 (reduced 'branched' pentasaccharide). The fractions corresponding to fractions 3 (R_G 0·29) and 5 (R_G 0·44) did not migrate during ionophoresis in molybdate solution.

Octasaccharide A. Octasaccharide A could be detected with the same spray reagents as pentasaccharide A. The complete hydrolysate contained only glucose whereas the partial hydrolysate contained glucose, isomaltohexaose, possibly isomaltoheptaose, and a component with R_G identical with that of heptasaccharide A.

Octasaccharide A alcohol (Table 1) was partially hydrolysed and fractionated as described for pentasaccharide A alcohol. In addition to the main products which had R_G values corresponding to those of the 'branched' heptasaccharide A, possibly isomaltoheptaose, isomaltohexaose, 'branched' hexasaccharide A, isomaltopentaose and glucose, components were found which had the same R_G values as those of the partial hydrolysate of pentasaccharide A alcohol (Table 4). Ionophoresis in molybdate solution of the fractions with R_G values 0·17, 0·29 and 0·44 gave the same results as the corresponding fractions of the partial hydrolysate of heptasaccharide A alcohol.

Structural examination of oligosaccharides produced by dextranase B

The tetra-, penta- and hexa-saccharides (series B) produced by the action of dextranase B on *L. mesenteroides* (Birmingham) dextran (40 g.) were fractionated and purified as described for those of series A. Determinations of the degree of polymerization by the method of Peat *et al.* (1956) gave values of 4·2, 4·9 and 6·0 respectively. The preliminary assignation of molecular size (Fig. 1 and Table 1) was thus correct.

Tetrasaccharide B. The methods used were as for pentasaccharide A. Tetrasaccharide B could be detected on paper chromatograms with spray reagents (*a*), (*b*), (*c*), (*d*) (green spot) and (*e*). During ionophoresis in borate solution, pH 10, it migrated with the same M_G as that of isomaltotetraose (0·65). Complete hydrolysis gave a single reducing component that was chromatographically identical with glucose, whereas a partial hydrolysate contained components corresponding to glucose and isomaltotriose, as the main products, and isomaltose and nigerose.

The component corresponding to isomaltotriose was isolated by paper chromatography. When benzoylated (Turvey & Whelan, 1957) it gave a crystalline product, which had m.p. 223–225° (Found: C, 69·2; H, 4·6. $C_{95}H_{76}O_{27}$ requires C, 69·2; H, 4·6%). Admixture with authentic undeca-*O*-benzoyl-β-isomaltotriose (m.p. 225–227°) caused no depression in melting point.

The disaccharide corresponding to nigerose maintained its identity during ionophoresis in borate solution, pH 10. When reduced with potassium borohydride it did not migrate during ionophoresis in molybdate solution, pH 5·5. This shows conclusively the presence of a (1→3)-glucosidic linkage (Bourne *et al.* 1961). The disaccharide was not hydrolysed by β-glucosidase, unlike laminaribiose, thus confirming the presence of an α-linkage.

Tetrasaccharide B consumed 7·1 mol.prop. of periodate. After destruction of the excess of periodate, the oxidation product was reduced with potassium borohydride and hydrolysed with hydrochloric acid (Bailey *et al.* 1961). The hydrolysate was fractionated by paper chromatography in solvent (*a*) when a component corresponding to glucose was revealed. This component was determined by the anthrone method (Yemm & Willis, 1954). The yield of glucose from tetrasaccharide B corresponded to 28% of the glucose present in a tetrasaccharide containing glucose only.

Tetrasaccharide B alcohol was partially hydrolysed by heating for 4 hr. at 100° in aq. 1% oxalic acid. By the methods described by Bailey *et al.* (1961) it was shown that the hydrolysate contained 6-α-isomaltosylsorbitol, 3^2-glucosylisomaltose, isomaltose, 6-α-D-glucopyranosylsorbitol, nigerose, glucose and sorbitol. 6-Nigerosylsorbitol and 3^1-glucosylisomaltose were absent from the hydrolysate.

Pentasaccharide B and hexasaccharide B. The R_G values and the compositions of the complete and partial hydrolysates were identical with those of pentasaccharide A and hexasaccharide A respectively. The partial hydrolysates of pentasaccharide B alcohol and hexasaccharide B alcohol (Table 1) contained the same components as those of the alcohols of pentasaccharide A and hexasaccharide A respectively.

DISCUSSION

The assignment of the structures of the oligosaccharides produced by the action of dextranases A and B on *L. mesenteroides* (Birmingham) dextran is based on the facts that: (*a*) in the solvents used the reducing sugars have the same R_G values as their reduction products; (*b*) oligosaccharides of the isomaltose series have slightly lower R_G values than their isomers containing (1→3)-linkages; (*c*) glucose and oligosaccharides of glucose do not migrate during ionophoresis in molybdate solution and can thus be separated from their reduction products, provided the latter are not 3-substituted sorbitols; (*d*) 3-substituted sorbitols do not migrate during ionophoresis in molybdate solution; (*e*) isomeric oligosaccharide alcohols containing the same substituted polyol component, e.g. 6-α-isomaltosylsorbitol and 6-α-nigerosylsorbitol, have identical M_S values during ionophoresis in molybdate solution.

The evidence presented shows that the oligosaccharides investigated in detail are tetra-, penta-, hexa- and hepta-saccharides in which one glucose unit is joined through a (1→3)-linkage to a glucose unit, other than the reducing one, of isomaltotriose, -tetraose, -pentaose and -hexaose respectively. It was also shown that pentasaccharide A and hexasaccharide A are identical with pentasaccharide B and hexasaccharide B respectively. As the (1→3)- as well as the (1→6)-glucosidic linkages in this dextran are of the α-type it is reasonable to assume that the (1→3)-glucosidic linkages in the oligosaccharides also are α-(1→3)-linkages. This is confirmed by the characterization of nigerose as one of the products of the hydrolysis of tetrasaccharide B and its reduction product.

Tetrasaccharide B was shown conclusively to be 3^3-α-glucosylisomaltotriose (I) (Fig. 3). The products expected from the 3^2-isomer were absent from the hydrolysate of tetrasaccharide B alcohol.

There are three possible structures, (II)–(IV) (Fig. 2), for a pentasaccharide containing one glucose unit joined through an α-(1→3)-linkage to a glucose unit, other than the reducing one, of isomaltotetraose. The structure was elucidated further by a partial hydrolysis of the reduction product (V), (VI) or (VII).

The products that would be expected from all three structures (Fig. 2), namely 6-isomaltotriosylsorbitol (VIII) (fraction 2), 6-isomaltosylsorbitol (XIV) (fraction 4ii), isomaltotriose (XV) (fraction 4i), 6-glucosylsorbitol (XIX) (fraction 6ii), isomaltose (XX) (fraction 6i), glucose (XXII) (fraction 7i) and sorbitol (XXIII) (fraction 7ii), were identified by paper chromatography and ionophoresis. Under the conditions used it was not possible to detect nigerose (XXI), which also would be expected from all three structures.

Structure (IV) is eliminated for the pentasaccharide since (XIII) and (XVII) have been shown to be absent from the partial hydrolysate of the pentasaccharide alcohol.

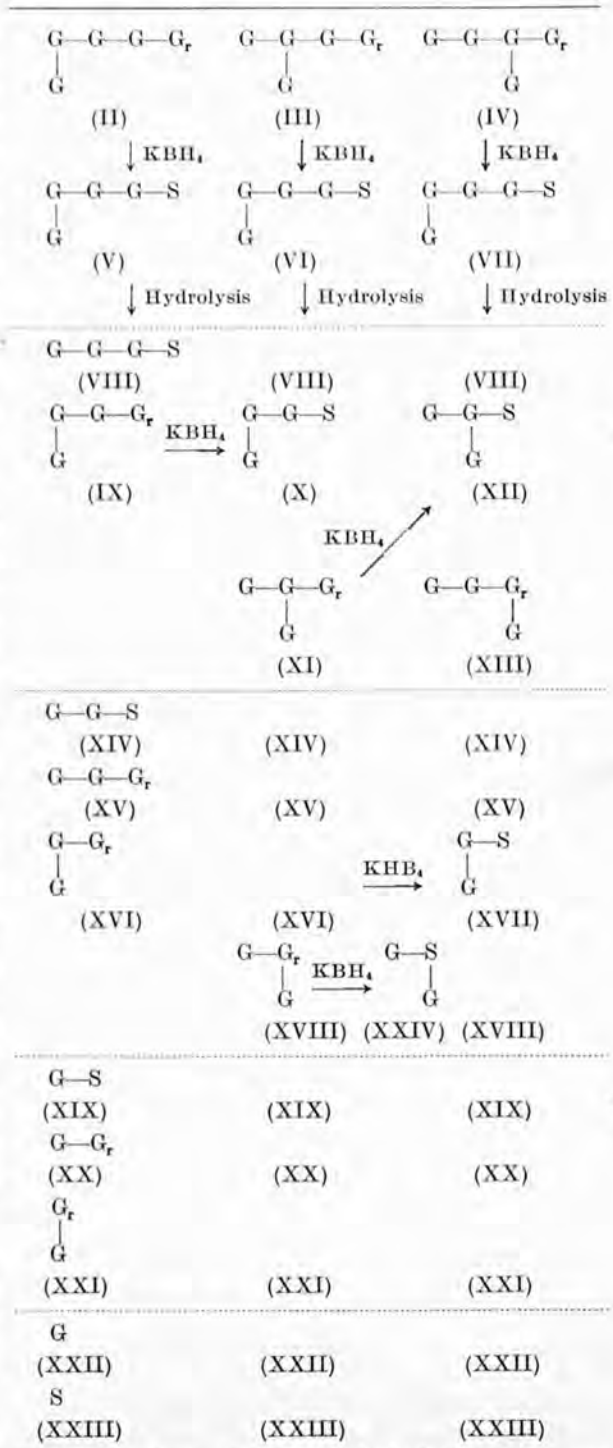

Fig. 2. Products of reduction–hydrolysis of pentasaccharides (II), (III) and (IV). —, α-(1→6)-Link; |, α-(1→3)-link; G_r, reducing glucose unit; G, glucosyl unit; S, sorbitol.

Reduction of the branched trisaccharide fragments (fraction 5) followed by ionophoresis in molybdate solution revealed the presence in the hydrolysate of (XVI) and (XVIII). As structure (IV) has been eliminated the presence of 3^1-glucosylisomaltose (XVIII) in the hydrolysate shows conclusively that the pentasaccharide contains structure (III). 3^3-Glucosylisomaltotriose (IX) is the only fragment derivable solely from structure (II) for the pentasaccharide. However, the method used did not distinguish between this and 3^2-glucosylisomaltotriose (XI) [from (III)]. Thus the pentasaccharides A and B are 3^3-glucosylisomaltotetraose (III), alone or in admixture with 3^4-glucosylisomaltotetraose (II).

Hexasaccharides A and B on partial hydrolysis gave glucose and isomaltopentaose. Their reduction products likewise gave isomaltopentaitol, a 'branched' pentasaccharide, pentasaccharide A alcohol and the same hydrolysis products as the pentasaccharide A alcohol. Thus it is concluded that each hexasaccharide is 3^3-glucosylisomaltopentaose (XXVI), alone or in admixture with 3^4-(XXV) or 3^5-glucosylisomaltopentaose or both.

The results obtained with heptasaccharide A show that it contained one glucose unit joined through a (1→3)-linkage to a glucose unit, other than the reducing one, of isomaltohexaose. Partial hydrolysis of heptasaccharide A alcohol produced in addition to isomaltohexaitol and its expected hydrolysis products components which had the same R_G values as those of the partial hydrolysate of pentasaccharide A alcohol. The structure of the heptasaccharide could thus have been similar to those of the penta- and hexa-saccharides. However, when the components with R_G values corresponding to 'branched' tri- and tetra-saccharides or their reduction products were subjected to ionophoresis in molybdate solution fragments with structures (X) or (XVII) could not be detected. Hence the component that had the same R_G and M_s values as pentasaccharide A alcohol must have structure (V). Thus it is concluded that heptasaccharide A is 3^4-glucosylisomaltohexaose (XXVII), alone or in admixture with isomers in which the branching occurs further towards the non-reducing end of the isomaltohexaose unit.

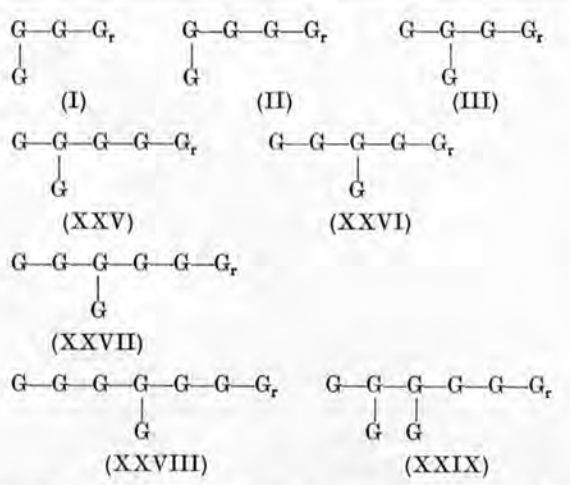

Fig. 3. Structures of oligosaccharides produced by the action of dextranases on *L. mesenteroides* (Birmingham) dextran. —, α-(1→6)-Link; |, α-(1→3)-link; G_r, reducing glucose unit; G, glucosyl unit.

Octasaccharide A consumed 14·3 mol.prop. of periodate. The theoretical consumption of periodate of an octasaccharide containing one glucose unit joined through a (1→3)-linkage to isomaltoheptaose (XXVIII) is 15 mol.prop., whereas that containing two glucose units joined through (1→3)-linkages to isomaltohexaose (XXIX) is 13 mol.prop. It is thus likely that octasaccharide A is a mixture of compounds with structures similar to (XXVIII) and (XXIX). This is supported by the fact that the main products of the partial hydrolysis of octasaccharide A were glucose, isomaltohexaose, possibly isomaltoheptaose and a component with R_G identical with that of heptasaccharide A. The results of the partial hydrolysis of octasaccharide A alcohol show that the octasaccharide contains a 3^4-glucosylisomaltotetraose unit as does heptasaccharide A. It is thus concluded that octasaccharide A is a mixture of 3^4-glucosylisomaltoheptaose (XXVIII), possibly isomers in which the branching occurs further towards the non-reducing end of the isomaltoheptaose unit, and octasaccharides with structures similar to that of (XXIX).

An interesting feature is the production of 3^3-glucosylisomaltotriose (I) by dextranase B but not by dextranase A. Dextranase B hydrolyses isomaltotriose appreciably faster than does dextranase A (Bourne *et al.* 1962). It is thus possible that dextranase B is capable of hydrolysing the main dextran chain closer to the non-reducing side of the branch point than dextranase A. Alternatively, dextranase B could be contaminated with an enzyme capable of hydrolysing terminal α-(1→6)-glucosidic linkages at the non-reducing end of the chain. The presence, or absence, of such an enzyme must be investigated before a full assessment of the above results can be made.

Further, the 'branching' of the oligosaccharides consists of only one glucosyl unit, as with the oligosaccharides produced by the action of *L. bifidus* dextranase on the same dextran (Bailey *et al.* 1961).

SUMMARY

1. The dextranases of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) (dextranase A) and *P. funiculosum* (I.M.I. 79195; NRRL 1132) (dex-

tranase B) have been shown to hydrolyse *Leuconostoc mesenteroides* (Birmingham) dextran to complex mixtures of oligosaccharides.

2. Both dextranases produced glucose, isomaltose, isomaltotriose and 'branched' penta-, hexa-, hepta- and octa-saccharides. Dextranase B produced, in addition, a 'branched' tetrasaccharide. Unresolvable material of degree of polymerization greater than 8 was produced by both dextranases. The 'branched' oligosaccharides were shown to be members of an homologous series.

3. The tetrasaccharide produced by dextranase B was shown to be 3^3-α-glucosylisomaltotriose.

4. The pentasaccharides produced by dextranases A and B were identical and shown to be a 3^3-glucosylisomaltotetraose, alone or in admixture with 3^4-glucosylisomaltotetraose. Likewise, the hexasaccharides were identical and shown to be 3^3-glucosylisomaltopentaose, alone or in admixture with isomers in which the branching occurred further towards the non-reducing end of the isomaltopentaose unit.

5. The heptasaccharide and octasaccharide produced by dextranase A were shown to contain 3^4-glucosylisomaltohexaose and 3^4-glucosylisomaltoheptaose respectively. Both saccharides could have been in admixture with isomers in which the branching occurred further towards the non-reducing end of the isomalto-hexaose and -heptaose units respectively. The octasaccharide also contained a saccharide in which two glucose units are joined through (1→3)-linkages to isomaltohexaose.

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Studies on Dextrans and Dextranases

4. MECHANISM OF THE ACTIONS OF INTRA- AND EXTRA-CELLULAR MOULD HYDROLASES*

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The extracellular endodextranases of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) (dextranase A) and *Penicillium funiculosum* (I.M.I. 79195; NRRL 1132) (dextranase B) were shown to hydrolyse readily the almost unbranched dextran of *Streptococcus bovis*, isomaltodextrins and modified isomaltodextrins containing not less than three α-glucosidic linkages, the hydrolysis being principally at glucosidic linkages other than the terminal ones; the extent of hydrolysis of various dextrans was markedly lowered with increasing degree of branching of the dextran molecule (Bourne, Hutson & Weigel, 1962). The (1→3)-branched dextran of *Leuconostoc mesenteroides* (Birmingham) was hydrolysed by each dextranase to glucose, isomaltose, isomaltotriose and a complex mixture of 'branched' oligosaccharides (Fig. 1) (Bourne, Hutson & Weigel, 1963). The smallest 'branched' oligosaccharide produced by dextranase A was a pentasaccharide [3^3-glucosylisomaltotetraose (II), alone or in a mixture with 3^4-glucosylisomaltotetraose (III)], whereas that produced by dextranase B was a tetrasaccharide [3^3-glucosylisomaltotriose (I)]. The production of the latter by dextranase B only could have been due to a difference in specificity of the two dextranases or to contamination of dextranase B with another, perhaps mainly intracellular, enzyme. We have now investigated cell extracts of the two moulds. An attempt has been made to interpret these and previous results with regard to both the mechanism of dextranase action and the structure of *Ln. mesenteroides* (Birmingham) dextran.

EXPERIMENTAL

Cell extracts. Washed mycelia of *P. lilacinum* (I.M.I. 79197; NRRL 896) and *P. funiculosum* (I.M.I. 79195; NRRL 1132), that had been grown in media containing *Streptococcus bovis* dextran (1%) and yeast extract (1%), were freeze-dried (Bourne *et al.* 1962). The freeze-dried material (0·4 g.) was suspended in 0·05M-citrate buffer, pH 5·5 (20 ml.), and shaken with cooling in a Mickle disintegrator (no. 4 glass balls) for a total of 45 min. The supernatant liquid was decanted and the solid material washed with 0·05M-citrate buffer, pH 5·5 (20 ml.). The fluids were combined and centrifuged at 10 000 *g* for 30 min. to remove debris. The resulting extracts were dialysed against 2 l. of 0·05M-citrate buffer, pH 5·5, after which they were used as such or stored at about 5°. Extracts of *P. lilacinum* (I.M.I. 79197) and *P. funiculosum* (I.M.I. 79195) are referred to as extracts A′ and B′ respectively.

Enzyme digests. Except where otherwise stated digests were made up from carbohydrate (10 mg.) in 0·2M-citrate buffer, pH 4.8 (1 ml.), and cell extract (1 ml.), and were incubated for 24 hr. at 37°. Deionization was effected with Amberlite IR-120 (H^+ form) and Amberlite IR-45 (OH^- form) resins.

Paper chromatography and ionophoresis. The solvent used for chromatography was the upper layer of ethyl acetate–water–pyridine (2:2:1, by vol.). Ionophoresis was carried out at about 50 V/cm. in molybdate solution at pH 5·5 (Bourne, Hutson & Weigel, 1961). The sugars were detected with silver nitrate in acetone–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950).

RESULTS

Action of extracts A′ and B′ on isomaltodextrins and isomaltodextrinols. The various compounds were separately incubated under standard conditions with extracts A′ and B′ respectively. The digests were analysed by paper chromatography and ionophoresis in molybdate solution. The results are shown in Table 1.

Effect of pH *and temperature on the action of extract A′ towards isomaltotriose.* Digests containing isomaltotriose (6 mg.) in 0·2M-citrate buffer of various pH values (1 ml.) and cell extract A′

Table 1. *Products of action of extract A′ or extract B′ on isomaltodextrins and isomaltodextrinols*

Experimental details are given in the text.

Substrate	Products
Isomaltose	None
Isomaltotriose	Glucose and isomaltose*
Isomaltotri-itol	None
Isomaltotetraose	Isomaltose
Isomaltotetraitol	Isomaltose and isomaltitol
Isomaltopentaose	Glucose, isomaltose and isomaltotriose (traces)

* Hydrolysis was complete after incubation for about 35 hr.

* Part 3: Bourne, Hutson & Weigel (1963).

(0·5 ml.) were incubated for 24 hr. at 33°. Digests containing the same components in 0·2 M-citrate buffer, pH 5·0, were incubated at various temperatures. The increase in reducing power of the solutions was determined by the method of Shaffer & Hartmann (1921). The results obtained showed that the extract possessed optimum activity towards isomaltotriose within the pH range 4–5 and temperature range 30–40°.

Action of extracts A′ and B′ on 'branched' oligosaccharides elaborated from Leuconostoc mesenteroides *(Birmingham) dextran by dextranases A and B.* The 'branched' oligosaccharides (see Fig. 1) were separately incubated for 24 hr. with extracts A′ and B′ respectively. Paper chromatography revealed that the tetra- (I) and penta-saccharides (II, or II and III) were not significantly hydrolysed. Both extracts produced from the 'branched' hexa-, hepta- and octa-saccharides, in addition to isomaltose, significant amounts of materials with R_G (migration with respect to glucose) values corresponding to 'branched' tetra-, penta- and hexa-saccharides.

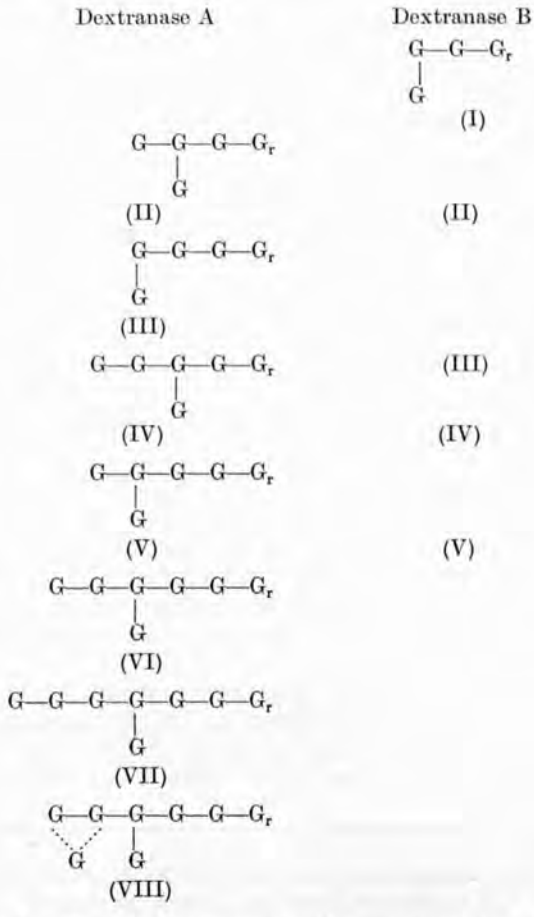

Fig. 1. 'Branched' oligosaccharides produced by the action of dextranases A and B on *Leuconostoc mesenteroides* (Birmingham) dextran. —, α-(1→6)-Link; |, α-(1→3)-link; G_r, reducing glucose unit; G, glucosyl unit; ⋮, alternative α-(1→3)-links. The penta-, hexa and hepta-saccharides were shown to be (II), (IV) and (VI), alone or in admixture with isomers in which the branching occurs further towards the non-reducing end of the isomaltodextrin unit, e.g. (III) and (V). Dextranase B also produced 'branched' hepta- and octa-saccharides, the structures of which were not elucidated.

DISCUSSION

The cell extracts A′ and B′ were made in a search for an enzyme that would cause the smallest 'branched' oligosaccharides to be different (see Fig. 1) when the extracellular dextranases A and B respectively acted on *Ln. mesenteroides* (Birmingham) dextran. The extracts A′ and B′ were found to contain a hydrolase that hydrolyses oligosaccharides of the isomaltose series containing at least three glucose units in the manner shown in Fig. 2. This is unlike the action of the extracellular-dextranase preparations A and B that required for ready hydrolysis at least three glucosidic linkages, thus hydrolysing 6-α-isomaltotriosylsorbitol and methyl α-isomaltotrioside (Bourne *et al.* 1962), but isomaltotriose only very slowly. Extracts A′ and B′ were found not to hydrolyse the 'branched' tetra- and penta-saccharides (Fig. 1), but to hydrolyse slowly the preparations of 'branched'

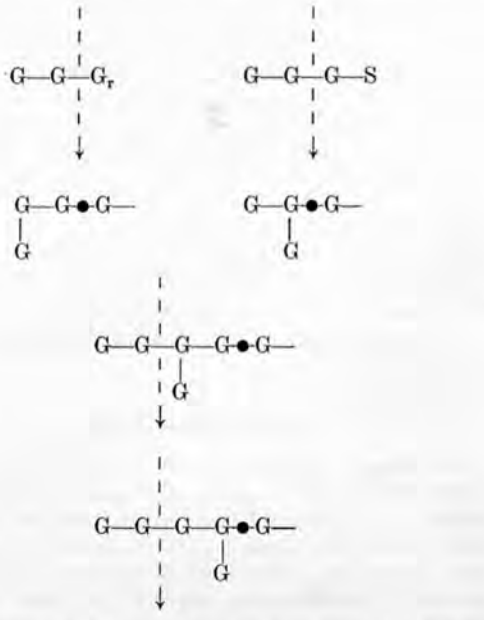

Fig. 2. Action of extracts A′ and B′. —, α-(1→6)-Link; |, α-(1→3)-link; G, glucosyl unit; G_r, reducing glucose unit; S, sorbitol; - - - →, point of hydrolysis; -●-, α-(1→6)-link resistant to hydrolysis due to presence of 'branch' point in isomaltosyl unit.

hexa-, hepta- and octa-saccharides. The latter three were known to contain 3^3-glucosylisomaltopentaose (IV), 3^4-glucosylisomaltohexaose (VI) and 3^4-glucosylisomaltoheptaose (VII) respectively. However, contamination with the isomers in which the branching occurs further towards the non-reducing end of the isomaltodextrin unit was not excluded. The main products were isomaltose and oligosaccharides that had R_G values corresponding to 'branched' tetra-, penta- and hexa-saccharides respectively. The structures of these 'branched' oligosaccharides are not necessarily the same as the 'branched' tetra-, penta- and hexa-saccharides produced by the action of the extracellular dextranases on the *Ln. mesenteroides* (Birmingham) dextran, as isomers of the type 3^2- or 3^3-glucosylisomaltotriose have the same R_G in the solvent used (Bailey, Hutson & Weigel, 1961). These results indicate that the mainly intracellular hydrolases acted only on those branched oligosaccharides (IV, VI and VII) that contained at the non-reducing end an unsubstituted isomaltose unit (Fig. 2).

Bailey & Robertson (1962) have shown that a cell extract of *Lactobacillus bifidus* contains an α-(1→6)-glucosidase that hydrolyses readily isomaltodextrins of degree of polymerization 2–9 to glucose, the hydrolysis proceeding by the cleavage of single glucose units from the non-reducing end of the molecule.

No difference between extracts A′ and B′ in their action on isomaltodextrins, isomaltodextrinols and 'branched' oligosaccharides was observed. Hence it is unlikely that the consistent differences in the nature of the 'branched' oligosaccharides produced from *Ln. mesenteroides* (Birmingham) dextran by preparations of dextranases A and B were caused by contamination with the mainly intracellular hydrolase. This is strengthened by the fact that these 'branched' oligosaccharides are formed within the first 1–2 hr. of incubation, after which no significant change in their concentration is observed, whereas the mainly intracellular hydrolases acted only slowly on some of these. We thus believe that the different actions of the two dextranase preparations A and B are inherent in the dextranases themselves and not due to other enzymes.

It has been shown that the extracellular dextranases A and B hydrolyse readily an almost unbranched dextran, isomaltodextrins or modified isomaltodextrins, containing not less than three α-glucosidic linkages, principally at glucosidic linkages other than the terminal ones (Bourne *et al.* 1962). The production of tetra-, penta-, hexa-, hepta- and octa-saccharides containing one glucose unit joined through an α-(1→3)-linkage to a glucose unit of the corresponding oligosaccharide of the isomaltose series (Fig. 1), when the extracellular dextranases act on the branched dextran of *Ln. mesenteroides* (Birmingham), suggests that this type of hydrolysis of dextran is inhibited by the presence of α-(1→3)-linkages. This is in contrast with the action of *Lb. bifidus* dextranase (Bailey *et al.* 1961.) The structure of these oligosaccharides indicates (see Fig. 3) that: (*a*) the enzymes cannot hydrolyse the α-(1→3)-linkages; (*b*) at least two α-(1→6)-linkages on the reducing side of the branch point of the main dextran chain are resistant to hydrolysis by both enzymes; (*c*) one α-(1→6)-link on the non-reducing side of the branch point of the main dextran chain is resistant to hydrolysis by dextranase A, i.e. dextranase B is capable of hydrolysing the main dextran chain closer to the non-reducing side of the branch point than dextranase A; and (*d*) one additional α-(1→6)-link on the non-reducing side of the branched oligosaccharides produced by the dextranases is resistant to hydrolysis by both enzymes.

Of the oligosaccharides shown in Fig. 1 the pentasaccharide (III), if produced by dextranase A, would preclude such a mechanism. The elucidation of the structure of the pentasaccharide produced by dextranase A established with certainty only the formation of (II); however, its contamination with (III) was not excluded (Bourne *et al.* 1963). It is thus possible that this pentasaccharide is 3^3-glucosylisomaltotetraose (II) alone.

The 'branching' of the oligosaccharides produced from *Ln. mesenteroides* (Birmingham) dextran by the action of either the bacterial dextranase of *Lb. bifidus* (Bailey *et al.* 1961) or the mould dextranases A and B (Bourne *et al.* 1963) consists of only one glucosyl unit. It was not possible to decide whether the dextranases are capable of hydrolysing α-(1→6)-linkages of branch chains, if such branch chains were present in the dextran molecule. However, oligosaccharides in which the 'branching' is an isomaltosyl unit, expected from branch chains containing two, four or more glucosyl units, have not been found in the hydrolysates. This suggests

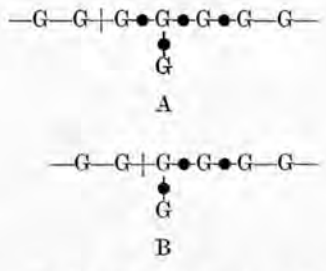

Fig. 3. Segments of the dextran molecule showing glucosidic linkages in the vicinity of the branch point which are resistant to hydrolysis by dextranase A and B. G, Glucosyl unit; —, α-(1→6)-link; ●, α-(1→3)-link; ●–, resistant glucosidic link in dextran and oligosaccharides; –, additional resistant link in oligosaccharides.

that these 'branched' oligosaccharides arise from parts of the dextran molecule where the branching is in fact a single glucosyl unit. It can thus be calculated from the yield of the isolated 'branched' penta-, hexa-, hepta- and octa-saccharides produced by the action of dextranase A (Bourne *et al.* 1963) that more than 18 % of all branches of *Ln. mesenteroides* (Birmingham) dextran consist of only one glucosyl unit. Bovey (1959), on the grounds of physical measurements, has suggested that 80 % of the branches in *Ln. mesenteroides* (NRRL B-512) dextran consist of only one glucosyl unit.

SUMMARY

1. Cell extracts from *Penicillium lilacinum* and *Penicillium funiculosum*, grown on *Streptococcus bovis* dextran, contain an enzyme that hydrolyses oligosaccharides of the isomaltose series with at least three glucose units. The effect of the presence of (1→3)-branch linkages on the hydrolysis is discussed.

2. The failure of the extracts to hydrolyse the smallest oligosaccharides produced by the action of the extracellular endodextranases of *P. lilacinum* (dextranase A) and *P. funiculosum* (dextranase B) on *Leuconostoc mesenteroides* (Birmingham) dextran permits certain conclusions to be reached about the specificity of these enzymes.

3. In the vicinity of the α-(1→6)-branch point, which the enzymes cannot hydrolyse, several α-(1→6)-linkages of the main dextran chain are resistant to hydrolysis by the dextranases. On the reducing side of this point at least two adjacent linkages are resistant to hydrolysis by dextranase A or dextranase B, and on the non-reducing side of this point one adjacent linkage is resistant to hydrolysis by dextranase A.

4. In the branched oligosaccharides produced from *Ln. mesenteroides* (Birmingham) dextran by dextranases A and B, one additional α-(1→6)-link on the non-reducing side of the branch point is resistant to hydrolysis by both enzymes.

5. The size of branches in *Ln. mesenteroides* (Birmingham) dextran is discussed.

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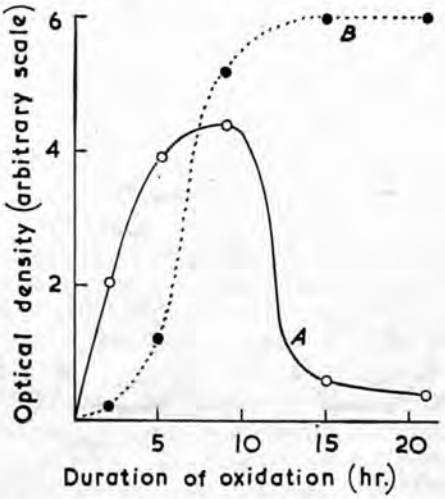
960. *Studies on Dextrans and Dextranases. Part V.*[1] *Synthesis of the Three Carboxylic Acids derived from Methyl β-Maltoside*

By D. Abbott and H. Weigel

The catalytic oxidation of methyl β-maltoside to give two monocarboxylic acids and one dicarboxylic acid is described. Evidence for their structures is presented.

In Part IV [1] we suggested that more than 18% of all branches of *Leuconostoc mesenteroides* (Birmingham) dextran consist of only one glucosyl unit. This view was based on the yields and structures of branched oligosaccharides produced when this α-1 ⟶ 3-branched dextran was treated with certain dextranases,[2] as well as on the mechanism of the action of the dextranases. A similar indication has now been obtained for the α-1 ⟶ 4-branched dextran of *L. mesenteroides* (NRRL B-1415).[3] It seemed to us that confirmatory evidence could be obtained by converting all primary hydroxyl groups of the dextrans into carboxyl groups and, after hydrolysis, isolation of the corresponding aldobiuronic acids. It thus became necessary to have available the uronic acids formed from disaccharides of D-glucose by oxidation of one or both primary hydroxyl groups. The recently reported synthesis of 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose [4] prompts us to describe our syntheses of all three uronic acids derived from maltose.[5]

The oxidation of methyl β-maltoside with oxygen in the presence of a platinum catalyst (Adams platinum dioxide reduced with hydrogen) yielded two acidic fractions (A and B, see Figure). Their mobilities during paper electrophoresis in phosphate solution

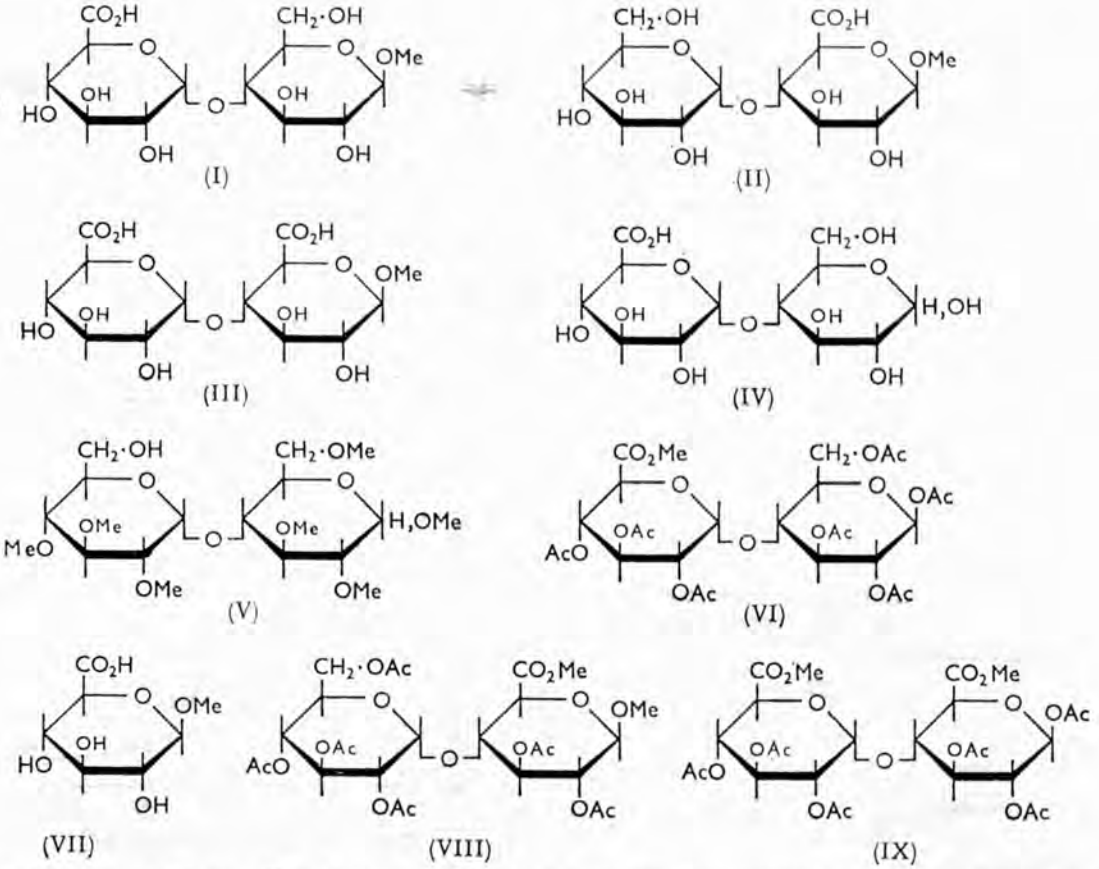


Relative concentrations of mono- (*A*) and dicarboxylic acids (*B*) produced from methyl β-maltoside

(pH 7·2) [A, $M_{GA}(P)$ 0·77; B, $M_{GA}(P)$ 1·33; for definition of all mobilities see Experimental section] and their rate of formation (and disappearance) on prolonged oxidation indicated that fraction A contained methyl 4-*O*-(α-D-glucopyranosyluronic acid)-β-D-glucopyranoside (I) and/or methyl 4-*O*-α-D-glucopyranosyl-β-D-glucopyranosiduronic acid (II) and that fraction B was methyl 4-*O*-(α-D-glucopyranosyluronic acid)-β-D-glucopyranosiduronic acid (III). For the preparation of the monocarboxylic acids (A) and the dicarboxylic acids (B) methyl β-maltoside was oxidised for 8·5 and 16 hr., respectively.

It was conceived that chromatographic resolution of the two monocarboxylic acids (I and II) might be possible after treatment with benzaldehyde since only acid (II) could easily form a benzylidene derivative (involving the hydroxyl groups on C-4 and C-6 of the D-glucose portion). Indeed, this method gave the expected two components. Partial hydrolysis of the benzylidene derivative gave acid (II).

The following evidence, in addition to the above, shows that the component remaining unaffected by benzaldehyde was in fact acid (I) contaminated with small quantities of acid (II). Hydrolysis gave, as the main product, 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (IV) and small quantities of materials which had R_G values identical with those of glucuronic acid, glucurone, glucose, and methyl β-D-glucopyranosiduronic acid (VII). Owing to the relative stabilities to acid hydrolysis of glycosides of D-glucuronic acid and D-glucose,[6] acid (IV) would indeed be expected as the main product from acid (I), whereas D-glucose and acid (VII) are expected from acid (II). Thus, the acid hydrolysis also degraded effectively the contamination (II), making the isolation of acid (IV) possible. Successive treatment of acid (IV) with (*a*) methanolic hydrogen chloride, (*b*) methyl iodide–silver oxide, and (*c*) lithium aluminium hydride gave a syrup which had OMe 47·5%, close to that expected for a methyl hexa-*O*-methylmaltoside (OMe, 49·3%). The hydrolysis products of this syrup were 2,3,6- and 2,3,4-tri-*O*-methyl-D-glucose. The former was



obtained crystalline. The latter, obtained as a syrup, was converted into the crystalline 2,3,4-tri-*O*-methyl-*N*-*p*-nitrophenyl-D-glucosylamine. The tri-*O*-methyl-D-glucoses were further characterised by reduction, periodate oxidation, and subsequent demethylation to give products which were chromatographically and electrophoretically identical with threose (from 2,3,6-tri-*O*-methyl-D-glucose) and xylose (from 2,3,4-tri-*O*-methyl-D-glucose). This showed that the syrup was the methyl hexa-*O*-methylmaltoside (V). Acid (IV) also gave a methyl ester hepta-acetate which exhibited absorption at 835 and 885 cm.$^{-1}$, indicative of α- and β-configurations at the anomeric carbon atoms [7] and expected for 1,2,3,6-tetra-*O*-acetyl-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)-β-D-glucopyranose (VI).

Chromatography of the acid hydrolysate of acid (II) revealed the expected components. Acid (II) was esterified, methylated, and then reduced. The hydrolysate of the product

contained two components which had migration rates (chromatography and electrophoresis) identical with those of 2,3,4,6-tetra- and 2,3-di-*O*-methyl-D-glucose. Acid (II) also gave a methyl ester hexa-acetate which exhibited absorption at 840 and 895 cm.$^{-1}$. These results show that acid (II) and its methyl ester hexa-acetate were in fact methyl 4-*O*-α-D-glucopyranosyl-β-D-glucopyranosiduronic acid (II) and methyl [4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)(methyl-2,3-di-*O*-acetyl-β-D-glucopyranosid)]uronate (VIII), respectively.

The equivalent weight (198) of the acid fraction B was close to that expected for a compound with structure (III) (192) and confirmed the conclusion drawn from electrophoresis. Hydrolysis with 90% formic acid produced a reasonable quantity of D-glucuronic acid (isolated as D-glucurone). Partial hydrolysis gave a dicarboxylic acid (electrophoretic evidence) which was converted into a diester (methyl) hexa-acetate. This exhibited absorption at 845 and 890 cm.$^{-1}$. Thus, acidic fraction B was methyl 4-*O*-(α-D-glucopyranosyluronic acid)-β-D-glucopyranosiduronic acid (III) and the di-ester hexa-acetate was methyl [1,2,3-tri-*O*-acetyl-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)-β-D-glucopyran]uronate (IX).

Experimental

General.—(i) *Paper chromatography.* The solvents used were (*a*) ethyl acetate–acetic acid–formic acid–water (18 : 8 : 3 : 6); (*b*) butan-1-ol–ethanol–water (40 : 1 : 19); (*c*) butan-1-ol–ethanol–water–ammonia (40 : 10 : 49 : 1, organic phase); (*d*) ethyl acetate–acetic acid–water (9 : 2 : 2). Migration rates are expressed relative to the movements of D-glucose (R_G) and 2,3,4,6-tetra-*O*-methyl-D-glucose (R_{TMG}).

(ii) *Paper electrophoresis.* Migration rates are expressed relative to the movements of D-glucuronic acid [$M_{GA}(P)$], sorbitol [$M_S(Mo)$], D-ribose [$M_R(As)$], and D-glucose [$M_G(B)$]. The symbols *P*, *Mo*, *As*, and *B* refer to the electrolytes used: (*a*) 0·2M-sodium phosphate, adjusted to pH 7·2 (*P*); (*b*) 2% sodium molybdate dihydrate, adjusted to pH 5 (*Mo*); [8] (*c*) sodium arsenite (*As*), As_2O_3 (19·8 g.) dissolved in 0·13N-sodium hydroxide (1 l.); [9] 0·2M-sodium borate (*B*).[10]

(iii) *Spray reagents.* The spray reagents used for the detection of compounds were: (*a*) silver nitrate in acetone–ethanolic sodium hydroxide; [11] (*b*) *p*-anisidine–hydrochloric acid; [12] (*c*) potassium periodatocuprate–rosaniline; [13] (*d*) aniline hydrogen phthalate.[14]

Rates of Formation of Mono- and Di-carboxylic Acids from Methyl β-Maltoside.—Platinum catalyst [Adams platinum dioxide (0·5 g.) reduced with hydrogen [15]] was added to a solution containing methyl β-maltoside [16] (1·025 g.) and sodium hydrogen carbonate (0·1 g.) in water (25 ml.). The mixture was stirred at 60° and oxygen passed through. Additional quantities of sodium hydrogen carbonate were added to maintain pH 7·5—8·5. Aliquot portions were withdrawn at time intervals, treated with Amberlite resin IR-120(H^+), and examined by paper electrophoresis in electrolyte (*a*) using spray reagent (*a*). The stained electrophoretograms were rendered translucent by impregnation with liquid paraffin. The relative optical densities of the spots with $M_{GA}(P)$ 0·77 (fraction A) and 1·25 (fraction B) were then measured with an EEL Scanner which incorporates a light source, a selenium cell, and a microammeter. The results are shown in the Figure.

Preparation of Monocarboxylic Acids from Methyl β-Maltoside.—(i) Methyl β-maltoside (4·1 g.) was oxidised in a manner similar to that described above except that the reaction was terminated after 8·5 hr. After cooling, the reaction mixture was filtered through "Celite." The solution was treated with Amberlite resin IR-120(H^+) followed by treatment with Duolite resin A4 (OH^-). From the latter acidic materials were eluted with N-sodium hydroxide. The eluate was freed from sodium ions by treatment with Amberlite resin IR-120(H^+) and then evaporated to a syrup. Preparative paper chromatography of the syrup using solvent (*a*) yielded two syrupy fractions: A (1·23 g.), with R_G 0·61 and $M_{GA}(P)$ 0·77; B (132 mg.), with R_G 0·73 and $M_{GA}(P)$ 1·25.

(ii) *Separation of monocarboxylic acids.* A mixture of the acidic fraction A (1·5 g.), benzaldehyde (4 g.), and freshly fused zinc chloride (1·5 g.) was shaken for 15 hr. Most of the excess of benzaldehyde was distilled off *in vacuo*. Ethanol (10 ml.) was added followed by light

petroleum (b. p. 100—120°; 20 ml.). The lower layer of the two-phase mixture was concentrated *in vacuo*. Preparative paper chromatography using solvent (*b*) and spray reagent (*a*) yielded methyl 4-*O*-(α-D-glucopyranosyluronic acid)-β-D-glucopyranoside (I; 900 mg.; R_G 0·79), contaminated with small quantities of unchanged methyl 4-*O*-α-D-glucopyranosyl-β-D-glucopyranosiduronic acid (II) (see below), and the *O*-benzylidene derivative of acid (II) [365 mg. R_G 3·2 (streak)].

The *O*-benzylidene derivative of acid (II) (360 mg.) was heated in 0·1N-methanolic hydrogen chloride (10 ml.) at 60° for 30 min. After neutralisation with N-sodium methoxide, the solution was evaporated to dryness *in vacuo*. The residue was extracted with ethanol, the extract concentrated, and the syrupy residue heated *in vacuo* at 125° for 30 min. Fractionation by paper chromatography using solvent (*a*) gave acid (II) (150 mg.), with R_G 0·61 and $M_{GA}(P)$ 0·77, as the main component. In addition, small amounts of components with R_G values identical with those of glucuronic acid, glucose, and methyl β-D-glucopyranosiduronic acid were present in the hydrolysate.

Characterisation of Acid (I).—(i) Acid (I) could be detected on paper chromatograms and electrophoretograms with spray reagent (*a*) but not with (*b*).

(ii) *Partial hydrolysis.* Acid (I) (900 mg.) was partially hydrolysed with N-sulphuric acid (25 ml.) at 90° for 2 hr. After cooling, sulphuric acid was removed by extraction with a 5% solution of *NN*-di-n-octylmethylamine in chloroform (4 × 20 ml.). Preparative paper chromatography of the concentrated aqueous layer, using solvent (*d*) and spray (*a*), gave 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (IV, 660 mg.) with R_G 0·29, $M_{GA}(P)$ 0·77, and $[\alpha]_D$ +113° (water). Small amounts of components with R_G values identical with those of glucuronic acid, glucurone, glucose [all revealed by spray (*a*)], and methyl β-D-glucopyranosiduronic acid [revealed by sprays (*b* and *c*)] were also present in the hydrolysate.

(iii) O-*Methyl-D-glucoses from acid* (IV). Acid (IV) (0·56 g.) was heated under reflux with 2% methanolic hydrogen chloride (150 ml.) for 6 hr. The solution was neutralised with silver carbonate, filtered, and concentrated *in vacuo*. The syrup was methylated according to the method of Kuhn, Baer, and Seeliger.[17] The methylated material was dissolved in a mixture of dioxan and ether (1 : 1 v/v; 80 ml.). A solution of lithium aluminium hydride (75 mg.) in the above solvent (80 ml.) was added dropwise with stirring. After *ca.* 18 hr. the excess of lithium aluminium hydride was destroyed by addition of ethyl acetate (1·5 ml.), followed by water (5 ml.). The reaction mixture was adjusted to pH 8 with N-sulphuric acid, filtered, and extracted with chloroform. Removal of the chloroform by distillation *in vacuo* left a clear syrup (0·49 g.) which had OMe, 47·5%.

The syrup (*ca.* 0·4 g.) was hydrolysed with 2N-hydrochloric acid (80 ml.) at 100° for 4 hr. The solution was neutralised with silver carbonate, filtered, and concentrated *in vacuo*. Paper chromatography of the residue using solvent (*c*) and spray (*b*) revealed the presence of only two, not well separated, components with R_{TMG} 0·90 and 0·88. The faster-moving component gave a yellow-brown stain, whereas the slower stained pink. The remainder of the residue was fractionated into two components on a charcoal–Celite column [18] (3·5 × 44 cm.) with aqueous ethyl methyl ketone as the eluant [linear gradient; 2·5% solution (1·5 l.), 5·5% solution (1·5 l.)].

The component which was eluted first stained pink with spray reagent (*d*) and was crystallised (90 mg.) from ether, m. p. 116°, $[\alpha]_D^{21}$ +70° (equilibrium, in water) (Found: C, 48·2; H, 8·0. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·2%). Admixture with authentic 2,3,6-tri-*O*-methyl-D-glucose,[19] m. p. 115—116°, caused no depression in m. p.

The second component (145 mg., syrup) stained yellow-brown with spray reagent (*b*) and gave a crystalline *N*-*p*-nitrophenylglycosylamine, m. p. 220—222°. Admixture with authentic 2,3,4-tri-*O*-methyl-*N*-*p*-nitrophenyl-D-glucosylamine,[20] m. p. 219—222°, caused no depression in m. p.

(iv) *Further characterisation of the tri-O-methyl-D-glucoses.* The tri-*O*-methyl-D-glucoses (4—5 mg.) were separately reduced with sodium borohydride. Treatment of the deionised solutions with sodium metaperiodate in the dark at room temperature gave the following results. The reduced 2,3,6- and 2,3,4-tri-*O*-methyl-D-glucose respectively consumed 0·92 and 1·05 moles of periodate and gave 0 and 1·04 moles of formaldehyde per mole of tri-*O*-methylhexitol.

The excess of periodate in each solution was destroyed by addition of ethylene glycol. The deionised solutions were concentrated, and the residues dried *in vacuo* and demethylated with boron trichloride.[21] Paper chromatography and electrophoresis of the demethylated materials

revealed in each case the presence of a single product. That from 2,3,6-tri-*O*-methyl-D-glucose had R_G 2·0 [solvent (*b*)] and $M_S(Mo)$ 0·56 identical with those of threose. The product from 2,3,4-tri-*O*-methyl-D-glucose had R_G 1·6 [solvent (c)] and $M_R(As)$ 0·18, identical with those of xylose.

(v) *Methyl ester hepta-acetate of acid* (IV). Acid (IV) (80 mg.) in methanol (7·5 ml.) was treated with a solution of diazomethane in ether [22] (*ca.* 30 ml.). After 30 min. the solution was evaporated to dryness. Acetic anhydride (15 ml.) and anhydrous sodium acetate (75 mg.) were added, the mixture was heated on the water-bath for 2 hr., and then poured into ice–water. The water was extracted with chloroform. The residue obtained after removal of chloroform was crystallised from methanol to give 1,2,3,6-tetra-*O*-acetyl-4-*O*-(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)-β-D-glucopyranose (VI, 60 mg.), m. p. 193—195°, $[\alpha]_D$ +79° (in chloroform) (Found: C, 48·8; H, 5·6; MeO, 4·9; Ac, 43·8. Calc. for $C_{27}H_{36}O_{19}$: C, 48·8; H, 5·5; MeO, 4·7; Ac, 45·3%). It exhibited absorption at 835 and 885 cm.$^{-1}$. Dutton and Slessor report m. p. 197—198°, $[\alpha]_D$ +77° (in chloroform).[4]

Characterisation of Acid (II).—(i) *Hydrolysis.* Acid (II) (5 mg.) was hydrolysed with N-sulphuric acid at 80° for 2 hr. Sulphuric acid was removed by extraction with 5% of *NN*-di-n-octylmethylamine in chloroform. Paper chromatography of the aqueous solution using solvent (*d*) revealed the presence of components with R_G values identical with those of acid (IV) (trace), glucuronic acid, glucose (large spot), methyl β-D-glucopyranosiduronic acid (large spot), and glucurone (trace).

(ii) *O-Methyl-D-glucoses from acid* (II). Acid (II) (150 mg.) was esterified by treatment with diazomethane, methylated, reduced, and hydrolysed under conditions similar to those described for acid (IV). Paper chromatography and electrophoresis of the hydrolysate revealed the presence of only two components which had R_{TMG} 1·0 and 0·68 [solvent (*c*)], M_G (*B*) 0 and 0·12, identical with those of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose, respectively.

(iii) *Methyl ester hexa-acetate of acid* (II). Acid (II) (40 mg.) was esterified and acetylated as described for acid (IV) to give methyl [4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)(*methyl* 2,3-*di*-O-*acetyl*-β-D-*glucopyranosid*)]*uronate* (VIII; 22 mg.), m. p. 173—177°, $[\alpha]_D$ +80° (in chloroform) (Found: C, 49·2; H, 5·7; MeO, 9·6; Ac, 38·5. $C_{26}H_{36}O_{18}$ requires C, 49·1; H, 5·7; MeO, 9·8; Ac, 40·6%). It exhibited absorption at 840 and 895 cm.$^{-1}$.

Preparation of Acid (III).—Methyl β-maltoside (2·5 g.) was oxidised with oxygen in the presence of platinum catalyst, as described above except that the oxidation was continued for 18 hr. when acid (III) was the main acidic product (181 mg.). Its equivalent weight, determined by titration with alkali,[23] was 198.

Characterisation of Acid (III).—Acid (III) (181 mg.) was hydrolysed with N-sulphuric acid (90 ml.) at 100° for 15 hr. Sulphuric acid was extracted with 5% *NN*-di-n-octylmethylamine in chloroform. Preparative paper chromatography of the concentrated aqueous layer using solvent (*a*) and spray (*b*) gave a component (90 mg.) with R_G 0·31 and $M_{GA}(P)$ 1·3. (The hydrolysate contained also a small quantity of a component with chromatographic and electrophoretic properties identical with those of glucuronic acid.) This was esterified and acetylated as described for acid (IV) to give *methyl* [1,2,3-*tri*-O-*acetyl*-4-O-(*methyl* 2,3,4-*tri*-O-*acetyl*-α-D-*glucopyranosyluronate*)-β-D-*glucopyran*]*uronate* (IX, 42 mg.), m. p. 215—220° (from methanol), $[\alpha]_D$ +88° (in chloroform) (Found: C, 48·4; H, 5·4; MeO, 9·7; Ac, 38·4. $C_{26}H_{34}O_{19}$ requires C, 48·0; H, 5·3; MeO, 9·5; Ac, 39·7%).

Another sample of acid (III) (70 mg.) was hydrolysed with 90% formic acid (10 ml.) at 100° for *ca.* 15 hr. The solution was evaporated to dryness and the residue further hydrolysed with N-sulphuric acid (2 ml.) at 100° for 2 hr. Sulphuric acid was removed by extraction with 5% *NN*-di-n-octylmethylamine in chloroform. The aqueous solution was treated with Amberlite resin IR-4B(OH^-) and the resin eluted with 10% aqueous formic acid. Formic acid was extracted from the eluate with ether. After removal of water by distillation *in vacuo* the residue was crystallised from glacial acetic acid to give a product (15 mg.) with m. p. 174—175°, $[\alpha]_D^{18}$ +16° (in water). Admixture with authentic D-glucurone,[24] m. p. 174—175°, caused no depression in m. p.

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22.

Studies on Dextrans and Dextranases. Part VI.[1] Type and Degree of Branching in Two Dextrans

By D. Abbott and H. Weigel

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Studies on Dextrans and Dextranases. Part VI.[1] Type and Degree of Branching in Two Dextrans

By D. Abbott and H. Weigel

The dextrans elaborated by *Leuconostoc mesenteroides* NRRL B-1415 (dextran A) and NRRL B-1416 (dextran B) have been shown to have branched structures. Dextran A contains 14% (α-1 ⟶ 4) branching glucose units. Dextran B contains 17% (10% α-1 ⟶ 3 and 7% α-1 ⟶ 4) branching glucose units.

PREVIOUS Reports in this Series have been concerned mainly with the α-1 ⟶ 3- branched dextran of *Leuconostoc mesenteroides* (Birmingham). By the action of various dextranases on this dextran we obtained branched oligosaccharides.[2] On the grounds of their structures and yields we reached certain conclusions regarding the detailed structure of this dextran. It seemed of interest to extend our studies to dextrans containing branch linkages other than the α-1 ⟶ 3, *e.g.*, an α-1 ⟶ 4-branch linkage. It was thus imperative to establish first the type and degree of branching in such dextrans. We chose for these investigations the dextrans elaborated by *L. mesenteroides* NRRL B-1415 (dextran A) and NRRL B-1416 (dextran B), since periodate-oxidation studies[3] had suggested that these contain 1 ⟶ 2- and/or 1 ⟶ 4-branch linkages.

The dextrans A and B were shown to be D-glucans. Paper-chromatographic analysis of the hydrolysates revealed trace quantities of a material which might have been fructose and could have arisen from dextran molecules terminated by a fructose unit.[4]

Partial hydrolysates of each dextran were shown by paper chromatography to contain the oligosaccharides of the isomaltose series and traces of maltose. In addition, nigerose was revealed in the partial hydrolysate of dextran B. Fractionation on charcoal–Celite columns of the partial hydrolysate of each dextran gave isomaltose and isomaltotriose, which were characterised as their acetate and benzoate, respectively.

Acetolysis.—The value of acetolysis of polysaccharides containing more than one type of glycosidic linkage has been demonstrated in several cases,[5] and disaccharides containing a linkage which would easily be cleaved under the conditions of acidic hydrolysis[6] have been obtained by this method. Accordingly, dextrans A and B were acetolysed and the products obtained were deacetylated. Both dextrans gave isomaltose and maltose, but dextran B gave, in addition, nigerose (all characterised as the octa-*O*-acetyl derivatives of their β-anomers). In the separation of isomaltose, maltose, and nigerose obtained from dextran B use was made

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of the ability of the reducing glucose unit of isomaltose and nigerose to form furanosides.[7]

Methylation.—Each dextran was methylated with sodium and methyl iodide in liquid ammonia.[8] Paper chromatography of a hydrolysate of methylated dextran A revealed three components which had properties identical with those of 2,3,4,6-tetra-, 2,3,4-tri-, and 2,3-di-*O*-methyl-D-glucoses. Their identity was confirmed on larger-scale fractionation of the hydrolysate on a charcoal–Celite column. The 2,3,4,6-tetra-*O*-methyl-D-glucose was obtained as its crystalline α-anomer and further characterised as its aniline derivative. The 2,3,4-tri-*O*-methyl-D-glucose was characterised by conversion into *N*-*p*-nitrophenyl-2,3,4-tri-*O*-methyl-D-glucosylamine. The 2,3-di-*O*-methyl-D-glucose was obtained as the crystalline α-anomer of its pyranose form and further characterised as its aniline derivative and methyl α-glycoside. Reduction of the sugar, followed by periodate oxidation and demethylation gave the results expected for a 2,3-di-*O*-methyl-D-glucose.

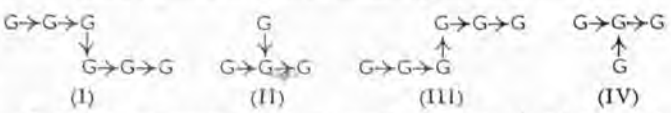
Paper chromatography of a hydrolysate of methylated dextran B revealed four components which had properties identical with those of 2,3,4,6-tetra-, 2,3,4-tri-, 2,3-di-, and 2,4-di-*O*-methyl-D-glucoses. They maintained their identity during electrophoresis in borate solution.[9] The identity of the 2,3- and 2,4-di-*O*-methyl-D-glucoses was confirmed by treatment with potassium borohydride, sodium metaperiodate, and finally with boron trichloride to give threose and xylose, respectively.

Periodate Oxidation.—Dextran A consumed 1·88 mol. of periodate, and produced 0·85 mol. of formic acid, per anhydroglucose unit. The corresponding figures for dextran B were 1·73 and 0·83.

Fragmentation of Periodate-oxidised Dextrans.—The controlled degradation of both dextrans by periodate oxidation, reduction, and hydrolysis, a procedure developed by Smith and co-workers,[10] gave glycerol (characterised as the tribenzoate) and erythritol (characterised as the tetrabenzoate). Dextran B gave, in addition, D-glucose which was characterised as the penta-*O*-acetyl-derivative of its β-anomer. No structural significance can reasonably be attached to the trace amounts of glucose detected in the hydrolysate of dextran A. The results obtained from dextran A, expressed as molecular proportions, were glycerol, 86·9; erythritol, 12·6; D-glucose, 0·5. The corresponding figures for dextran B were 83·2, 6·7, and 10·1.

Structure of Dextran A.—The isolation of isomaltose, isomaltotriose, and maltose from dextran A (and the detection of the oligosaccharides of the isomaltose series in a partial hydrolysate) shows that this dextran contains α-1 ⟶ 6- and α-1 ⟶ 4-glucosidic linkages. Maltose could arise from a segment of an unbranched dextran molecule where a continuous chain of α-1 ⟶ 6-linked glucose units is intersected by an α-1 ⟶ 4-linkage (I). Such a segment should give rise, on methylation and hydrolysis, to 2,3,6-tri-*O*-methyl-D-glucose. However, we were able to detect (and isolate) only one tri-*O*-methyl-D-glucose, namely the 2,3,4-isomer, which results from 1 ⟶ 6-linked glucose units (cf. detection and separation of 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucoses reported in Part V [1]). We are thus led to the conclusion, which is supported by the isolation of 2,3-di-*O*-methyl-D-glucose, that this dextran has a branched structure (II) where the branch linkages are of the α-1 ⟶ 4-type.

Possible segments of dextran containing anomalous linkages.



G, D-Glucopyranosyl unit; →, α-1 ⟶ 6-linkage; ↓, α-1 ⟶ 4-linkage; ↑, α-1 ⟶ 3-linkage.

It is now possible to calculate the percentage of branching glucose units in this dextran from (*a*) the ratio of the *O*-methyl-D-glucoses produced by hydrolysis of the methylated dextran A; (*b*) the amounts of periodate consumed and formic acid produced on treatment of the dextran with sodium metaperiodate; and (*c*) the ratio of the acyclic polyols (glycerol and erythritol) produced by the procedure of Smith and co-workers [10] (see Table). These independent determinations are in good agreement, and show that, on average, the repeating unit of dextran A contains *ca.* 7 anhydroglucose units.

Percentage of branching glucose units in dextrans

		Method [a]					
Dextran	Type of branching	(i)	(ii)	(iii)	(iv)	Average	No. of units [b]
A	α-1 ⟶ 4	11·2	13·5	18	12·6	14·3	7
	α-1 ⟶ 3			−3	0·5	Negligible	
B	α-1 ⟶ 4	17·1	14·9	7	6·7	6·9	6
	α-1 ⟶ 3			10	10·1	10·1	

[a] (i) Methylation, from molecular proportion of di-*O*-methyl-ether. (ii) Methylation, from molecular proportion of tetra-*O*-methyl ether. (iii) Periodate oxidation. (iv) Smith degradation. [b] No. of anhydroglucose units in average repeating unit of dextran.

Structure of Dextran B.—Dextran B possessed structural features similar to those of dextran A, *i.e.*, α-1 ⟶ 4-branch linkages (cf. maltose, 2,3-di-, and 2,3,4-tri-*O*-methyl-D-glucose). Acetolysis of this dextran yielded nigerose, in addition to maltose and isomaltose. Nigerose could arise from a segment of the dextran molecule such as (III). However, as was the case with dextran A, 2,3,4-tri-*O*-methyl-D-glucose was the only triether of D-glucose detectable in the hydrolysate of methylated dextran B. This hydrolysate contained, in addition to the ethers named above, 2,4-di-*O*-methyl-D-glucose. This was identified by paper chromatography and, after

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isolation, by reduction, periodate oxidation, and finally demethylation. Thus, dextran B contains α-1 ⟶ 4-(II) and α-1 ⟶ 3-branch linkages (IV). The percentage of branching glucose units in this dextran (Table) has been calculated from experiments similar to those described for dextran A. On average, the repeating unit of dextran B contains *ca.* 6 anhydroglucose units.

EXPERIMENTAL

General.—(i) *Paper chromatography.* The solvents used were (*a*) ethyl acetate–pyridine–water (2 : 1 : 2, organic phase); (*b*) butan-1-ol–ethanol–water–ammonia (40 : 10 : 49 : 1, organic phase); (*c*) butan-1-ol–ethanol–water (40 : 11 : 19). Migration rates are expressed relative to the movement of D-glucose [R_G (solvent)] and 2,3,4,6-tetra-*O*-methyl-D-glucose [R_{TMG} (solvent)].

(ii) *Paper electrophoresis.* The electrolytes used were (*a*) 0·2M-sodium borate (*B*);[9] (*b*) 2% sodium molybdate, adjusted to pH 5 (*Mo*).[11] The symbols used to express rates of migration have been described in Part V.[1]

(iii) *Spray reagents.* The spray reagents used for the detection of compounds were (*a*) silver nitrate in acetone–ethanolic sodium hydroxide;[12] (*b*) Aniline hydrogen-phthalate;[13] (*c*) *p*-anisidine–hydrochloric acid.[14]

Preparation and Purification of Dextrans.—The dextrans were isolated by precipitation with excess of acetone from culture media [1·2 l. containing sucrose (10%), yeast extract (1%), peptone (1%), $NH_4NaHPO_4.4H_2O$ (0·5%), and KH_2PO_4 (0·1%)] of *Leuconostoc mesenteroides* NRRL B-1415 (dextran A) and *L. mesenteroides* NRRL B-1416 (dextran B) after incubation for 3—4 days at 25°. The crude dextrans were dissolved in boiling water (1·5 l.) and the insoluble material removed by centrifuging (10 min. at 1500 r.p.m.). Ethanol (1·18 l.) was added to the supernatants and the precipitated dextrans were redissolved in boiling water (1·5 l.). After cooling, protein was removed by the method of Sevag *et al.*[15] The dextrans were again precipitated with ethanol, redissolved in water, dialysed, freeze-dried, and finally dried *in vacuo* over phosphoric oxide at 60°. The average yields (based on sucrose used) of dextrans A and B were 9 and 14% respectively. The dextrans A and B had, respectively, ash, 1·2 and 0·1; N, <0·1%; $[\alpha]_D^{20}$ +202° and +204° (*c* 1 in N-NaOH); $[\alpha]_D^{20}$ +215° and +216° (*c* 1 in $HCONH_2$).

Acidic Hydrolysis of Dextrans.—(i) Dextrans A and B (*ca.* 1·2 g.) were separately hydrolysed with 1·5N-sulphuric acid (10 ml.) at 100° for 9 hr. Paper chromatography using solvent (*a*) of each deionised hydrolysate revealed, as the main component, a material which had migration and staining properties identical with those of glucose. A trace component in each hydrolysate had properties similar to those of fructose. Preparative paper chromatography of each hydrolysate gave α-D-glucose (0·94 g. from dextran A; 0·86 g. from dextran B), having m. p. and mixed m. p. 144—146°, $[\alpha]_D^{20}$ +110° (3·5 min.) ⟶ 52° (equil.) (*c* 0·4 in H_2O).

After hydrolysis of another sample of each dextran (20 mg.) the amount of glucose was determined by cuprimetric titration.[16] The results, corrected for ash and loss in reducing power of glucose under the above conditions,[17] were dextran A, 98·0%; dextran B, 98·5%.

(ii) Each dextran (*ca.* 2·5 g.) was separately hydrolysed with 1·5N-sulphuric acid (100 ml.) at 100° for 2 hr. Paper chromatography, using solvent (*a*), of each deionised hydrolysate revealed the presence of glucose and the oligosaccharides of the isomaltose series. In addition, the hydrolysate of dextran A contained traces of a component with R_G identical with that of maltose, while that of dextran B contained traces of components with R_G identical with those of maltose and nigerose.

The remainder of each hydrolysate was fractionated on charcoal–Celite columns (3·5 × 40 cm.)[18] to give samples of glucose and the oligosaccharides of the isomaltose series (chromatography and optical rotatory evidence). Those of isomaltose and isomaltotriose were respectively converted into octa-*O*-acetyl-β-isomaltose[19] {m. p. and mixed m. p. 143—145°; $[\alpha]_D^{20}$ +100° (*c* 0·4 in $CHCl_3$)} and undeca-*O*-benzoyl-β-isomaltotriose[19] {m. p. and mixed m. p. 226—228°; $[\alpha]_D^{20}$ +131° (*c* 0·35 in $CHCl_3$)}.

Acetolysis of Dextrans.—(i) Dextrans A (4·2 g.) and B (4·6 g.) were separately treated with a mixture (*ca.* 52 ml.) of acetic anhydride, glacial acetic acid, and concentrated sulphuric acid (24 : 16 : 3, v/v) at 30° for 7 days. The mixtures were then heated at 80° for 30 min. and, after cooling, poured on to crushed ice (*ca.* 100 g.). The precipitated acetates were dissolved in chloroform (*ca.* 370 ml.), the chloroform solutions washed with sodium hydrogen carbonate solution, and dried (Na_2SO_4). The syrups obtained after evaporation of chloroform were dissolved in absolute methanol (50 ml.). Deacetylation was effected by addition of a small piece of sodium to each solution. After 2 days the solutions were neutralised with hydrochloric acid and evaporated *in vacuo*. The residues were dissolved in water, deionised and evaporated to syrups. A portion of each syrup was fractionated by paper chromatography using solvent (*a*). That from dextran A gave materials which had R_G (*a*) identical with those of fructose (1·30), glucose (1·0), maltose (0·75), isomaltose (0·69), and isomaltotriose (0·39). The syrup obtained from dextran B gave, in addition to the above, a material corresponding to nigerose [R_G(*a*) 0·81]. The disaccharide fractions maintained their identity during electrophoresis in electrolyte (*a*) and, after treatment with potassium borohydride, had M_s(*Mo*) identical with those of the corresponding reduction products, *i.e.*, nigeritol (0), maltitol (0·46), and isomaltitol (0·78).

(ii) *Fractionation of disaccharides from dextran A on charcoal–Celite column.* The remainder of the above syrup was dissolved in water (80 ml.) and the solution applied to a charcoal–Celite column[18] (3 × 50 cm.). Elution with water (2 l.) removed glucose and trace amounts of fructose (chromatographic evidence). The column was then eluted with mixtures of ethanol and aqueous borate

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solution [20] {linear gradient; ethanol (1 l.), aqueous sodium borate [boric acid (0·75%), sodium hydroxide (0·4%), pH 10; 1 l.]}. The fractions obtained with 1·5—3% and 5—10% ethanol contained isomaltose and maltose, respectively, as the major component. The fractions containing only a single component were deionised and then freeze-dried to give isomaltose (365 mg.) and maltose (368 mg.). They were respectively converted into octa-*O*-acetyl-β-isomaltose [19] {m. p. and mixed m. p. 140—142°, $[\alpha]_D^{22}$ +99° (*c* 0·35 in $CHCl_3$)} and octa-*O*-acetyl-β-maltose [21] {m. p. and mixed m. p. 158—161°, $[\alpha]_D^{20}$ +62° (*c* 0·2 in $CHCl_3$)}.

(iii) *Fractionation of disaccharides from dextran B on charcoal–Celite column.* The remainder of the above syrup was dissolved in 4% methanolic hydrogen chloride (60 ml.) and the solution left at room temperature for *ca.* 3 days. The solution was neutralised with silver carbonate. Methanol was removed by distillation *in vacuo* (below 35°) over barium carbonate. The residue was extracted with cold water (30 ml.) and the solution applied to a charcoal–Celite column (acid-free, 3·5 × 50 cm.). Elution with water (2 l.) removed glucose and traces of fructose as glycosides (chromatographic evidence).

Elution of the column with 2% aqueous ethanol (800 ml.) gave fractions, the major component of which had $M_G(B)$ 0·53. The residue, obtained after evaporation of the solvent, was dissolved in 0·01N-hydrochloric acid (100 ml.) and the solution kept at 45—55° for 2 days. The component of this reaction mixture which migrated in solvent (*a*) at the same rate as isomaltose (R_G 0·69) was isolated by paper chromatography to give chromatographically pure material (136 mg.). This was converted into octa-*O*-acetyl-β-isomaltose,[19] m. p. and mixed m. p. 143—145°, $[\alpha]_D^{22}$ +99° (*c* 0·4 in $CHCl_3$).

Fractions obtained with 5% ethanol (900 ml.) contained a major component with $R_G(b)$ 0·33 and $M_G(B)$ 0·31, identical with those of maltose. Purification by paper chromatography gave a material (198 mg.) which was converted into octa-*O*-acetyl-β-maltose,[21] m. p. and mixed m. p. 157—159°, $[\alpha]_D^{20}$ +62° (*c* 0·4 in $CHCl_3$).

Elution of the column with 10—15% ethanol gave fractions the major component of which had $R_G(b)$ 1·25 (streak) and $M_G(B)$ 0·30. They were treated as those obtained with 2% ethanol to give chromatographically pure material (118 mg.) with $R_G(a)$ 0·81 and $M_G(B)$ 0·69. This was converted into octa-*O*-acetyl-β-nigerose,[5b] m. p. and mixed m. p. 149—150°, $[\alpha]_D^{21}$ +82° (*c* 0·2 in $CHCl_3$).

Methylation of Dextrans.—Dextrans A (4 g.) and B (2 g.) were separately methylated by several treatments with sodium and methyl iodide in liquid ammonia until the OMe contents had become constant.[8] The methylated dextrans A (2·61 g.) and B (1·38 g.) had OMe, 44·0 and 44·1%; ash, 0·4 and 0·5%, respectively.

Isolation and Determination of Methylated D-*Glucoses obtained from Methylated Dextran A.*—Methylated dextran A (2·1 g.), in dry chloroform (6 ml.), was treated with 8% methanolic hydrogen chloride (6 ml.) in a sealed tube at 100° for 7·5 hr., and then, after removal of the solvent *in vacuo*, with N-hydrochloric acid (30 ml.) at 100° for 6·5 hr. Paper chromatography [solvent (*b*)] of the deionised reaction mixture revealed three components which had migration rates and staining properties identical with those of 2,3,4,6-tetra-*O*-methyl-D-glucose [$R_{TMG}(b)$ 1·0; orange-pink and red-pink stains with spray reagents (*b*) and (*c*), respectively], 2,3,4-tri-*O*-methyl-D-glucose [$R_{TMG}(b)$ 0·90; salmon pink and yellow-brown stains with spray reagents (*b*) and (*c*), respectively], and 2,3-di-*O*-methyl-D-glucose [$R_{TMG}(b)$ 0·68; orange-brown and yellow-brown stains with spray reagents (*b*) and (*c*), respectively].

A portion of the above hydrolysate was used for the quantitative determination of each component by the hypoiodite method.[22] The results, expressed as molecular proportions, were as follows: tetra-*O*-methyl-D-glucose, 13·5; tri-*O*-methyl-D-glucose, 75·3; di-*O*-methyl-D-glucose, 11·2.

The remainder of the hydrolysate was fractionated on a charcoal–Celite column (3·5 × 46 cm.) by the method of Lindberg and Wickberg.[23] Three fractions were obtained. The main component of each was removed by paper chromatography using solvent (*b*) to give chromatographically pure syrups C [$R_{TMG}(b)$ 0·69; 320 mg.], D [$R_{TMG}(b)$ 0·90; 985 mg.] and E [$R_{TMG}(b)$ 1·0; 295 mg.].

Characterisation of Methylated D-*Glucoses obtained from Methylated Dextran A.*—(i) Syrup C (*ca.* 315 mg.), crystallised from ether, gave 2,3-di-*O*-methyl-α-D-glucopyranose [24] (260 mg.), m. p. 86—87°, $[\alpha]_D^{18}$ +50° (equil.) (*c* 0·4 in Me_2CO). The residue obtained after evaporation of the mother-liquors was converted into *N*-phenyl-2,3-di-*O*-methyl-D-glucosylamine [25] (18 mg.), m. p. 131—133° (Found: MeO, 21·4. Calc. for $C_{14}H_{21}O_5N$: MeO, 21·9%). A portion (200 mg.) of the crystalline 2,3-di-*O*-methyl-D-glucose was converted into methyl 2,3-di-*O*-methyl-α-D-glucopyranoside [24] (120 mg.), m. p. 80—82°, $[\alpha]_D^{21}$ +145° (*c* 0·4 in H_2O) (Found: MeO, 41·0. Calc. for $C_9H_{18}O_6$: MeO, 41·9%).

Another portion of the crystalline 2,3-di-*O*-methyl-D-glucose (30 mg.; concentration determined by hypoiodite method [22]) was dissolved in water (100 ml.) and reduced with potassium borohydride (100 mg.). The solution was deionised [by treatment with Amberlite resin IR-120(H^+) followed by repeated distillation with dry methanol] and then treated with sodium periodate. The results, expressed per mole of di-*O*-methylhexitol, were as follows: periodate consumed, 1·94 moles; formic acid and formaldehyde produced, 0·98 and 0·96 moles, respectively.

Ethylene glycol was added to the above reaction mixture, the solution deionised with Amberlite resins IR-120(H^+) and IR-45(OH^-), and then evaporated to dryness *in vacuo*. The residue was treated with boron trichloride in dichloromethane.[26] Paper chromatography and electrophoresis of the product revealed a single component with migration rates identical with those of threose [R_G (*c*) 2·0; $M_S(Mo)$ 0·56].

(ii) Syrup D (*ca.* 100 mg.) was converted into *N*-*p*-nitrophenyl-2,3,4-tri-*O*-methyl-D-glucosylamine (65 mg.) by the method of Van Cleve *et al.*,[27] m. p. and mixed m. p. 224—226°, $[\alpha]_D^{22}$ −250° (equil.) (*c* 0·5 in pyridine).

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(iii) Syrup E (295 mg.), crystallised from ether, gave 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranose [28] (136 mg.), m. p. and mixed m. p. 87—88°, $[\alpha]_D^{22}$ +83·5° (equil.) (*c* 0·3 in H_2O). The residue obtained after evaporation of the mother-liquors was converted into 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucosylamine [28] (22 mg.), m. p. and mixed m. p. 135—137°, $[\alpha]_D^{19}$ +228·5° (equil.) (*c* 0·4 in Me_2CO).

Identification and Determination of Methylated D-*Glucoses obtained from Methylated Dextran B.*—Methylated dextran B (1·1 g.) was methanolysed and hydrolysed as described for methylated dextran A. Paper chromatography [solvent (*b*)] of a portion of the hydrolysate revealed the presence of components F, G, H, and J which had $R_{TMG}(b)$ identical with those of 2,3,4,6-tetra- (1·0), 2,3,4-tri- (0·90), 2,3-di- (0·68), and 2,4-di-*O*-methyl-D-glucose (0·64), respectively. Each was removed from chromatograms (only the faster and slower edges of components H and J, respectively, were removed) and shown to maintain their identity during electrophoresis in electrolyte (*a*).

The concentrations of the aqueous solutions of components H and J were determined by the hypoiodite method.[22] They were then treated with potassium borohydride, sodium metaperiodate and finally with boron trichloride as described for 2,3-di-*O*-methyl-D-glucose obtained from dextran A. Component H consumed 1·94 mol. of periodate, and produced 0·98 and 0·96 mol. of formic acid and formaldehyde, respectively. The corresponding figures for component J were: periodate, 0·96; formic acid, 0; formaldehyde, 0·92. The fragments obtained from component H and J by the above treatment had chromatographic and electrophoretic migration rates identical with those of threose and xylose, respectively.

A portion of the hydrolysate of methylated dextran B was used for the quantitative determination of its components. The di-*O*-methylglucoses, *i.e.*, components H and J, were determined together. The results, expressed as molecular proportions, were: tetra-*O*-methyl-D-glucose, 14·9; tri-*O*-methyl-D-glucose, 68·0; di-*O*-methyl-D-glucoses, 17·1.

Periodate Oxidation of Dextrans A and B.—To separate solutions of dextrans A and B (*ca.* 0·2—0·4 g.) in water (250 ml.) was added 0·3M-sodium metaperiodate (*ca.* 10—20 ml.). Each solution was made up to 500 ml. and kept in the dark at 25°. At intervals portions were withdrawn for the estimation of periodate consumed (by spectrophotometry [29] and titration with arsenite and iodine solutions [30]) and formic acid liberated (by titration with sodium hydroxide solution). The values became constant after *ca.* 100 hr. The number of moles of periodate consumed (average of both methods) per unit of anhydroglucose of dextrans A and B were then 1·88 and 1·73, respectively. The corresponding figures for formic acid liberated from dextrans A and B were 0·85 and 0·83, respectively.

Fragmentation of Periodate-oxidised Dextrans.—Dextrans A and B (*ca.* 9—10 g.) were separately oxidised with sodium metaperiodate (43 g.) as described above. Each solution was worked up in the following manner. Ethylene glycol (25 ml.) was added to destroy the excess of periodate. The solutions were then dialysed against running water for 3 days. Potassium borohydride (2·2 g.) in water (30 ml.) was added over 24 hr. After a further 6 hr. the solutions were neutralised with acetic acid and then concentrated *in vacuo* to *ca.* 300 ml. each. Concentrated hydrochloric acid was added until the pH was 1. The solutions were heated at 60—70° for 10 hr. After treatment with Amberlite resin IR-120(H^+) the solutions were concentrated *in vacuo* to syrups. These were repeatedly distilled with dry methanol. Paper chromatography (Whatman No. 3) revealed each syrup to contain four components with $R_G(c)$ 4—5, 3·2, 2·2, and 1·0 (only trace from dextran A), identical with those of glycollic aldehyde, glycerol, erythritol, and glucose, respectively.

A portion (*ca.* 1 g.) of each of the above syrups was fractionated by paper chromatography (Whatman No. 3MM) in solvent (*c*). The amounts of glycerol and erythritol in the fractions were determined from the quantities of formaldehyde (estimated by chromotropic acid method [31]) produced on oxidation with sodium metaperiodate. Those of glucose were determined by the cuprimetric method.[16] The results obtained from dextran A, expressed as molecular proportions, were glycerol, 86·9; erythritol, 12·6; D-glucose, 0·5. The corresponding figures for dextran B were 83·2, 6·7, and 10·1.

Fractionation by paper chromatography [Whatman No. 3; solvent (*c*)] of the remainder of each syrup gave syrupy samples of glycerol (4·28 and 4·09 g. from dextran A and B, respectively), erythritol (0·68 and 0·40 g. from dextran A and B, respectively), and glucose (0·68 g. from dextran B). They were respectively converted into tri-*O*-benzoylglycerol (m. p. and mixed m. p. 71·5°), tetra-*O*-benzoylerythritol (m. p. and mixed m. p. 184—185°), and penta-*O*-acetyl-β-D-glucopyranose {m. p. and mixed m. p. 130—132°, $[\alpha]_D^{20}$ +4·0° (*c* 0·4 in $CHCl_3$)}.

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23.

Studies on Dextrans and Dextranases. Part VII.[1] Structures of Oligosaccharides from an α-1 → 4-Branched Dextran

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Studies on Dextrans and Dextranases. Part VII.[1] Structures of Oligosaccharides from an α-1 → 4-Branched Dextran

By D. Abbott and H. Weigel

Dextranases produce from the dextran of *Leuconostoc mesenteroides* NRRL B-1415 oligosaccharides which contain one glucose unit joined by an α-1 → 4-link to a glucose unit of a homologue of isomaltose. A method for locating the branching glucose unit in branched oligosaccharides is described.

DEXTRANASES have been shown to produce complex mixtures of branched oligosaccharides from the α-1 → 3-branched dextran of *Leuconostoc mesenteroides* (Birmingham).[2] These oligosaccharides contain one glucose unit joined through an α-1 → 3-linkage to a glucose unit of a homologue of isomaltose. We now report the structural analysis of branched oligosaccharides produced by the action of the dextranases of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) (dextranase A), *P. funiculosum* (I.M.I. 79195; NRRL 1132) (dextranase B), and *Lactobacillus bifidus* (dextranase C) on the α-1 → 4-branched dextran of *L. mesenteroides* NRRL B-1415. (It is appreciated that some of these oligosaccharides and degradation products described below possess only one non-reducing end-group. Thus, the term branched is used to indicate the attachment of a glucose unit to isomaltose or its homologues by an α-1 → 4-link.) The repeating unit of this dextran has been shown [1] to contain, on average, *ca.* 7 anhydroglucose units, *i.e.*, its size is similar to that of the *L. mesenteroides* (Birmingham) dextran.[3]

The rates and amounts of reducing sugars liberated from the dextran by the dextranases (Figure 1) are digests revealed, in addition to the products expected from unbranched sections of the dextran,[4] the presence of branched oligosaccharides (series A, B, and C from dextranase A, B, and C, respectively). The latter were

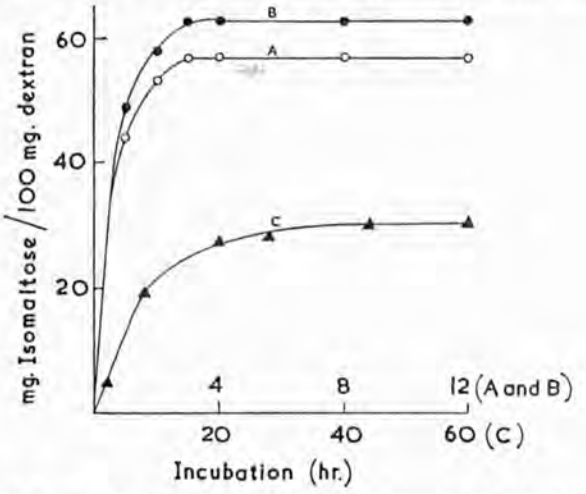

FIGURE 1 Rates of liberation of reducing sugars (as isomaltose) in dextran–dextranase digests. A, Dextranase A; B, dextranase B; C, dextranase C

TABLE 1

Yields and properties of branched oligosaccharides produced by dextranases A, B, and C

Branched oligosaccharide	Yield (g. from 100 g. dextran *) A	B	C	Carbohydrate content (%) A	B	C	Reducing power † (% of theoretical) A	B	C
Tetra		2·33	2·67		98·0	99·2		98·0	98·8
Penta	1·94	1·90	2·01	97·2	98·0	99·0	95·7	98·5	100·0
Hexa	4·97	4·82	1·18	96·5	95·2	99·2	96·9	102·9	91·7
Hepta	6·34	5·83		96·0	96·0		102·1	94·4	
Octa	4·73	4·03		95·0	96·0		101·6	99·0	

Branched oligosaccharide	Periodate oxidation: Periodate consumed (mol.) Found A	B	C	Calc.‡	HCO_2H produced (mol.) Found A	B	C	Calc.‡	Glycerol/erythritol (mol. prop.) Found A	B	C	Calc.‡
Tetra		7·9	7·9	8		3·9	3·9	4		2·8	2·9	3
Penta	10·2	10·1	9·9	10	5·2	4·9	4·9	5	3·9	3·8	3·8	4
Hexa	12·0	12·1	11·9	12	6·1	6·2	5·9	6	4·9	5·2	4·9	5
Hepta	13·9	14·1		14	7·1	6·8		7	5·8	5·8		6
Octa	15·5	15·4		16	7·6	7·4		8	6·1	6·2		7

* The actual quantities of dextran treated with dextranases A, B, and C were 30, 30, and 6·8 g., respectively. † Corrected for carbohydrate content. ‡ Calculated for oligosaccharides of the isomaltose series to which one glucopyranosyl unit is joined by an 1 → 4-linkage.

those found, under the same conditions, with dextrans possessing a similar degree of branching.[4]

Paper chromatography of the dextran–dextranase isolated by methods similar to those used for α-1 → 3-branched oligosaccharides.[2] The properties of the branched oligosaccharides (Tables 1 and 2 and Figure 2)

[1] Part VI, D. Abbott and H. Weigel, preceding Paper.
[2] E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, 1963, **86**, 555.
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show that they are members of a homologous series. The tetra-, penta-, hexa-, and hepta-saccharides (see below for structures of octasaccharides) are, in fact, compounds (with n glucose units) in which one D-glucopyranosyl unit is joined through an α-1 ⟶ 4-linkage to a glucose unit, other than the reducing one, of a homologue of isomaltose (isomaltodextrin) (with $n - 1$ glucose units). This assignment is based on the following data: (*a*) paper-chromatographic migration rates (Figure 2); (*b*) optical rotations (Figure 2); (*c*) identification of maltose in the partial hydrolysate of each branched oligosaccharide or its reduction product (branched oligosaccharide alcohol); (*d*) amounts of periodate reduced and formic acid liberated on treatment of the saccharides with sodium periodate (Table 1); (*e*) ratios of glycerol/erythritol found after periodate fragmentation (Table 1); (*f*) mobilities of the branched oligosaccharide alcohols during electrophoresis in molyb-

TABLE 2

Paper chromatography of glucamylase-saccharide digests

Saccharide	Component revealed in digest	Time of incubation (days): 0·5	1	2·5
		Relative density of spots (visual estimation)		
Amylose	Glucose	+	++	++++
Maltose	Glucose	+	+	
	Maltose	—	—	
Tetrasaccharide B	Glucose	++	+++	
	Isomaltotriose	++	+++	
	Tetrasaccharide B	+	—	
Pentasaccharide A	Glucose	+	++	+++
	Isomaltotetraose	+	++	+++
	Pentasaccharide A, B	++++	+++	+
Hexasaccharide A	Glucose	+	++	+++
	Isomaltopentaose	+	++	+++
	Hexasaccharide A, B	++++	+++	+

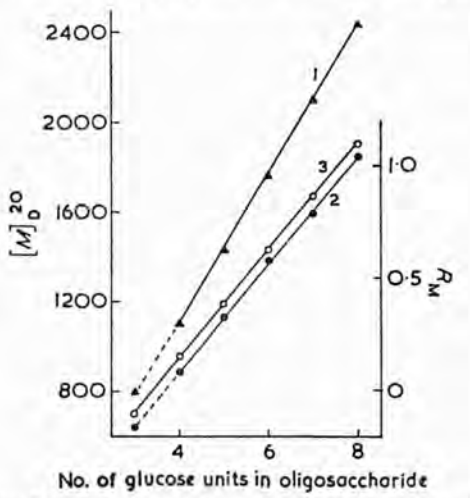

FIGURE 2 Optical rotations and paper-chromatographic migration rates of oligosaccharides

1, Molecular rotation, $[M]_D^{30}$, of branched oligosaccharides of series A, B, and C. 2, R_M values [$R_M = \log (1/R_{IM} - 1)$] of branched oligosaccharides of series A, B, and C. The branched tetrasaccharides are only those of series B and C, whereas the branched hepta- and octa-saccharides are only those of series A and B. 3, R_M values of homologues of isomaltose. The R_{IM} values have been measured using solvent (*a*). The molecular rotation (D. French, P. M. Taylor, and W. J. Whelan, *Biochem. J.*, 1964, **90**, 616) of 6-*O*-α-maltosyl-D-glucose and the R_M value of branched trisaccharides (Table 3, fraction 11a) have been included for comparison

TABLE 3

Products of hydrolysis of branched oligosaccharide alcohols

Oligosaccharide alcohol	Fraction	R_{IM} (*a*)	M_s (*Mo*)	Identity
1	1	0·09	0·31	Branched octasaccharide alcohol
2	2	0·12	0·35	Isomaltoheptaitol
3	3a	0·14	0	Branched heptasaccharide
4	3b	0·14	0·35	Branched heptasaccharide alcohol
	4a [a]	0·19	0	Isomaltohexaose
	4b	0·19	0·39	Isomaltohexaitol
5	5a	0·23	0	Branched hexasaccharide
	5b	0·23	0·39	Branched hexasaccharide alcohol
	6a [b]	0·29	0	Isomaltopentaose
	6b	0·29	0·44	Isomaltopentaitol
	7a	0·32	0	Branched pentasaccharide
	7b	0·32	0·44	Branched pentasaccharide alcohol
	8a [c]	0·40	0	Isomaltotetraose
	8b	0·40	0·53	Isomaltotetraitol
	9a	0·45	0	Branched tetrasaccharide
	9b [d]	0·45	0·53	Branched tetrasaccharide alcohol
	10a [e]	0·56	0	Isomaltotriose
	10b	0·56	0·62	Isomaltotri-itol
	11a	0·59	0	Branched trisaccharide
	11b [f]	0·59	0·62	Branched trisaccharide alcohol (X1)
	12a	1·0	0	Isomaltose
	12b	1·0	0·77	Isomaltitol
	13	1·08	0	Maltose
	14a	1·45	0	Glucose
	14b	1·45	1·0	Glucitol

1, Branched octasaccharide alcohols A and B. 2, Branched heptasaccharide alcohols A and B. 3, Branched hexasaccharide alcohols A, B, and C. 4, Branched pentasaccharide alcohols A, B, and C. 5, Branched tetrasaccharide alcohols B and C.

[a] Only from branched octasaccharide alcohols. [b] Only from branched hepta- and octa-saccharide alcohols. [c] Only from branched hexa-, hepta-, and octa-saccharide alcohols. [d] Not from branched hepta- and octa-saccharide alcohols. [e] Not from branched tetrasaccharide alcohols. [f] Only from branched tetra-, penta-, and hexa-saccharide alcohols of series C.

date solution [5] (Table 3); (*g*) formation of isomaltodextrins with $n-1$ glucose units and glucose on partial hydrolysis or glucamylolysis of branched oligosaccharides with n glucose units (Table 2); and (*h*) formation of reduced isomaltodextrins (isomaltodextrinols) with $n-2$ glucose units on partial hydrolysis of branched oligosaccharide alcohols with $n-1$ glucose units (Table 3).

Methylation of tetrasaccharide B, followed by hydrolysis, furnished 2,3,4,6-tetra-, 2,3,4-, and 2,3,6-tri-*O*-methyl-D-glucose. The molecular ratio of tri-/tetra-ethers was 2·85. This, together with the evidence above, shows conclusively that this saccharide is in fact 4^3-*O*-α-D-glucopyranosyl-isomaltotriose * (XII).

Methylation studies would, however, not distinguish between all possible structures of the higher branched saccharides, *e.g.*, identical results would be obtained from saccharides (XV) and (XVI). We feel that the method eventually adopted, a development of one used earlier,[2] can be applied, with minor modifications, to other types of branched or complex oligosaccharides and we therefore describe it in detail.

Location of Branching Glucose Unit in Oligosaccharides.—(i) *General procedure.* The method is based on the following facts: (*a*) branched oligosaccharides, the main segment of which is an isomaltose homologue unit, and homologues of isomaltose of the same molecular size can be separated chromatographically; (*b*) in the solvent used reducing saccharides have the same R_{IM} values (see Experimental section for definition of migration rates) as the corresponding saccharide alcohols; (*c*) glucose and its oligosaccharides do not form complexes with molybdate or tungstate [5] and, thus, do not migrate during electrophoresis in molybdate or tungstate solutions; (*d*) 1- and 3-mono-, and 1,3-di-*O*-glycopyranosyl-L-gulitols form complexes with molybdate and tungstate; and (*e*) the electrophoretic mobilities [6] of 1-*O*-glycopyranosyl-L-gulitols are expressed by equations (1) and (2):

$$1/M_R(Mo) = 1 + 0{\cdot}31n, \quad (1)$$

$$1/M_R(W) = 1 + 0{\cdot}32n, \quad (2)$$

whereas those of 3-mono- and 1,3-di-*O*-glycopyranosyl-L-gulitols are given by equations (3) and (4):

$$1/M_R(Mo) = 1{\cdot}52 + 0{\cdot}65n, \quad (3)$$

$$1/M_R(W) = 4{\cdot}07 + 1{\cdot}81n, \quad (4)$$

where n = number of D-glucopyranosyl units.

In this method four essential steps, outlined in Figure 3, erode the saccharide from its reducing end: (*a*) reduction (R) of the branched oligosaccharide [(I), n glucose units] to the corresponding alcohol [(II), $n-1$ glucose units]; (*b*) partial hydrolysis (H) of the branched saccharide alcohol (II); (*c*) chromatographic separation (C) of the next lower branched oligosaccharide [(III), $n-1$ glucose unit] and/or branched oligosaccharide alcohol [(IV), $n-2$ glucose units] from other products

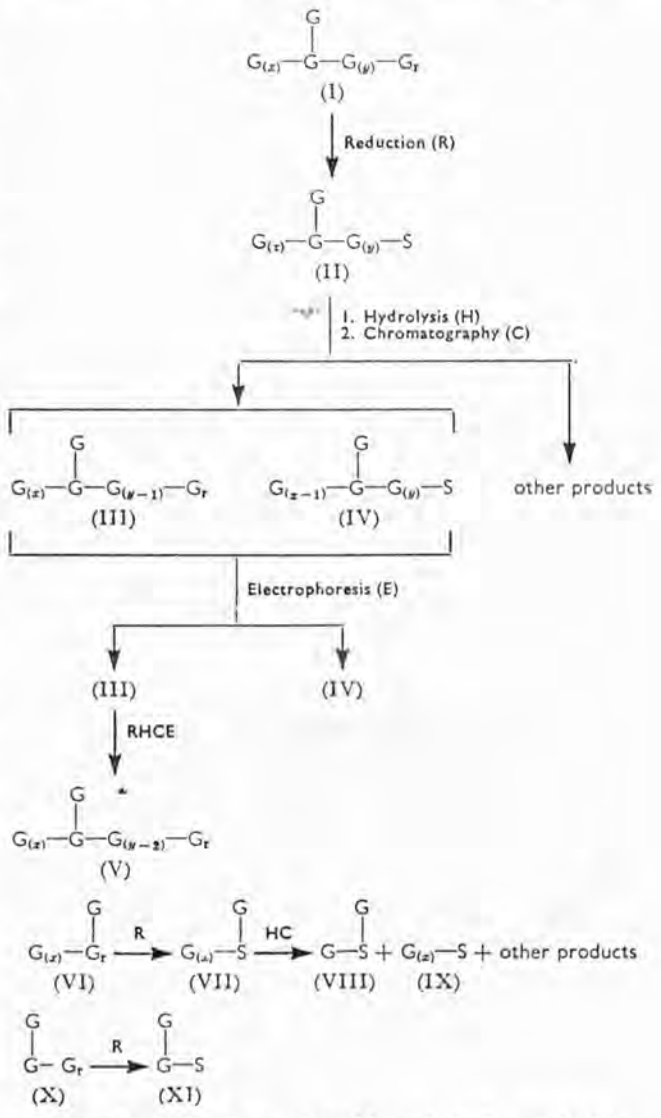

FIGURE 3 Reductive erosion of branched oligosaccharides

G, D-Glucopyranosyl unit; G_r, reducing D-glucose unit; S, L-gulitol (sorbitol); *x* and *y*, number of D-glucopyranosyl units; —, α-1 → 6- and α-1 → 1-link between D-glucose units and D-glucose and L-gulitol, respectively; |, α-1 → 4- and α-1 → 3-link between D-glucose units and D-glucose and L-gulitol, respectively.

of the hydrolysis; (*d*) electrophoretic separation (E) in molybdate or tungstate solutions of the branched oligosaccharide (III) from the branched oligosaccharide alcohol (IV).

One or more reductive erosions give, depending on the structure of the original branched oligosaccharide, the products (VI) or (X). The latter, 6-*O*-α-maltosyl-D-glucose, is obtained only from saccharides in which a

* The nomenclature used is essentially that suggested by W. J. Whelan (*Ann. Rev. Biochem.*, 1960, **29**, 105). In this system the compounds are named as derivatives of disaccharides or their homologues (root chain), *e.g.* homologues of isomaltose. The position number of the oxygen atom of the monosaccharide unit in the root chain to which the substituent is attached precedes the name of the substituent. The superscript numeral indicates the serial number of the monosaccharide unit of the root chain involved in substitution, the reducing unit being No 1.

[5] H. Weigel, *Adv. Carbohydrate Chem.*, 1963, **18**, 61.
[6] H. J. F. Angus and H. Weigel, *J. Chem. Soc.*, 1964, 3994.

glucose unit is joined by an α-1 ⟶ 4-link to the non-reducing end-group of an isomaltodextrin, *e.g.*, (XII). Its reduction product, 1-*O*-α-maltosyl-L-gulitol (XI), has $M_s(Mo)$ 0·62 and $M_s(W)$ 0·61. Reduction of the saccharide (VI) gives the branched saccharide alcohol (VII), the electrophoretic mobilities of which are expressed by equations (3) and (4). Partial hydrolysis of the alcohol (VII), followed by chromatographic separation of the products, yields the isomaltodextrinol (IX) with electrophoretic mobilities given by equations (1) and (2) ($n = x$), and 1,3-di-*O*-α-D-glucopyranosyl-L-gulitol (VIII) with $M_s(Mo)$ 0·38 and $M_s(W)$ 0·13.

The branched penta-, hexa-, hepta-, and octa-saccharides A were shown to be identical with the corresponding saccharides of series B. The corresponding saccharides (AB) are thus described together.

(ii) *Tetrasaccharide B* (XII). One reductive erosion gave a branched trisaccharide (Table 3, fraction 11a). The corresponding alcohol had $M_s(Mo)$ 0·62 and $M_s(W)$ 0·61, identical with those of 1-*O*-α-maltosyl-L-gulitol (XI). The tetrasaccharide was thus 4^3-*O*-α-D-glucopyranosyl isomaltotriose (XII), confirming the conclusion reached above.

(iii) *Branched pentasaccharide AB* (XV). Two reductive erosions gave a branched trisaccharide. The reduction product of this material had $M_s(Mo)$ 0·38 and $M_s(W)$ 0·13, identical with those of 1,3-di-*O*-α-D-glucopyranosyl-L-gulitol (VIII). The branched pentasaccharide AB was thus 4^3-*O*-α-D-glucopyranosylisomaltotetraose (XV).

(iv) *Branched hexasaccharide AB* (XVIII *and* XIX). Two reductive erosions of the saccharide gave a branched tetrasaccharide fraction. Reduction of this material, followed by fractionation in electrolyte (*c*) gave two branched tetrasaccharide alcohols with $M_s(W)$ 0·53 and 0·08. The former yielded, on partial hydrolysis, a branched trisaccharide, the alcohol of which had mobilities identical with those of 1,3-di-*O*-α-D-glucopyranosyl-L-gulitol (VIII) [$M_s(Mo)$ 0·38 and $M_s(W)$ 0·13]. Partial hydrolysis of the branched tetrasaccharide alcohol with $M_s(W)$ 0·08 gave the alcohol (VIII) and 1-*O*-α-isomaltosyl-L-gulitol (isomaltotri-itol) (IX; $x = 2$). The branched hexasaccharide AB was thus a mixture of the isomeric 4^3- (XIX) and 4^4-*O*-α-D-glucopyranosylisomaltopentose (XVIII).

(v) *Branched heptasaccharide AB* (XXI). Three reductive erosions gave a branched tetrasaccharide, the alcohol of which had $M_s(Mo)$ 0·33 and $M_s(W)$ 0·08. Hydrolysis of this alcohol gave 1,3-di-*O*-α-D-glucopyranosyl-L-gulitol (VIII) [$M_s(Mo)$ 0·38 and $M_s(W)$ 0·13] and isomaltotri-itol (IX; $x = 2$). Structure (XXI) (4^4-*O*-α-D-glucopyranosylisomaltohexaose) is thus assigned to the branched heptasaccharide AB.

(vi) *Branched octasaccharide AB* (XXII *and* XXIII). The results of the periodate oxidation of the saccharide (Table 1) are intermediate between those expected of octasaccharides containing (*a*) one glucose unit joined through a 1 ⟶ 4-linkage to isomaltoheptoase and (*b*) two glucose units joined through 1 ⟶ 4-linkages to isomaltohexaose. Partial hydrolysis of the branched octasaccharide alcohol AB gave, in addition to 1-*O*-α-isomaltohexaosyl-L-gulitol (isomaltoheptaitol) (IX; $x = 6$) and a branched heptasaccharide, products which had the same chromatographic and electrophoretic mobilities as those produced from branched heptasaccharide

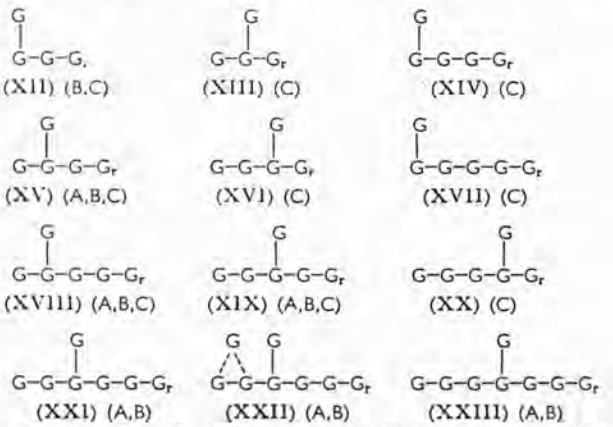

FIGURE 4 Branched oligosaccharides produced by the action of dextranases on dextran of *L. mesenteroides* NRRL B-1415

G, D-Glucopyranosyl unit; G_r, reducing D-glucose unit; —, α-1 ⟶ 6-link; |, α-1 ⟶ 4-link; ⋮, alternative α-1 ⟶ 4-links. The letters A, B, and C indicate the dextranase producing the oligosaccharide

alcohol AB (Table 3). The smallest branched saccharide alcohol present in the hydrolysate was a branched pentasaccharide alcohol (Table 3, fraction 7b; cf. branched heptasaccharide alcohol AB). Hence, the first three glucose units on the reducing end of the octasaccharide do not carry branches, whereas the fourth unit (from the reducing end) is a branch point. It is thus suggested that the branched octasaccharide AB is a mixture of 4^4-*O*-α-D-glucopyranosylisomaltoheptaose (XXIII) and saccharides (XXII) in which two glucose are joined by an α-1 ⟶ 4-linkage to glucose units of isomaltohexaose.

(vii) *Branched tetrasaccharide C* (XII) *and* (XIII). One reductive erosion gave a branched trisaccharide fraction (Table 3, fraction 11a). Electrophoresis in electrolyte (*a*) showed this to be a mixture of 6-*O*-α-maltosyl-D-glucose (X) and 4,6-di-*O*-α-D-glucopyranosyl-D-glucose (VI; $x = 1$) with $M_G(B)$ 0·66 and 0·29, respectively. The reduction products of the branched trisaccharide fraction were the alcohols (VIII) and (XI). This shows that the branched tetrasaccharide C is a mixture of the isomeric 4^2- (XIII) and 4^3-*O*-α-D-glucopyranosylisomaltotriose (XII).

(viii) *Branched pentasaccharide C* (XIV), (XV), *and* (XVI). One reductive erosion gave a branched tetrasaccharide fraction (Table 3, fraction 9a). Reduction of this material, followed by electrophoresis in electrolyte (*c*) gave the alcohol (VII; $x = 2$) [$M_s(W)$ 0·08] and another branched tetrasaccharide alcohol fraction with $M_s(W)$ 0·53. Hydrolysis of the latter furnished 1-*O*-α-maltosyl-L-gulitol (XI) and a branched trisaccharide fraction. The reduction products of the latter

were the trisaccharide alcohols (VIII) and (XI). It is thus concluded that branched pentasaccharide C is a mixture of 4^2- (XVI), 4^3- (XV), and 4^4-*O*-α-D-glucopyranosylisomaltotetraose (XIV).

(ix) *Branched hexasaccharide C.* Partial hydrolysis of branched hexasaccharide C alcohol gave, *inter alia*, 1-*O*-α-maltosyl-L-gulitol (XI) and a branched tetrasaccharide fraction (Table 3, fractions 11b and 9a, respectively). The latter was shown to contain the three branched tetrasaccharides that were obtained from the branched pentasaccharides C. The branched hexasaccharide C is thus a mixture of 4^2-*O*-α-D-glucopyranosylisomaltopentaose (XX) and isomers in which the branching occurs further towards the non-reducing end of the isomaltopentaose unit, *i.e.*, (XVII), (XVIII), and/or (XIX).

Mechanism of Actions of Dextranases.—(i) *Dextranases A and B.* The structures of the branched oligosaccharides produced by the action of dextranases A [(XV), (XVIII), (XIX), (XXI,) (XXII), and (XXIII)] and B [(XII), (XV), (XVIII), (XIX), (XXI), (XXII), and (XXIII)] on the α-1 → 4-branched dextran of *L. mesenteroides* NRRL B-1415 are similar to those produced by the same dextranases from an α-1 → 3-branched dextran.[2] The fact that the branched pentasaccharides A and B of the present investigation were 4^3-*O*-α-D-glucopyranosylisomaltotetraose (XV) strengthens our previous suggestion [7] that 3^4-*O*-α-D-glucopyranosylisomaltotetraose [(III) in ref. 7] is not produced when the dextranases act on the α-1 → 3-branched dextran of *L. mesenteroides* (Birmingham). Thus, we reach conclusions regarding the mechanism of the actions of the dextranases in the vicinity of the α-1 → 4-branch linkages in the dextran of the present investigation, and the structure of this dextran, similar to those made when the branch linkages were of the α-1 → 3-type.[7]

(ii) *Dextranase C.* Previously,[8] we suggested that dextranase C hydrolyses dextran or an isomaltodextrin containing not less than seven glucose units at a point not less than three glucosidic linkages from a chain end, but that the α-1 → 6-link on the reducing side of the branch point, and an α-1 → 3-branch link, are resistant to hydrolysis. The structures of the branched oligosaccharides of series C (XII—XX) allow a similar conclusion to be made when the dextran contains α-1 → 4-branch linkages.

EXPERIMENTAL

General.—(i) *Paper chromatography.* The solvents used were (*a*) and (*b*) of Part VI; [1] (*c*) acetone–water (1 : 1). Reference compounds used to express migration rates were isomaltose [R_{IM} (solvent)] and 2,3,4,6-tetra-*O*-methyl-D-glucose [R_{TMG} (solvent)].

(ii) *Paper electrophoresis.* The electrolytes used were (*a*) and (*b*) of Part VI; [1] (*c*) 2% sodium tungstate (IV).[6]

(iii) *Spray reagents.* The spray reagents used for the detection of compounds were (*a*), (*b*), and (*c*) of Part VI; [1] (*d*) Amido-black.[9]

Dextran.—The dextran used is elaborated by *L. mesenteroides* NRRL B-1415 and has been described in Part VI.[1]

Dextranases.—The dextranase preparations of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) (dextranase A), *P. funiculosum* (I.M.I. 79195; NRRL 1132) (dextranase B), and *Lactobacillus bifidus* (dextranase C) used in the present investigation were as described previously.[4,8]

Rates of Liberation of Reducing Sugars.—The rates of liberation of reducing sugars (as isomaltose) in dextran–dextranase digests were determined as described previously.[4,8] The results are shown in Figure 1.

Degradation of Dextran by Dextranases to Branched Oligosaccharides.—The dextran was treated separately with the dextranases A, B, and C in a manner similar to that described for the dextran of *L. mesenteroides* (Birmingham).[2,8] The branched oligosaccharides produced (series A, B, and C) were isolated by the same methods as those obtained from *L. mesenteroides* (Birmingham) dextran. Their yields are shown in Table 1. The molecular size of the oligosaccharides was assigned from properties determined as described before,[2] *i.e.*, R_M (Figure 2), $[M]_D$ (Figure 2), and reducing power (Table 1) of the oligosaccharides, and $M_s(Mo)$ of the reduced oligosaccharides which are expressed by equation (1). The dextranases A and B produced, in addition, glucose, isomaltose, isomaltotriose, and unresolvable material, whereas the additional products obtained with dextranase C, were isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose, isomaltoheptaose, and unresolvable material (chromatographic evidence).

Acidic Hydrolysis of Branched Oligosaccharides.—Each oligosaccharide (*ca.* 1 mg.) was hydrolysed with 1·5N-hydrochloric acid (2 ml.) at 100° for 4 hr. Chromatography of the deionised hydrolysate showed in each case a single component identical with glucose.

Partial hydrolysates of each branched oligosaccharide, obtained by heating for 45 min. at 100° in N-hydrochloric acid, were shown by paper chromatography in solvent (*a*), using spray reagents (*a*—*c*), to contain a small quantity of a component which had migration and staining properties identical with those of maltose. The main products from the branched tetra-, penta-, hexa-, and hepta-saccharides (*n* glucose units) were glucose and a homologue of isomaltose (*n* − 1 glucose units) whereas those from the branched octasaccharides were glucose and isomaltohexaose. Small quantities of isomaltoheptaose and a component with R_{IM} identical with that of the branched heptasaccharides were detected in the partial hydrolysates of the branched octasaccharides.

Periodate Oxidation of Branched Oligosaccharides.—Aqueous solutions of each oligosaccharide (15—25 mg.; concentrations determined by the anthrone method [10]) were separately treated with sodium metaperiodate. At time intervals portions were withdrawn for the estimation of periodate consumed [11] and formic acid liberated.[12] The values, which became constant after *ca.* 24 hr., are shown in Table 1.

The remainder of each solution was worked up (with

[7] D. H. Hutson and H. Weigel, *Biochem. J.*, 1963, **88**, 588.
[8] R. W. Bailey, D. H. Hutson, and H. Weigel, *Biochem. J.*, 1961, **80**, 514.
[9] O. Smithies, *Biochem. J.*, 1955, **61**, 629.
[10] E. W. Yemm and A. J. Willis, *Biochem. J.*, 1954, **57**, 508.
[11] G. O. Aspinall and R. J. Ferrier, *Chem. and Ind.*, 1957, 1216.
[12] T. G. Halsall, E. L. Hirst, and J. K. N. Jones, *J. Chem. Soc.*, 1947, 1427.

minor modifications) as described in Part VI.[1] The amounts of erythritol and glycerol produced were quantitatively determined by oxidation with periodate and estimation of the formaldehyde thus produced (by the chromotropic acid method [13]). The results, expressed as molecular proportions, are shown in Table 1.

Glucamylolysis of Branched Oligosaccharides.—(i) *Purification of glucamylase.* A glucamylase preparation obtained by the method of Barker and Fleetwood [14] from a culture (4 l.) of *Aspergillus niger* " 152 " was fractionated by paper chromatography (Whatman No. 3 MM) at 0° using spray reagent (*d*) to give fractions with $R_F(c)$ 0·04—0·14 and 0·47—0·57. (The latter was shown, by a method essentially the same as that described below, to contain α-1 ⟶ 6-glucosidase activity, since it liberated an appreciable quantity of glucose from isomaltose). The component with $R_F(c)$ 0·04—0·14 was eluted from the chromatograms with 0·02M-phosphate buffer (pH 6·18).

(ii) *Effect of glucamylase on saccharides.* To separate solutions of kojibiose, sophorose, nigerose, laminaribiose, cellobiose, isomaltose, isomaltotriose, gentiobiose, and the saccharides listed in Table 2 (*ca.* 15 mg.) in water (1 ml.) were added 0·1M-acetate buffer (pH 4·5; 2 ml.) and the eluate (5 ml.) of the glucamylase fraction with $R_F(c)$ 0·04—0·14. The digests were incubated at 40°. Samples were withdrawn at intervals and, after deionisation with Amberlite resins IR-120(H^+) and IR-45(OH^-), examined by paper chromatography in solvent (*a*) using spray reagents (*a*) and (*b*). Of the saccharides examined only those listed in Table 2 were appreciably affected by glucamylase. Very small traces of glucose were revealed after 2·5 days incubation in the digests containing isomaltose or isomaltotriose; these, however, did not increase on prolonged incubation.

(iii) *Characterisation and determination of* D-*glucose and isomaltotriose produced by action of glucamylase on tetrasaccharide B.* Tetrasaccharide B (100 mg.) in water (50 ml.) and 0·1M-acetate buffer (pH 4·5; 100 ml.), and glucamylase preparation (25 ml.) were incubated at 40° for 36 hr. The digest was deionised with Amberlite resins IR-120(H^+) and IR-45(OH^-) and fractionated by paper chromatography using solvent (*a*). The fraction corresponding to glucose was converted into 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (30 mg.), m. p. and mixed m. p. 131°, $[\alpha]_D^{21}$ +3·5° (*c* 0·4 in $CHCl_3$). The material corresponding to isomaltotriose was converted into undeca-*O*-benzoyl-β-isomaltotriose (43 mg.), m. p. and mixed m. p. 225—227°, $[\alpha]_D^{23}$ +132° (*c* 0·4 in $CHCl_3$).

The amounts of D-glucose and isomaltotriose (separated as above) produced from tetrasaccharide B (15·2 mg.) by glucamylase preparation (5 ml.) were determined by cuprimetric titrations.[15] The results, expressed as molecular proportions, were: D-glucose, 1·02; isomaltotriose, 1.

Methylated D-*Glucoses from Tetrasaccharide B.*—The details of the methylation procedure were kindly supplied by Professor R. Kuhn. The saccharide (500 mg.) was dissolved in dimethyl formamide (25 ml.) which had been dried for 3 days over barium oxide. To the cooled solution (0°) were added barium oxide (2·42 g., dried at 200°) and barium hydroxide octahydrate (1·66 g.). Dimethyl sulphate (3·2 ml.) was added dropwise under nitrogen with continuous stirring and cooling in an ice-bath. The temperature of the reaction mixture rose to 20°. After 24 hr. at 20° concentrated ammonia solution (1·5 ml.) was added and the reaction mixture stirred at 20° for 3 hr. It was extracted with chloroform (100 ml.) and the chloroform extract washed with water until this was neutral to litmus. The chloroform solution was dried (Na_2SO_4) and the solvent removed by distillation *in vacuo*. The residue (510 mg.) was subjected to another methylation. The material thus obtained was distilled at 240° (oil-bath)/0·02 mm. to give the *methylated tetrasaccharide B* (456 mg.) (Found: MeO, 49·5. $C_{38}H_{70}O_{21}$ requires MeO, 50·3%).

The methylated saccharide (425 mg.), in dry chloroform (5 ml.), was treated with 4% methanolic hydrogen chloride (40 ml.) in a sealed tube at 100° for 4 hr. The cooled solution was neutralised with silver carbonate, clarified in the centrifuge, and evaporated *in vacuo*. The residue was hydrolysed with 0·5N-hydrochloric acid at 100° for 4 hr. The solution was neutralised with silver carbonate (as before) and evaporated *in vacuo* to a syrup. Paper chromatography of the syrup revealed components with $R_{TMG}(b)$ 1·0 and 0·87—0·91 (tri-*O*-methyl-D-glucose).

A portion of the hydrolysate was used for the quantitative determination [16] of the above components. The results, expressed as molecular proportions, were as follows: tetra-*O*-methyl-D-glucose, 1; tri-*O*-methyl-D-glucose, 2·85.

The remainder of the above hydrolysate was fractionated by paper chromatography (Whatman No. 3 MM) using solvent (*b*) to give 2,3,4,6-tetra-*O*-methyl-D-glucose (65 mg.), m. p. and mixed m. p. 84—86°, $[\alpha]_D^{22}$ +84·5° (equil.) (*c* 0·4 in water) and the tri-*O*-methyl-D-glucose fraction (325 mg.).

The tri-*O*-methyl-D-glucose fraction was resolved by the method described in Part V.[17] This gave 2,3,6-tri-*O*-methyl-D-glucose [18] (42 mg.), m. p. and mixed m. p. 118°, $[\alpha]_D^{22}$ +71° (equil.) (*c* 0·4 in water) and syrupy 2,3,4-tri-*O*-methyl-D-glucose (181 mg.). The latter was converted into 2,3,4-tri-*O*-methyl-*N*-*p*-nitrophenyl-D-glucosylamine,[19] m. p. and mixed m. p. 223—225°.

Reductive Erosion of Branched Oligosaccharides.—The branched oligosaccharide (*n* glucose units) (*ca.* 0·02—1 g.) was reduced with potassium borohydride (*ca.* 3—160 mg.) (Figure 3, step R). The reaction mixture was deionised by treatment with Amberlite resin IR-120(H^+) and repeated distillation with dry mthanol. The branched oligosaccharide alcohol obtained (electrophoretic mobility shown in Table 3) was then partially hydrolysed in 1% oxalic acid at 100° for 3—4 hr. (Figure 3, step H). The deionised [with Amberlite resin IRA-400 (CO_3^{2-})] hydrolysate was fractionated by paper chromatography (Figure 3, step C) using solvent (*a*). Each fraction was examined by paper electrophoresis in electrolyte (*b*) (see Table 3). The fraction containing the next lower branched oligosaccharide (*n* − 1 glucose units) [$M_s(Mo)$ 0] and branched oligosaccharide alcohol (*n* − 2 glucose units) [electrophoretic mobility expressed by equation (1), see Table 3] was further fractionated by paper electrophoresis (Figure 3, step E) in electrolyte (*b*). The fraction contining the branched oligosaccharide (*n* − 1 glucose units) was deionised by treatment with Amberlite resin IR-120(H^+) and IRA-400

[13] L. H. Adcock, *Analyst*, 1957, **82**, 427.
[14] S. A. Barker and J. G. Fleetwood, *J. Chem. Soc.*, 1957, 4857.
[15] P. A. Shaffer and A. F. Hartmann, *J. Biol. Chem.*, 1921, **45**, 365.
[16] E. L. Hirst, L. Hough, and J. K. N. Jones, *J. Chem. Soc.*, 1949, 928.
[17] D. Abbott and H. Weigel, *J. Chem. Soc.*, 1965, 5157.
[18] E. Schlüchterer and M. Stacey, *J. Chem. Soc.*, 1945, 776.
[19] J. W. van Cleve, W. C. Schaefer, and C. E. Rist, *J. Amer. Chem. Soc.*, 1956, **78**, 4435.

(CO_3^{2-}). Where applicable the procedure was repeated with the saccharide thus obtained.

The fraction corresponding to maltose (Table 3, fraction 13) maintained its identity during paper electrophoresis in electrolyte (*a*) [$M_G(B)$ 0·31]. When reduced with potassium borohydride it had $M_s(Mo)$ 0·46 and $M_s(W)$ 0·17, identical with those of maltitol.

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Studies on Dextrans and Dextranases. Part VIII.[1] Size and Distribution of Branches in Some Dextrans

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Studies on Dextrans and Dextranases. Part VIII.[1] Size and Distribution of Branches in Some Dextrans

By D. Abbott, E. J. Bourne, and H. Weigel

Catalytic oxidation of the dextrans of *Leuconostoc mesenteroides* NRRL B-1375 (Birmingham) and NRRL B-1415, followed by hydrolysis, furnished 3- and 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose, respectively. Glucamylase liberates D-glucose from the dextrans of *L. mesenteroides* NRRL B-1415 and NRRL B-1416. Quantitative evaluation of the results, and the effect of a dextranase on dextrans pretreated with glucamylase, show that the branches in the above dextrans consist mainly, if not exclusively, of single glucose units. More than 17% of all branches in the dextran of *L. mesenteroides* NRRL B-1375 (Birmingham) are joined to two adjacent α-1 ⟶ 6-linked glucose units.

THE structures of branched oligosaccharides produced from dextrans of strains of *Leuconostoc mesenteroides* by the action of bacterial or mould dextranases suggested to us that a large portion of the branches in these dextrans consist of only one D-glucopyranosyl unit.[1-3] Several workers arrived at similar conclusions on the grounds of physical measurements and these have been reviewed.[4] We now report [5] what we believe to be the first chemical evidence for the size of branches in the dextrans elaborated by the strains NRRL B-1415 (dextran A), NRRL B-1416 (dextran B), and NRRL B-1375 (Birmingham) (dextran C). The type and degree of branching in these dextrans are shown in Table 1.

TABLE 1

Type and degree of branching in dextrans

Strain of *L. mesenteroides*	Dextran	Type of branching	Branching glucose units (%)	Anhydroglucose content *	Ref.
NRRL B-1415	A	α-1 ⟶ 3	Negligible	7·0	12
		α-1 ⟶ 4	14·3		
NRRL B-1416	B	α-1 ⟶ 3	10·1	5·9	12
		α-1 ⟶ 4	6·9		
NRRL B-1375 (Birmingham)	C	α-1 ⟶ 3	15·7	6·4	13
		α-1 ⟶ 4	Negligible		

* Number of anhydroglucose units in the average repeating unit of dextran.

Some possible structures of a segment of a dextran molecule in the vicinity of a branch point are (I—IV) (Figure 1). However, for the above dextrans, structures (III) and (IV) can be eliminated since 2,3,6- and/or 2,4,6-tri-*O*-methyl-D-glucose were not present in the hydrolysates of the corresponding methylated dextrans. Indeed, evidence from methylation has shown that a glucose unit linked through position 3 (or 4) is always linked also through position 6.

It was conceived that a distinction between structures (I) and (II) could be made by modifying the non-reducing end-groups of dextrans and, after hydrolysis,

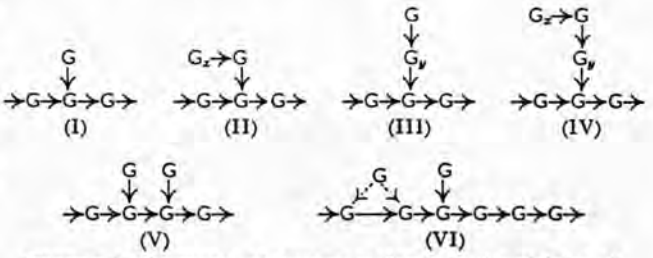


FIGURE 1 Segments of dextran molecule in the vicinity of possible branch points

G, D-Glucopyranosyl unit; →, α-1 ⟶ 6-link; *x* and *y*, number of G units; (Ia), (IIa), (IIIa), (IVa): ↓, α-1 ⟶ 4-link; (Ib), (IIb), (IIIb), (IVb), (V), (VI): ↓, α-1 ⟶ 3-link; ⇣, alternative α-1 ⟶ 3-link

isolation of the disaccharides containing the modified group. Since the relative stabilities to acid hydrolysis [6] of glycosides of D-glucuronic acid and D-glucose facilitate the isolation of aldobiuronic acids from acidic polysaccharides, the non-reducing end-groups of dextrans A and C were oxidised catalytically to D-glucopyranosyluronic acid groups. The properties (Table 2) of the products (carboxy-dextrans A and C) show that *ca.* 72 and 80% of the non-reducing end-groups of dextrans A and C, respectively, had been oxidised. Hydrolysis of each carboxy-dextran furnished, *inter alia*, D-glucurone and an aldobiuronic acid.

The aldobiuronic acid obtained from carboxy-dextran

[1] Part VII, D. Abbott and H. Weigel, preceding Paper.
[2] R. W. Bailey, D. H. Hutson, and H. Weigel, *Biochem. J.*, 1961, **80**, 514.
[3] D. H. Hutson and H. Weigel, *Biochem. J.*, 1963, **88**, 588.
[4] W. B. Neely, *Adv. Carbohydrate Chem.*, 1960, **15**, 341.
[5] Presented at Internat. Symp. über die Chemie der Kohlenhydrate, Münster (Germany), July 1964.
[6] D. B. Easty, *J. Org. Chem.*, 1962, **27**, 2102.

A was in fact 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (VII), which was characterised as its methyl ester hepta-acetate (VIII).[7] Esterification of acid (VII) followed by reduction gave 3-*O*-α-D-glucopyranosyl-L-gulitol (maltitol) but not 1-*O*-α-D-glucopyranosyl-L-gulitol (isomaltitol). This shows that the acid (VII)

TABLE 2

Yields and properties of carboxy-dextrans

Carboxy-dextran	Yield (g. from 5 g. of dextran)	Equiv. Found *	Equiv. Calc.‡	CO_2H (%) Found †	CO_2H (%) Calc.‡
A	3·6	1619	1149	2·84	3·92
C	3·8	1350	1052	3·43	4·28

* Determined by the method of R. L. Whistler and M. S. Feather ("Methods in Carbohydrate Chemistry," ed. R. L. Whistler, Academic Press, New York, 1962, vol. I, p. 467. † Determined by the method of Johansson *et al.*[16] ‡ Based on size of average repeating unit of dextran (Table 1).

was not contaminated by detectable quantities of 6-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (IX) [cf. (I), $R_G(d)$ 0·29; (IX),[8] $R_G(d)$ 0·27].

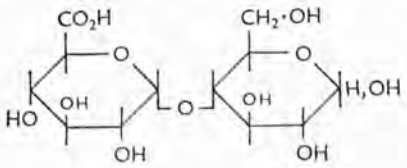
The aldobiuronic acid obtained from carboxy-dextran C was shown to be 3-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (X). This assignment is based on the data: (*a*) reduction of acid (X) with sodium borohydride, followed by hydrolysis gave D-glucitol and D-glucuronic acid; (*b*) reduction of the ester of acid (X) gave a material which was chromatographically and electrophoretically identical with 3-*O*-α-D-glucopyranosyl-D-glucitol (nigeritol); (*c*) esterification of acid (X), followed by methylation, reduction, and hydrolysis, gave 2,3,4- and 2,4,6-tri-*O*-methyl-D-glucose; and (*d*) the high optical rotation of acid (X) and the fact that the branch linkages in dextran C are of the α-1 ⟶ 3-type. Again, the acid (X) was not contaminated by detectable quantities of acid (IX).

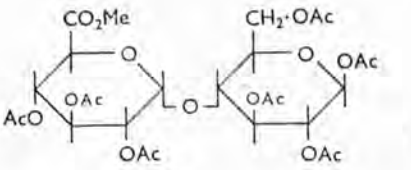
The yields of acid (VII) and (X) represent *ca.* 50 and 62%, respectively, of those expected from the sizes of the average repeating units (Table 1) of the corresponding dextrans. Alternatively, the yields of acid (VII) and (X), when corrected for incomplete oxidation of the corresponding dextrans, are *ca.* 70 and 78%, respectively. Thus, we reach the conclusion that the branches in dextrans A and C consist mainly, if not exclusively, of single D-glucopyranosyl units, *i.e.*, (Ia) and (Ib), respectively.

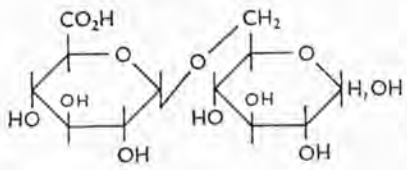
Oxidation of dextran B, followed by hydrolysis would give rise to a mixture of the aldobiuronic acids (VII) and (X) if the branches in this dextran also consist of single glucose units. Since the resolution of such a mixture would complicate the structural analysis, it was of interest to examine the effect of glucamylase (known to remove single α-1 ⟶ 4-linked glucose units stepwise from non-reducing chain ends [9]) on dextrans containing α-1 ⟶ 4-branch linkages (dextrans A and B). Paper chromatography of the digests showed indeed that glucose was liberated from both dextrans.

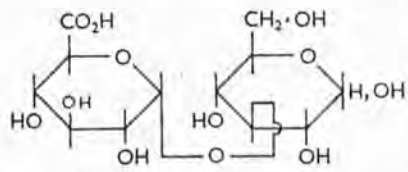
[7] D. Abbott and H. Weigel, *J. Chem. Soc.*, 1965, 5157.
[8] D. Abbott and H. Weigel, unpublished results.

The extents of conversion of dextrans A and B into glucose by glucamylase were shown by cuprimetric


(VII)


(VIII)


(IX)


(X)

titration to 9·4 and 5·5%, respectively. Thus, based on the degree of branching of the α-1 ⟶ 4 type in these dextrans, *ca.* 66 and 80% of all α-1 ⟶ 4-linked glucose units of dextrans A and B, respectively, are liberated by glucamylase and, hence, constitute chain ends (Ia) or (IIIa). Since methylation evidence has eliminated structure (IIIa) the branch must be of the (Ia) type.

The extent of conversion of the residual dextran A′ (*i.e.*, dextran A pretreated with glucamylase) into reducing sugars (estimated as isomaltose) by a dextranase (dextranase B of Part II [10]) was shown to be 95%. It thus seems that dextran A′ is virtually unbranched (cf. dextran of *Streptococcus bovis* [10]). This suggestion is supported by an analysis of the dextran A′–dextranase B digest, which revealed the presence of glucose, isomaltose, and isomaltotriose, but none of the branched oligosaccharides produced [1] from dextran A by dextranase B.

The extent of conversion of the residual dextran B′ (*i.e.*, dextran B pretreated with glucamylase) into reducing sugars (59%, estimated as isomaltose) by dextranase B is that expected [10] of a dextran with *ca.* 10% branching glucose units (cf. degree of α-1 ⟶ 3-branching in this dextran). The dextran B′–dextranase B digest was shown by paper chromatography to

[9] J. Larner in "The Enzymes," eds. P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York, 1960, vol. IV, p. 370.
[10] E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, 1962, **85**, 158.

contain the same components as were produced [11] when dextranase B acted on dextran C, *i.e.*, glucose, isomaltose, isomaltotriose, and branched oligosaccharides. We thus suggest that the α-1 ⟶ 4- and α-1 ⟶ 3-linked branches in dextran B consist mainly, if not exclusively, of single D-glucopyranosyl units, *i.e.*, (Ia) and (Ib), respectively. Since it has been shown that the branches in three dextrans consist mainly of single glucose units, it is possible that this structural feature is common to many other branched dextrans.

Previously, we suggested [2] that the pattern of branching in the α-1 ⟶ 3-branched dextran C is random, *i.e.*, the structure cannot be represented by a regular repetition of an average repeating unit. Confirmatory evidence has now been obtained by fragmentation of the periodate-oxidised dextran. This furnished 1-*O*-α-D-glucopyranosyl- and 1-*O*-α-isomaltosyl-glycerol, the glucose units of which originate from the branch points of the dextran. It can now be calculated from the yield of 1-*O*-α-isomaltosylglycerol that more than 17% of all branches in this dextran are joined to two adjacent, α-1 ⟶ 6-linked glucose units (V).

It is interesting to note that a dextranase (dextranase A of Part II [10]) produced from dextran C, *inter alia*, a branched octasaccharide corresponding to (VI).[11] The yield of this material (calculated from the total yield of branched octasaccharides and results of their periodate oxidation) indicates that a segment as (VI) represents *ca.* 2% of all glucose units in dextran C.

EXPERIMENTAL

General.—(i) *Paper chromatography.* The solvents used were (*a*), (*b*), and (*c*) of Part VI; [12] (*d*) ethyl acetate–acetic acid–water (9 : 2 : 2).

(ii) *Paper electrophoresis.* The electrolytes used were (*a*) and (*b*) of Part V,[7] and (*c*) of Part VII.[1]

(iii) *Spray reagents.* The spray reagents used for the detection of compounds were those described in Part VI.[12]

(iv) *Dextrans.* The dextrans used were elaborated by the following strains of *Leuconostoc mesenteroides*: NRRL B-1415 (dextran A),[12] NRRL B-1416 (dextran B),[12] and NRRL B-1375 (Birmingham) (dextran C).[13]

(v) *Dextranase.* The dextranase preparation used was that of *Penicillium funiculosum* (I.M.I. 79195; NRRL 1132) (dextranase B) and was described in Part II.[10]

(vi) *Glucamylase preparation.* The purified glucamylase preparation described in Part VII [1] was used.

Catalytic Oxidation of Dextrans to Carboxy-dextrans.—A typical experiment was as follows. Separate solutions of dextran (5 g.) in water (150 ml.) and sodium hydrogen carbonate (0·5 g.) in water (100 ml.) were shaken with platinum catalyst (10 mg.; Adams platinum dioxide reduced with hydrogen [14]) at room temperature for *ca.* 2 hr. to remove possible poisons. The catalyst was removed from each suspension by centrifugation. The solutions were combined and fresh platinum catalyst (1 g.) added. The mixture was stirred at 70° for 23 days while oxygen was passed through. Water was added at intervals to compensate for that lost by evaporation. After cooling, the catalyst was removed by centrifugation and the polysaccharide precipitated with ethanol (750 ml.). The precipitate was dissolved in water (250 ml.), the solution treated with Amberlite resin IR-120(H^+), and the polysaccharide reprecipitated with ethanol (500 ml.). The polysaccharide, after dissolution in water, was then freeze-dried. Yields and properties of the carboxy-dextrans are shown in Table 2.

D-*Glucuronic Acid from Carboxy-dextrans.*—The method used for the hydrolysis of carboxy-dextrans A (800 mg.) and C (800 mg.), and the isolation of D-glucurone, was that of Jones and Perry.[15] The specimen from carboxy-dextran A (45 mg.) had m. p. and mixed m. p. 175—176°, $[\alpha]_D^{18}$ +18° (*c* 0·2 in water), whereas that from carboxy-dextran C (51 mg.) had m. p. and mixed m. p. 173—176°, $[\alpha]_D^{20}$ +17° (*c* 0·2 in water).

Aldobiuronic Acid (VII) *from Carboxy-dextran A.*—Carboxy-dextran A (7·5 g.) was hydrolysed with N-sulphuric acid (100 ml.) at 100° for 6 hr. Sulphuric acid was removed by extraction with 5% *NN*-di-n-octylmethylamine in chloroform (4 × 30 ml.). The aqueous solution was evaporated *in vacuo* to a syrup. Fractionation by paper chromatography gave 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (VII) (835 mg.), with $R_G(d)$ 0·29, $M_{GA}(P)$ 0·77, identical with those of an authentic specimen.[7] A portion (60 mg.) of the acid was converted into 4-*O*-(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose [7] (VIII) (41 mg.), m. p. and mixed m. p. 193—196°, $[\alpha]_D^{20}$ +75° (*c* 0·2 in $CHCl_3$).

Another portion (100 mg.) of the acid (VII) was converted, as described previously,[7] into its methyl ester by treatment with diazomethane. A solution of the ester [M_{GA} (*P*) 0] in water (10 ml.) was added, over 18 hr., to a solution of sodium borohydride (50 mg.) in water (6 ml.). The solvent was removed *in vacuo* and the residue treated with more sodium borohydride (50 mg.) as above. Paper electrophoresis and chromatography of the deionised solution [with Amberlite resin IR-120(H^+) and repeated distillation with dry methanol] revealed a component with migration rates identical with those of maltitol [$M_s(Mo)$ 0·46, R_G (*a*) 0·75] and traces of a material with $R_G(a)$ 1·2 [presumably 3-*O*-(methyl α-D-glucopyranosyluronate)-L-gulitol].

From another hydrolysate of carboxy-dextran A (1 g.) the acid (VII) was quantitatively removed by paper chromatography. The amount isolated, when estimated by the method of Johansson, Lindberg, and Theander,[16] was 156 mg.

Aldobiuronic Acid (X) *from Carboxy-dextran C.*—Carboxy-dextran C (7·5 g.) was hydrolysed as described for carboxy-dextran A. Preparative paper chromatography of the hydrolysate gave 3-O-(α-D-*glucopyranosyluronic acid*)-D-*glucose* (X) (730 mg.), with $R_G(d)$ 0·33, M_{GA} (*P*) 0·76, $[\alpha]_D$ 113° (*c* 0·5 in water), Equiv., 361.

From another hydrolysate of carboxy-dextran C (0·95 g.) the acid (X) was quantitatively removed by paper chromatography. The amount isolated was estimated [16] to be 198 mg.

[11] E. J. Bourne, D. H. Hutson, and H. Weigel, *Biochem. J.*, 1963, **86**, 555.
[12] D. Abbott and H. Weigel, *J. Chem. Soc.* (*C*), 1966, 816.
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[14] K. Heyns and H. Paulsen, *Adv. Carbohydrate Chem.*, 1962, **17**, 169.
[15] J. K. N. Jones and M. B. Perry, *J. Amer. Chem. Soc.*, 1957, **79**, 2787.
[16] A. Johansson, B. Lindberg, and O. Theander, *Svensk Papperstidn.*, 1954, **57**, 41.

Characterisation of Aldobiuronic Acid (X).—(i) *Hydrolysis.* A portion of the acid (X) (*ca.* 5 mg.) in 90% formic acid (5 ml.) was heated at 100° for 20 hr. The residue, obtained after removal of formic acid by distillation *in vacuo*, was further hydrolysed with N-sulphuric acid (1 ml.) at 100° for 2 hr. After cooling, sulphuric acid was removed by extraction with 5% *NN*-di-n-octylmethylamine in chloroform (2 × 5 ml.). Paper chromatography of the aqueous layer revealed the presence of components with staining properties and migration rates identical with those of glucose [$R_G(d)$ 1·0], glucuronic acid [$R_G(d)$ 0·90], and glucurone [$R_G(d)$ 2·90].

(ii) *D-Glucitol and D-glucuronic acid from aldobiuronic acid* (X). A solution of the acid (X) (100 mg.) in water (100 ml.) was treated with sodium borohydride (150 and 20 mg.) as described for the methyl ester of aldobiuronic acid (VII). After deionisation, the product was shown to be a single component with $R_G(d)$ 0·34, $M_{GA}(P)$ 0·77, and $M_s(Mo)$ 0·77. This was hydrolysed with 90% formic acid (10 ml.) and N-sulphuric acid (2·5 ml.) as described above. After treatment with Amberlite resin IR-120(H^+), the hydrolysate was passed through a column of Amberlite resin IR-4B(OH^-). The eluate contained a single component with $R_G(d)$ 1·0, $M_{GA}(P)$ 0, and $M_s(Mo)$ 1·0, which was converted into hexa-*O*-acetyl-D-glucitol (38 mg.), m. p. and mixed m. p. 98—99°, $[\alpha]_D^{20}$ +9·0° (*c* 0·2 in $CHCl_3$). Elution of the column with 10% aqueous formic acid (100 ml.), and removal of formic acid from the eluate by extraction with ether (3 × 30 ml.), gave materials with $R_G(d)$ 0·90 and 2·90, and $M_{GA}(P)$ 1·0 and 0, identical with those of an equilibrium mixture of glucuronic acid and glucurone. Crystallisation from glacial acetic acid gave D-glucurone (12·5 mg.), m. p. and mixed m. p. 173—175°, $[\alpha]_D^{20}$ +18° (in water).

(iii) *Reduction of ester of aldobiuronic acid* (X). The acid (X) (*ca.* 5 mg.) was converted into its methyl ester and then reduced with sodium borohydride as described for aldobiuronic acid (VII). The reaction product was a single component with $R_G(c)$ 0·55, $R_G(d)$ 0·82, and $M_s(Mo)$ 0, identical with those of nigeritol.

(iv) *O-Methyl-D-glucoses from aldobiuronic acid* (X). The acid (X) (500 mg.) was esterified by treatment with methanolic hydrogen chloride, methylated, reduced, and hydrolysed under conditions described for 4-*O*-(α-D-glucopyranosyluronic acid)-D-glucose (VII) in Part V.[7] Paper-chromatographic analysis of the hydrolysate revealed the presence of two components with $R_{TMG}(b)$ 0·85 and 0·89. The hydrolysate was fractionated on a charcoal–Celite column (44 × 3·5 cm.) as described previously.[7] Fractions containing both components were further fractionated by paper chromatography using solvent (*b*).

The component (186 mg.; syrup) which was eluted first had $R_{TMG}(b)$ 0·89 and was converted into 2,3,4-tri-*O*-methyl-*N*-*p*-nitrophenyl-D-glucosylamine,[17] m. p. and mixed m. p. 222—223°, $[\alpha]_D^{20}$ −242° (*c* 0·2 in pyridine).

The second component (180 mg.; syrup) had $R_{TMG}(b)$ 0·85 and was crystallised from ether to give 2,4,6-tri-*O*-methyl-D-glucose [18] (68 mg.), m. p. and mixed m. p. 117—120°, $[\alpha]_D^{20}$ +72·5° (equil., *c* 0·2 in MeOH).

Rates of Liberation of Reducing Sugars from Dextrans A and B by Glucamylase.—Dextrans A and B [100 mg. (corrected for ash), dried *in vacuo* over P_2O_5 at 50° for 2 days] were separately dissolved in water (10 ml.). Acetate buffer (0·1M; pH 4·5; 2 ml.) and the eluate (5 ml.) of the glucamylase fraction of Part VII [1] were added to each solution which was then made up to 25 ml. and incubated at 40° for 8 days. Portions (2 ml.) were withdrawn at intervals and the reducing sugars estimated by the method of Shaffer and Hartmann,[19] with D-glucose as a standard. The results are shown in Figure 2. After 8 days incubation, paper chromatography revealed the presence of glucose in each digest.

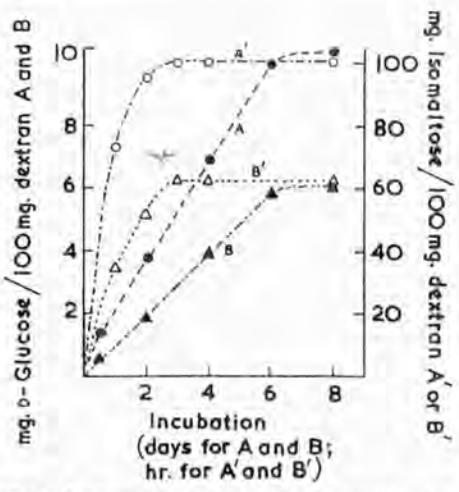

FIGURE 2 Enzymic liberation of reducing sugars from dextrans

A and B, D-glucose liberated from dextrans A and B, respectively, by glucamylase. A′ and B′, reducing sugars (as isomaltose) liberated from dextrans A′ and B′, respectively (*i.e.*, dextrans A and B after pretreatment with glucamylase) by dextranase B [from *P. funiculosum* (I.M.I. 79195; NRRL 1132)]

Action of Dextranase on Dextrans Pretreated with Glucamylase (Dextrans A′ and B′).—The remainder of each of the above digests (*ca.* 15 ml.) was dialysed against running water for 2 days, when glucose was shown to be absent (chromatographic evidence). They were then freeze-dried to give the modified dextrans A′ (49 mg.) and B′ (52 mg.).

The modified dextrans A′ and B′ were dissolved in water (25 ml.). Citrate buffer (0·2M; pH 5·0; 25 ml.) and dextranase B [10] (100 mg.) in water (10 ml.) were added. The digests were incubated at 37°. Portions (1 ml.) were withdrawn at intervals and the reducing sugars estimated by the method of Shaffer and Hartmann,[19] with isomaltose as a standard. The results are shown in Figure 2.

The remainder of each digest was then deionised by treatment with Amberlite resins IR-120(H^+) and IR-45(OH^-) and concentrated *in vacuo*. Paper chromatography of the digests of dextrans A′ and B′ revealed the presence of components with $R_{IM}(a)$ values identical with those of glucose (1·45), isomaltotriose (0·56), and isomaltose (1·0) in concentrations increasing in that order (visual estimation). The digest of dextran B′ contained, in addition, components with $R_{IM}(a)$ values 0·49, 0·36, 0·24, 0·16, and 0·09, identical with those of the branched tetra-, penta-, hexa-, hepta-, and octa-saccharides, respectively, produced from dextran C by dextranase B.[11]

Isolation of D-Glucose Liberated from Dextran A by

[17] J. W. van Cleve, W. C. Schaefer, and C. E. Rist, *J. Amer. Chem. Soc.*, 1956, **78**, 4435.

[18] E. J. Bourne and S. Peat, *Adv. Carbohydrate Chem.*, 1954, **5**, 145.

[19] P. A. Shaffer and A. F. Hartmann, *J. Biol. Chem.*, 1921, **45**, 365.

Glucamylase.—Dextran A (500 mg.) and the eluate (25 ml.) of the glucamylase fraction of Part VII [1] were incubated under the conditions described above. The component (41 mg., syrup) corresponding to glucose was isolated from the deionised digest by paper chromatography using solvent (*a*) and converted into 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (30 mg.), m. p. and mixed m. p. 131—132°, $[\alpha]_D^{20}$ +3·8° (*c* 0·4 in $CHCl_3$).

Fragmentation of Periodate-oxidised Dextran C.—(i) *Glycerol and* 1-O-α-D-*glucopyranosylglycerol.* Dextran C (1·85 g.) was treated with sodium metaperiodate (6·42 g.) and then with potassium borohydride (0·6 g.) by the method described earlier.[12] Concentrated hydrochloric acid was added to the solution of the resulting material to give pH 1. The solution was kept at 20° for 12 hr., treated with Amberlite resin IR-120(H^+), and concentrated *in vacuo*. The residue was repeatedly distilled with dry methanol. It was then dissolved in a small volume of water, the solution treated with Amberlite resin IR-400 (CO_3^{2-}), and evaporated *in vacuo* to a syrup. Fractionation of the syrup by paper chromatography gave glycerol [$R_G(c)$ 3·2, 362 mg. (concentrate)], characterised as the crystalline tris-*p*-nitrobenzoate (m. p. and mixed m. p. 186—187°), and 1-*O*-α-D-glucopyranosylglycerol [$R_G(c)$ 1·0; 152 mg.]. The syrup also contained a small quantity of a material with $R_G(c)$ 0·75 (1-*O*-α-isomaltosylglycerol).

Treatment of 1-*O*-α-D-glucopyranosylglycerol (65·0 mg. in 7·5 ml. of water; concentration determined by the anthrone method [20]) with 0·1M-sodium metaperiodate (15 ml.) in the dark at room temperature gave the following results: periodate consumption, 3·15 mol., formic acid and formaldehyde produced, 0·90 and 0·86 mol., respectively. The glucoside was not degraded when incubated with β-glucosidase.

(ii) 1-O-α-*Isomaltosylglycerol.* Dextran C (5 g.) was fragmented as described above. 1-*O*-α-Isomaltosyl glycerol [$R_G(c)$ 0·75; 175 mg.] was isolated by paper chromatography. Periodate oxidation of this material (conditions as above) gave the following results: periodate consumption: 4·62 mol.; formic acid and formaldehyde produced; 1·80 and 0·75 mol., respectively. Incubation with β-glucosidase caused no degradation of the material.

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[23] Bacterial Polysaccharides

Extraction with Chloral Hydrate

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Introduction

Aqueous chloral hydrate is an excellent solvent for starch (*1*) and lowers very materially its gelatinization point (*2*). Whereas solvents such as ethylenediamine and hydrazine hydrate cause appreciable hydrolysis of the polysaccharide (*3*), treatment with chloral hydrate solution has a negligible effect upon the blue value of potato starch (*4*). Bourne and co-workers reported a procedure for the extraction of protein-free amylose from a pea variety (*5*) and starch from the protozoon *Polytomella coeca* (*4*). This is based on a method described by Meyer and Bernfeld (*3*) who utilized the above properties of chloral hydrate in their studies on starch. By this procedure, polysaccharides of the amylopectin type have been extracted from cells of *Clostridium butyricum* (*6*), *Cycloposthium* (*7*), and *Holotrich ciliates* (*8*). Although chloral hydrate has been used principally for the extraction of polysaccharides of the amylose and amylopectin type, it might find wider application since it readily dissolves a complex polysaccharide such as gum arabic (*9*).

"Methods in Carbohydrate Chemistry."
Academic Press, New York, 1965, vol. V, p. 78

Procedure

Extraction of Starch

Polytomella coeca cells, cultured in a synthetic medium containing ethanol or acetic acid as the carbon source, are collected in a Sharples centrifuge. The cells are ruptured by grinding with sand. The material obtained is suspended in 0.01 *M* phosphate or 0.02 *M* citrate buffer (pH 7). Centrifuging at low speed yields a sediment which consists mainly of starch, together with small amounts of minerals, proteins, and other cellular debris.

The starch-containing sediment (dry weight, 2 g.) is stirred 1 hr. at 80° with a 33% solution (100 ml.) of chloral hydrate in water. The insoluble residue, which is collected in a centrifuge, is stirred with two fresh portions (20 ml. each) of warm chloral hydrate solution.

The combined extracts are filtered through fritted glass and injected in a fine stream into acetone (250 ml.). The flocculent polysaccharide is collected and hardened by trituration with acetone. The last traces of chloral hydrate are removed by extraction in a Soxhlet apparatus with acetone for 2 hr. and then with ether for 1 hr. The product is dried at 60° in a vacuum over phosphorus pentaoxide; yield about 1.7 g.

Reprecipitation of Starch

A suspension of the starch in a small volume of ethanol is heated in a boiling water bath and stirred at high speed while water (150 ml. per 1 g. of polysaccharide) is added. The stirring and heating are continued for 20 min. At this stage most of the polysaccharide is dissolved, the rest remaining as a very fine suspension. The addition of a few drops of 10% sodium hydroxide solution causes rapid completion of the solution process. After 5 min., the solution is allowed to cool and then neutralized with hydrochloric acid. The polysaccharide is precipitated with 1.5 volumes of ethanol, triturated with ethanol and then with ether, and dried as above.

The product contains about 0.8% ash but no detectable protein. It has a blue value of 0.36, $[\alpha]^{15}D + 160°$ (0.5 *N* sodium hydroxide), and gives only D-glucose when hydrolyzed with acid; with soya bean β-amylase, the limiting conversion into maltose is 54% (see Vol. IV [57]).

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METAL CHELATES OF POLYHYDROXY COMPOUNDS

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Polyhydroxy compounds have long been known to form chelates with a number of polyvalent metallic ions in alkaline media.[1–13] They can also be precipitated as heavy metal chelates by barium hydroxide. However, the products obtained with sodium hydroxide redissolve with excess alkali.[7] Metal chelates of polyhydric alcohols and sugars have been isolated,[14] some as their water-insoluble alkaline earth salts[7, 15, 16] and shown to contain the polyhydroxy compound and the metal in varying ratio. Traube[17] found that the amount of copper oxide dissolved in an alkaline solution of glycerol, mannitol, or sucrose is dependent upon the ratio of alkali to polyhydroxy compound and on the concentration of the solution, and he postulated that alkali alcoholates were intermediates in the formation of the copper chelates.

We have examined the chelation of metals with polyhydroxy compounds in sodium hydroxide solution at pH 12. With D-mannitol as the chelating agent, the metallic ions which formed soluble chelates were: Cu^{++}, Ti^{++++}, Zr^{++++}, Pb^{++}, Sb^{+++}, Bi^{+++}, Fe^{++}, Fe^{+++}, Co^{++}, Ni^{++}, Th^{++++} and UO_2^{++}. No soluble chelates were obtained with Ag^{+}, Ca^{++}, Ba^{++}, Cd^{++}, and Hg^{++}. The results of a quantitative determination of the power of some polyhydroxy compounds to form water-soluble chelates with Cu^{++}, Fe^{+++}, Co^{++} and Ni^{++} are shown in the Table. The chelating power was determined by adding an excess of a solution of the metal salt to a 0·125 M solution of the chelating agent adjusted to pH 12 with sodium hydroxide, immediate centrifugation and determination of the amount of precipitated metal ion with ethylenediamine tetra-acetic acid (EDTA). Similar results were obtained by titration of the alkaline solution of the chelating agent with the solution of the metal salt until the appearance of a visible precipitate. In two cases (*) the results of the latter method only are reported. The values of the chelating power of D-mannitol with Co^{++} and Ni^{++} could not be reproduced accurately. The analytical method used will need further refinement.

The chelating power (g.-atom metal/mole chelating agent) of the polyhydroxy compounds for Cu^{++}, Co^{++}, and Ni^{++} is not greater than expected if a pair of hydroxyl groups is involved in chelate formation with each metal ion. The results for the copper chelates indicate a similarity between these chelates and the copper salts of polyhydroxy compounds prepared by Lieser and Ebert.[14] However, the immediate products are not, in a number of cases, the same as those formed after a prolonged reaction time. Iron chelates with hexitols to a much greater extent that the other three metals. It is well known that ferric hydroxide, as long as it is kept under water, is composed of molecular aggregates which are separated by layers of water.[18] It is thus suggested that in the chelation of ferric ion with hexitols in alkaline solution, the hexitols are incorporated into these aggregates and thereby confer solubility, a behaviour which makes the hexitols superior to other chelating agents.

Applications of metal chelates of polyhydroxy compounds, in addition to those already described,[9–13, 16] may be found in the fields of plant and animal nutrition, where a deficiency of metals can give rise to serious problems, e.g. iron chlorosis in plants. It has been pointed out[19] that the high cost of chelates in practical use, e.g. ethylenediamine tetra-acetic acid (EDTA) or closely related compounds, is perhaps the biggest deterrent to their widespread use in soil application. It is suggested that the chelates of polyhydroxy compounds might remedy this difficulty. It is also envisaged that these chelates will find a wider use in the separation of mixtures of metallic salts,[10, 20] in fields where trace metals are not desirable, and in the growth of micro-organisms (*cf.* Basu, *et al.*[21] who found that the presence of mannitol promotes growth of a copper-tolerant penicillium species in a saturated copper sulphate solution).

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Table

Chelating Power of Polyhydroxy Compounds

	Cu^{++}		*Fe*$^{+++}$		*Co*$^{++}$		*Ni*$^{++}$	
Chelating agent	*mg. Cu per g. chelating agent*	*g.-atoms Cu per mole chelating agent*	*mg. Fe per g. chelating agent*	*g.-atoms Fe per mole chelating agent*	*mg. Co per g. chelating agent*	*g.-atoms Co per mole chelating agent*	*mg. Ni per g. chelating agent*	*g.-atoms Ni per mole chelating agent*
D-Glucitol	988	2·83	3295	10·75	140	0·43	298	0·92
D-Mannitol	668	1·91	2302	7·51	224–719	0·69–2·22	114–666	0·35–2·07
Dulcitol	546	1·57	2319	7·56	35	Negligible	69	Negligible
Pentaerythritol	19	Negligible	43	Negligible	23	,,	12	,,
D-Glucose	684*	1·94*	30	,,	17	,,	—	—
D-Fructose	649*	1·84*	248	0·80	366	1·12	321	0·99

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PAPER IONOPHORESIS OF CARBOHYDRATES IN MOLYBDATE SOLUTIONS

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It is known[1] that molybdate reacts with polyhydroxy compounds and causes changes in their specific rotations, particularly at pH values less than about 2·5.[2] A comparison of the rates of elution of oligosaccharides from charcoal–'Celite' columns, impregnated with borate and molybdate separately, suggested that borate and molybdate complex across different positions and might therefore be used in separations of different pairs of sugars. We have examined this phenomenon further and found that the ionophoresis of carbohydrates in molybdate solution is a method which may well find wide application; it is a technique complementary to ionophoresis in other electrolytes.[3–5]

Ionophoresis was carried out on Whatman No. 3MM paper at 15 volts/cm. in 0·1 M sodium molybdate adjusted to pH 5 with sulphuric acid. The compounds were located with acetone silver nitrate-alcoholic sodium hydroxide.[6] D-Glucitol was used as a standard for the comparison of rates of migration, and glycerol as a non-migrating marker for the correction of electro-osmosis. Hence the migration rate is expressed as an M_S value. Under the above conditions D-glucitol migrated 6 cm. per hour, i.e. the rates are not as slow as might have been expected at pH 5. Some of the results are shown in the Table.

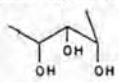
Table

M_S Values of Carbohydrates in Molybdate Solution

Compound	*M_S value*	*Compound*	*M_S value*
D-Glucitol	1·0	Turanose	0·14
D-Mannitol	1·0	Leucrose	0·43
Dulcitol	1·0	Sophorose	<0·1
Ribitol	1·1	Nigerose	<0·1
D-Arabitol	1·1	Laminaribiose	<0·1
Xylitol	1·1	Maltose	<0·1
D-Altrose	<0·1	Cellobiose	<0·1
D-Glucose	<0·1	Lactose	<0·1
D-Mannose	0→0·9	Isomaltose	<0·1
D-Gulose	1·1	Gentiobiose	<0·1
D-Galactose	<0·1	Melibiose	<0·1
D-Talose	0·7	Sophoritol (β-1:2)*	0·9
D-Ribose	0·4	Nigeritol (α-1:3)*	0
D-Arabinose	<0·1	Laminaribiitol (β-1:3)*	0
D-Xylose	<0·1	Maltitol (α-1:4)*	0·4
D-Lyxose	1·1	Cellobiitol (β-1:4)*	0·4
D-Erythrose	0·9	Lactitol (β-1:4)*	0·4
D-Threose	0·6	Isomaltitol (α-1:6)*	0·8
D-Fructose	0·5	Gentiobiitol (β-1:6)*	0·8
L-Sorbose	0·3	Melibiitol (β-1:6)*	0·8

*Glycosidic link in parentheses

By this method a very quick and efficient separation of some pentoses and hexoses from the corresponding alcohols is effected. The separation within the series of monosaccharides is complementary to separations in other electrolytes.[3–5] It is of interest to note that, of the aldohexoses and aldopentoses examined, only those which possess a *cis-cis*-1 : 2 : 3-triol system and thus, in at least one of their conformational isomers, have one equatorial hydroxyl group neighboured by two axial hydroxyl groups[7] (I), complex significantly with molybdate. Similar results have been obtained with inositols.



(I)

The technique has proved most useful in characterising the position at which the glucose reducing end-group of oligosaccharides is linked. This arose from studies of the changes of the optical rotation of derivatives of D-glucitol. 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 the M_S values of *O*-glycosyl D-glucitols obtained by 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, allowing thus an efficient separation (see Table). We have used this method also to distinguish the two trisaccharides, *O*-α-D-glucopyranosyl-(1 → 3)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucose (A) and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucose (B). obtained on hydrolysis of nigeran.[8] Reduction of the trisaccharides followed by ionophoresis in molybdate effected marked separation, (B) being immobile.

Frahn and Mills[5] have briefly examined the paper ionophoresis of hexitols and monosaccharides in molybdate solution but reported that, under their conditions, some did not separate and others streaked too badly for measurement.

Further work is in progress to determine the behaviour of other *O*-glycosyl polyalcohols.

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PAPER IONOPHORESIS OF GLUCOPYRANOSYL-FRUCTOSES AND OTHER SUBSTITUTED FRUCTOSES

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Oligosaccharides possessing fructose as the reducing unit form a class of carbohydrates which is now more frequently encountered, but they are not easy to characterise. Fructose has a greater tendency to complex with inorganic oxy-acids than the common aldoses and their glycosides and therefore these oligosaccharides should readily lend themselves to characterisation by ionophoresis in a number of separate electrolytes.

Ionophoresis of sugars in borate solution has been well established by Foster,[1] and a technique using molybdate solution was reported recently.[2, 3] In addition, Frahn and Mills[2] have investigated the behaviour of many compounds in a number of electrolytes. Borate, molybdate, arsenite, and sodium hydroxide solutions were chosen for the present work. The electrolytes were prepared as described in the references quoted above. Ionophoresis was carried out at 80 volts/cm. for *ca.* 1 hour. It has not been found possible to find a reference compound and a non-migrating marker generally applicable to all the electrolytes.[2] Those used are shown in the Table. The ketoses were located with the urea phosphate reagent,[4] and reference compounds and non-migrating markers with acetone silver nitrate-alcoholic sodium hydroxide,[5] except levoglucosan on papers impregnated with arsenite, which was located by heating with acidic molybdate.

The results (see Table) reveal that the glucopyranosyl-fructoses can be clearly distinguished by measurement of their rates of migration in the four electrolytes. The non-reducing glucopyranosyl moiety of a disaccharide molecule and the nature of the glucosidic linkage (i.e. α or β) have only little effect on the mobility.[1–3] Hence the α- and β-isomers of the above compounds will behave more or less identically. Higher saccharides, e.g. maltosyl-fructoses, should migrate slightly slower than the disaccharides but with the same relative differences.

In molybdate solution, 1-*O*-methyl-D-fructose (M_S 0·30) and 1-*O*-β-D-glucopyranosyl-D-fructose (M_S 0·25) have similar mobilities, as also do 6-deoxy-D-fructose (M_S 0·70) and isomaltulose (M_S 0·64). A striking feature is that the migration rates for the latter pair of compounds are higher than that of fructose itself (M_S 0·50), which indicates that molybdate complexes more readily when a fructopyranose ring cannot be formed.

Mobility during paper ionophoresis in electrolytes of the above type depends very much more on structure than on molecular size (contrast paper chromatography) and it is clear that ionophoresis in several electrolytes may be used as a much firmer characterisation of the above oligosaccharides. The method is also of general value in distinguishing mono-*O*-substituted fructoses as long as the substituent plays no part in complexing. The mono-*O*-methyl-fructoses, for example, should be readily separated and characterised by ionophoresis in these electrolytes.

Table

Migration rates of some glucopyranosyl-fructoses

		Borate (0·1M, pH 10)	Molybdate (0·1M, pH 5·0)	Arsenite (0·2M, pH 9·6)	NaOH (0·1N)
Reference Compound		D-Glucose	Sorbitol	D-Ribose	D-Ribose
Non-migrating marker		Levo-glucosan	Glycerol	Levo-glucosan	Glycerol
Compound	*Linkage*	M_G *value*	M_S *value*	M_R *value*	M_R *value*
D-Fructose		0·89	0·50	0·75	0·89
1-*O*-β-D-Glucopyranosyl-D-fructose	β-1,1	0·74	0·25	0·78	0·35
Sucrose	α-1,2	0·10	0	0·25	0·15
Turanose	α-1,3	0·69	0·10	0·80	0·28
Maltulose	α-1,4	0·63	0·15	0·76	0·60
Leucrose	α-1,5	0·56	0·35	0·62	0·28
Isomaltulose	α-1,6	0·60	0·64	0·72	0·73

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831. *Paper Ionophoresis of Sugars and Other Cyclic Polyhydroxy-compounds in Molybdate Solution.*

By E. J. Bourne, D. H. Hutson, and H. Weigel.

Paper ionophoresis in molybdate solution at pH 5 has proved a useful analytical method for carbohydrates, complementary to the technique in other electrolytes. Examination of aldoses, derivatives of aldoses, and cyclitols has revealed that compounds with a six-membered ring system form complexes with molybdate if they possess three hydroxyl groups in a *cis-cis*-1,2,3-triol arrangement.

The fact, long known,[1] that molybdate forms complexes with polyhydroxy-compounds found little practical use until Richtmyer and Hudson [2] attempted to use quantitatively the greatly increased specific rotations of the hexitols in acidified ammonium molybdate solutions. Barker *et al.*,[3] comparing the rates of elution of the oligosaccharides of the maltose series from charcoal columns impregnated with borate and molybdate severally, suggested that the two ions formed complexes in different manners and might be used for separations of different pairs of sugars. Complex-formation from polyhydroxy-compounds and borate has been of great value as the complexes, possessing a negative charge, migrate in an electric field,[4] and borate ionophoresis has been studied extensively and brought into general use by Foster.[5]

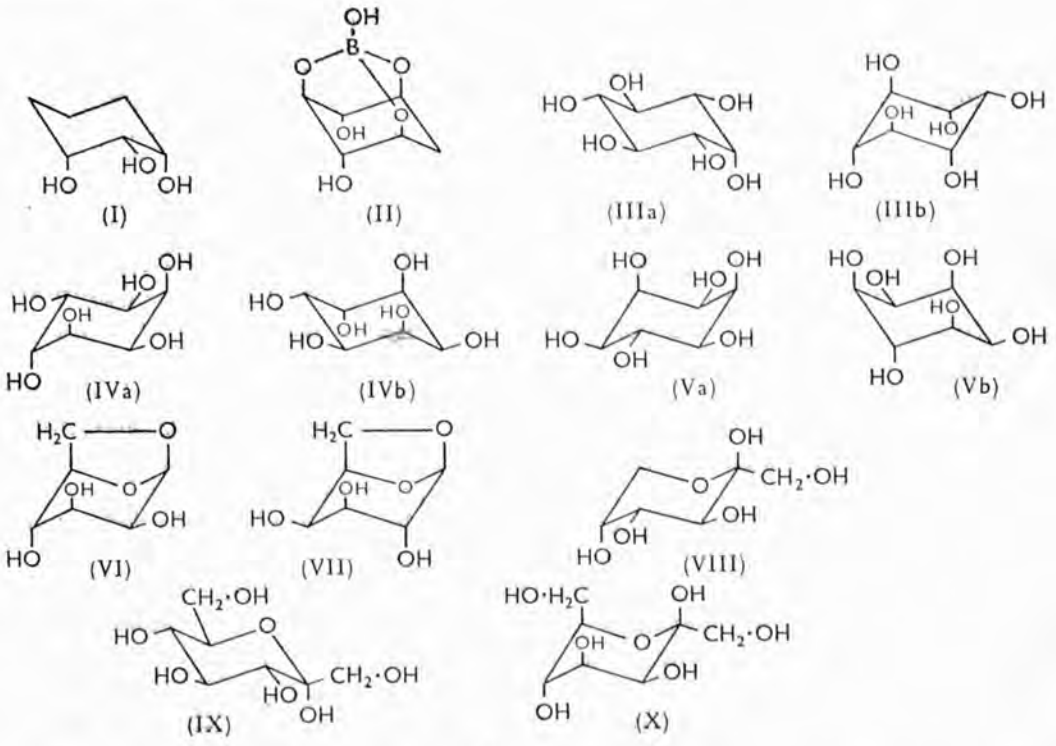
Use of molybdate complexes in paper ionophoresis was studied for a few sugars by Frahn and Mills.[6] We have examined the paper ionophoresis of a number of cyclic carbohydrates in molybdate solution at pH 5·0. This pH was chosen after trials had confirmed that molybdate forms complexes more strongly in acidic than in alkaline solution.[1,2] The investigation has given some insight into the structure of the molybdate–carbohydrate complex, a hitherto unexplored field, and interpretation was aided by dividing the compounds into groups according to their structure. Sorbitol was used as a standard for the comparison of rates of migration, and glycerol as a non-migrating marker for correction of electro-osmosis. Hence, the migration rates are expressed as M_S values.

Aldoses and Derivatives.—M_S values of aldoses and their derivatives are given in the Table. Glyceraldehyde and the tetroses were included for completeness. The separation of the two tetroses, which takes *ca.* 20 min. by this technique, is difficult by any other simple method.

Glycoside formation reduced the tendency to migrate to a negligible or very low level (cf. D-mannose, D-ribose, and D-lyxose, and the corresponding methyl glycosides), and this suggested that complex formation may have been due to small amounts of *aldehydo*-forms, as in the case of borate-complex formation.[5] However, the amounts of these forms in aqueous solution (*e.g.*, D-ribose 8·5% and D-lyxose 0·4% [7]) would not explain satisfactorily the relative rates of migration. The possibility of the formation of a " tridentate " complex was then examined and it was found that only those aldohexoses and aldopentoses which possess a *cis-cis*-1,2,3-triol system and thus, in at least one of their conformations,[8,9] one equatorial hydroxyl group neighboured by two axial hydroxyl groups (cf. I), migrated during ionophoresis. This type of " tridentate " complex is believed to occur between periodate and D-ribose and other compounds possessing the *cis-cis*-1,2,3-triol system, *e.g.*, 1,6-anhydro-β-D-allopyranose.[10] Similar structures have been postulated for scyllo-quercitol borate [11] (II) and pentaerythritol arsenite.[12]

In all cases, except D-ribopyranose and D-talopyranose, the *cis-cis*-1,2,3-triol system includes the hydroxyl group on the anomeric carbon atom. Substitution in, or replacement of, at least one of the hydroxyl groups of this system destroys the ability to form a " tridentate " complex. This is exemplified by 3,4-di-*O*-methyl-D-mannose, methyl

β-D-mannopyranoside, methyl α- and β-D-lyxopyranoside, and 2-deoxy-D-ribopyranose. D-Ribopyranose possesses the 1(*ax*),2(*eq*),3(*ax*)-triol system (I) in both the C1 and the 1C conformation (Reeves' nomenclature). Methyl α-D-ribopyranoside, however, has this system only in the 1C conformation.



Of the D-*glycero*-aldoheptoses examined, the D-*gulo*-, D-*allo*-, and L-*manno*-compounds possess the 1(*ax*),2(*eq*),3(*ax*)-triol system (I) in one of their conformations of the pyranose ring. The 4-, 6-, and 7-hydroxyl groups of all heptoses can be brought, without distortion of bond angles, into the same relative positions as those of the 1(*ax*),2(*eq*),3(*ax*)-triol system (I).

Cyclitols.—Less ambiguous results might be obtained from an examination of the behaviour of the cyclitols where the possibility of open-chain and five-membered ring structures does not arise. Of the eleven cyclitols examined, only myoinositol (III), alloinositol (IV), and epi-inositol (V) (Angyal and Anderson's nomenclature [13]) possess the *cis-cis*-1,2,3-triol system and hence migrated. Their rates of migration can be related to the instability factors [8] of their conformations which have the 1(*ax*),2(*eq*),3(*ax*)-triol system (I). Of the two chair conformations of myoinositol (IIIa and b) only the less favoured conformation (IIIb), which has five axial hydroxyl groups, has the 1(*ax*),2(*eq*),3(*ax*)-triol system. The two chair conformations of alloinositol (IVa and b) are mirror images and both have three axial hydroxyl groups. Both chair conformations of epi-inositol (Va and Vb) possess the 1(*ax*),2(*eq*),3(*ax*)-triol system, but (Va) is very much more favoured than (Vb) and it has only two axial hydroxyl groups.

1,6-*Anhydro*-β-D-*aldopyranoses.*—Of those examined (Table) only 1,6-anhydro-β-D-mannopyranose (VI) migrated. The steric arrangement of the three hydroxyl groups (*eq, ax, ax*) occurs also in 1,6-anhydro-β-D-galactopyranose (VII). However, a unique feature of 1,6-anhydro-β-D-mannopyranose is the *ax, eq, ax* relation of the 2- and 3-hydroxyl groups and the anhydro-ring oxygen atom. A possible explanation for complex formation in this compound is that a cyclic complex across the 2- and 3-hydroxyl groups is stabilised by hydrogen bonding between a molybdenum-hydroxyl group and the oxygen atom of the

M_S Values of polyhydroxy-compounds.

Complex-forming	M_S value	Not complex-forming (M_S <0·1)	
Aldoses and their derivatives.			
		Glyceraldehyde	
D-Erythrose	0·9	2-Deoxy-D-ribose	Methyl α-D-glucopyranoside
L-Threose	0·6	D-Arabinose	Phenyl β-D-glucopyranoside
D-Ribose	0·4	Methyl α-D-arabopyranoside	Catechol β-D-glucopyranoside
Methyl α-D-ribopyranoside	0·1	Methyl β-D-arabopyranoside	3,4-Di-*O*-methyl-D-mannose
		1,2-Dideoxy-D-arabinose	Methyl α-D-mannopyranoside
D-Lyxose	1·1	D-Xylose	Methyl β-D-mannopyranoside
D-Mannose	0→0·9	Methyl α-D-xylofuranoside	D-Galactose
D-Gulose	1·1	Methyl α-D-lyxopyranoside	Sophorose
D-Talose	0·7	Methyl β-D-lyxopyranoside	Nigerose
L-Rhamnose	0→0·6	D-Altrose	Laminaribiose
D-*glycero*-D-*allo*Heptose	0·9	D-Glucose	Maltose
D-*glycero*-L-*gluco*Heptose	0·2	3-*O*-Methyl-D-glucose	Cellobiose
D-*glycero*-L-*manno*Heptose	0·8	2,3,4-Tri-*O*-methyl-D-glucose	Lactose
D-*glycero*-D-*gulo*Heptose	1·1	2,3,6-Tri-*O*-methyl-D-glucose	Isomaltose
D-*glycero*-D-*ido*Heptose	1·0	2,3,4,6-Tetra-*O*-methyl-D-glucose	Gentiobiose
D-*glycero*-D-*gala*Heptose	0·4	2-Deoxy-D-glucose	Melibiose
D-*glycero*-L-*gala*Heptose	0·4		
Cyclitols.			
Myoinositol (III)	0·2	Mucoinositol	Quebrachitol
Alloinositol (IV)	0·4	Scylloinositol	(−)-Viboquercitol
Epi-inositol (V)	1·1	Mytilitol	Scylloquercitol
		Pinitol	(+)-Protoquercitol
1,6-*Anhydro*-β-D-*aldopyranoses.*			
1,6-Anhydro-β-D-mannopyranose (VI)	0·5	1,6-Anhydro-β-D-glucopyranose 1,6-Anhydro-β-D-gulopyranose 1,6-Anhydro-β-D-galactopyranose (VII)	
Ketoses.			
D-Fructose (VIII)	0·5	Sucrose	
L-Sorbose	0·3		
D-Glucosone	0·9		
D-*gluco*Heptulose (IX)	1·0		
D-*manno*Heptulose (X)	0·4		
Leucrose	0·4		
Turanose	0·1		

anhydro-ring. There was no evidence from the behaviour of other compounds to refute this suggestion.

Ketoses.—The general tendency of ketoses in this section to migrate (see Table) suggests that an examination of the behaviour of more ketoses is warranted. Comparison with the aldoses shows that several ketose–aldose separations are possible. These may be of particular use in the oligosaccharide field. The nature of the molybdate–ketose complex is uncertain but three possibilities could arise: (*a*) complex-formation by an open-chain form; (*b*) slight enolisation of the keto-group and complex-formation by the resultant ene-diol with molybdate;[14] and (*c*) "tridentate" complex formation with either the furanose or the pyranose ring form. For example, in the furanose and pyranose form of D-fructose (VIII) and L-sorbose, the 1-, 2-, and 3-hydroxyl groups are able to form a structure of the same relative spacings as those of the 1(*ax*),2(*eq*),3(*ax*)-triol system (I). It is probable, by virtue of the α–β-equilibrium and the free rotation of the 2-hydroxymethyl group, that unsubstituted 1-, 2-, and equatorial 3-hydroxyl groups of the pyranose form of any 2-ketose can form a structure approximating to that of (I). The same applies for unsubstituted 1-, 2-, and 3-hydroxyl groups of the furanose form of any 2-ketose. The large difference in M_S values of D-*gluco*heptulose (IX) and D-*manno*heptulose (X) confirms this, as the latter, when in the pyranose form, has an equatorial 3-hydroxyl group only in its unfavoured conformation (X). The relatively high migration rate of leucrose (5-*O*-substituted fructopyranose), the low migration rate of turanose (3-*O*-substituted fructopyranose), and the immobility of sucrose (2-*O*-substituted fructofuranose) support this.

General Observations.—It is thus established that sugars and other six-membered cyclic polyhydroxy-compounds form complexes significantly with molybdate only if they possess a *cis-cis*-1,2,3-triol system, or can assume an equivalent system. It can be seen from the Table that several useful separations are obtained by ionophoresis in molybdate solution which are very difficult by chromatography or ionophoresis in borate solution. The rapidly migrating sorbitol, used as a standard, is very quickly separated from glucose and the aldose–alditol separation is fairly general. In an investigation of the action of Fenton's reagent on sorbitol,[15] where the main products are D-glucose, L-gulose, D-fructose, and L-sorbose, the technique was most useful. Ionophoresis of such a mixture in borate solution was virtually useless. Also molybdate forms its complexes most strongly in acid, whereas the borate complexes are more stable in alkali. The two methods can thus be regarded as complementary.

It is generally accepted that when a borate ion reacts with a polyhydroxy-compound a cyclic diester is usually formed.[5] Angyal and McHugh [11] postulated the formation of a " tridentate " borate complex (II) with certain cyclitols. Molybdate, however, seems to form complexes with sugars and other six-membered cyclic polyhydroxy-compounds only when three or more hydroxyl groups are available in the correct relative positions. There is one exception to this rule. Compounds containing the " ene-diol " group, *e.g.*, ascorbic acid and *o*-dihydroxybenzene, form stable complexes with molybdate at pH *ca.* 5·0, giving deep orange solutions. This complex has been used by Pridham [16] and by Halmekoski [17] for the ionophoresis and chromatography of phenolic compounds. *m*-Dihydroxybenzene does not form a complex with molybdate, and an " ene-diol " group seems to be essential. The participation of only two hydroxyl groups in the " ene-diol " system need not be at variance with our suggestion. The intense colour of the complex of " ene-diol " compounds indicates a conjugated system. Thus the diester structure could be resonance-stabilised. When only two suitable hydroxyl groups were involved in a sugar complex, as in 1,6-anhydro-β-D-mannopyranose (VI), the complex was probably stabilised by hydrogen-bonding.

Ionophoresis of sugars and other six-membered cyclic polyhydroxy-compounds in molybdate solution is not only a tool for the separation of such compounds. Applications might be found in the determination of structures. Affinity for molybdate can be regarded as a diagnosis for the ability of a compound possessing a *cis-cis*-1,2,3-triol system to adopt a conformation with a 1(*ax*),2(*eq*),3(*ax*)-triol system. For example, α-D-ribopyranose possesses this system in the C1 and the 1C conformation. β-D-Ribopyranose has this spatial arrangement of three hydroxyl groups in the 1C conformation. The equilibrium mixture of D-ribose thus migrates during ionophoresis in molybdate solution. However, methyl α-D-ribopyranoside possesses the 1(*ax*),2(*eq*),3(*ax*)-triol system only in the 1C conformation and its low rate of migration suggests that it will not easily adopt this.

In the discussion above only " chair " conformations of six-membered ring compounds have been considered. Reeves [8] concluded that a " chair " conformation is adopted in preference to any " boat " conformation whenever both are structurally possible. However, complex formation with molybdate could also easily occur with " boat " conformations of the compounds possessing the *cis-cis*-1,2,3-triol system, as at least one of their " boat " conformations possesses a 1(*bax*),2(*bs*),3(*bax*)-triol system (Angyal and Mills's nomenclature [18]), which is spatially identical with the 1(*ax*),2(*eq*),3(*ax*)-triol system.

Experimental

Paper Ionophoresis.—The apparatus used was built according to a design kindly provided by Dr. D. Gross of Tate and Lyle, Ltd., and was capable of delivering up to 5000 v at 100 mA. Ionophoresis was carried out on 10 cm. wide sheets of Whatman No. 3MM filter paper. The electrolyte was prepared by dissolving sodium molybdate dihydrate (25 g.) in water (1200 ml.) and adjusting the whole to pH 5·0 with concentrated sulphuric acid. Ionophoretograms were prepared by applying a voltage of 30—60 v/cm. for 1—2 hr. Compounds were detected by

spraying with acetone–silver nitrate–ethanolic sodium hydroxide [19] or *p*-anisidine hydrochloride in butan-1-ol.[20] Migration rate was expressed relative to the movement of sorbitol which migrated *ca.* 25 cm. in 2 hr. at 60 v/cm. By comparison with 2,3,4,6-tetra-*O*-methyl-D-glucose, glycerol was shown not to form a complex and was used for the correction of electro-osmosis.

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7. *Complexes between Molybdate and Acyclic Polyhydroxy-compounds.*

By E. J. Bourne, D. H. Hutson, and H. Weigel.

The compositions of complexes between molybdate and some acyclic polyhydroxy-compounds have been determined. Ionophoresis of reduced oligosaccharides in molybdate solution has been shown to be a useful method for determining the position of the glycosidic linkage to the reducing group of the original oligosaccharide.

It is known [1] that complexes of molybdate with polyhydroxy-compounds in aqueous solution have a maximum stability at acidic pH values and are decomposed by alkali. In a previous paper [2] we discussed the behaviour of sugars and other cyclic polyhydroxy-compounds, with six atoms in the ring, during ionophoresis in molybdate solution. We now report studies on the complexes between molybdate and acyclic polyhydroxy-compounds.

The effect of pH on the specific rotations of some acyclic polyhydroxy-compounds in molybdate solution was examined in order to find suitable conditions for ionophoresis. It can be seen from the results (Fig. 1) that maximal changes in specific rotation, and hence maximal complex-formation, occurred at *ca.* pH 2, but that there was some complex-formation over the whole range of pH 1—8. Ionophoresis was therefore carried out at pH 5, a value which is low enough to assure presence of sufficient complex and high enough to allow reasonable ionisation. The M_S values of acyclic polyhydroxy-compounds are shown in Table 1.

Table 1. *M_S Values of acyclic polyhydroxy-compounds.*

$$M_S = \frac{\text{true distance of migration of compound}}{\text{true distance of migration of sorbitol}}$$

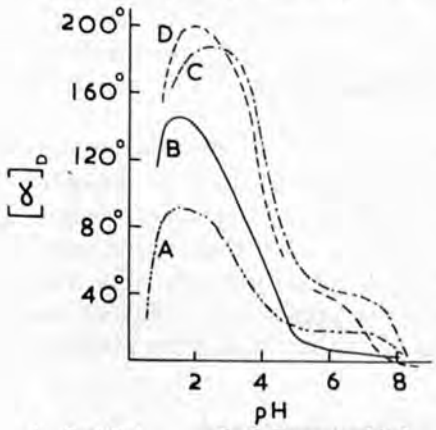
Compound	M_S	Compound	M_S
Ethane-1,2-diol	<0·1	2,3-Di-*O*-methylsorbitol	<0·1
Propane-1,2-diol	<0·1	4-*O*-α-D-Glucopyranosylsorbitol	0·4
Propane-1,3-diol	<0·1	4-*O*-β-D-Glucopyranosylsorbitol	0·4
Butane-2,3-diol	<0·1	4-*O*-β-D-Galactopyranosylsorbitol	0·4
Butane-1,3-diol	<0·1	4-*O*-α-Isomaltosylsorbitol	0·4
Butane-1,4-diol	<0·1	4-*O*-α-Nigerosylsorbitol	0·4
Pentane-1,5-diol	<0·1	5-*O*-α-D-Glucopyranosylsorbitol	0·8
Hexane-1,6-diol	<0·1	6-*O*-α-D-Glucopyranosylsorbitol	0·8
2-Methylpentane-2,4-diol	<0·1	6-*O*-β-D-Glucopyranosylsorbitol	0·8
2-Methylhexane-1,3-diol	<0·1	6-*O*-α-D-Galactopyranosylsorbitol	0·8
Pentaerythritol	<0·1	6-*O*-α-Isomaltosylsorbitol	0·7
Glycerol	<0·1	6-*O*-α-Isomaltotriosylsorbitol	0·6
Erythritol	1·0	6-*O*-α-Isomaltotetraosylsorbitol	0·5
D-Threitol	0·5	6-*O*-α-Isomaltopentaosylsorbitol	0·4
Ribitol	1·1	6-*O*-α-Isomaltohexaosylsorbitol	0·3
D-Arabitol	1·1	6-*O*-α-Isomaltoheptaosylsorbitol	0·25
3-*O*-α-D-Galactopyranosyl-D-arabitol	<0·1	D-Mannitol	1·0
Xylitol	1·1	1-Deoxy-D-mannitol	1·0
Sorbitol	1·0	2-*O*-Methyl-D-mannitol	1·0
2-Deoxysorbitol	1·0	2-*O*-α-D-Glucopyranosyl-D-mannitol	0·8
2-*O*-β-D-Glucopyranosylsorbitol	0·9	2-*O*-α-D-Mannopyranosyl-D-mannitol	0·8
3-*O*-Methylsorbitol	<0·1	3-*O*-α-D-Mannopyranosyl-D-mannitol	<0·1
3-*O*-α-D-Glucopyranosylsorbitol	<0·1	1,2-Di-*O*-methyl-D-mannitol	1·0
3-*O*-β-D-Glucopyranosylsorbitol	<0·1	Galactitol	1·0
3-*O*-α-Maltosylsorbitol	<0·1	6-Deoxy-D-galactitol	1·0

A further examination was made of the effect of the relative concentrations of the polyhydroxy-compounds and molybdate on the specific rotations of the former at pH values between 2 (Fig. 2) and 5. The rotation became constant when either one or two

mol. of molybdate had been added. This revealed the compositions of the complexes which were found to be unchanged over this pH range, although the specific rotations were lower at the higher pH value. The specific rotations, when based on the polyhydroxy-compounds, were also found to be independent of the absolute concentrations of the solutions.

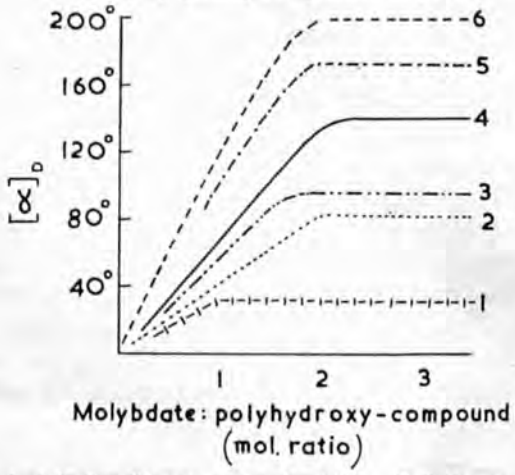
The behaviour of the complexes between polyhydroxy-compounds and molybdate during paper chromatography was examined as a possible analytical method. Impregnation of the paper with acidic molybdate solution [3] before chromatography did not yield reproducible results. When the complexes were formed by dissolving sodium molybdate and the polyhydroxy-compounds in the molar ratio of 2 : 1, and the sodium ions removed

FIG. 1. *Effect of* pH *on* $[\alpha]_D$ *of polyhydroxy-compounds in molybdate solutions (molar ratio molybdate/polyhydroxy-compound = 3).*



A, *Sorbitol.* C, 2-*Deoxysorbitol.*
B, *Mannitol.* D, D-*Arabitol.*

FIG. 2. *Effect of relative concentrations of polyhydroxy-compounds and molybdate on* $[\alpha]_D$ *of polyhydroxy-compounds at* pH2.



1, 4-O-β-D-*Glucopyranosylsorbitol.* 2, 6-O-α-D-*Galactopyranosylsorbitol.* 3, *Sorbitol.* 4, D-*Mannitol.* 5, 2-*Deoxysorbitol.* 6, D-*Arabitol.*

with an ion-exchange resin, the resulting solutions could be chromatographed. Paper chromatography in acidic or neutral solvents revealed (Table 2) a trace of molybdic acid remaining on the origin line and traces of the polyhydroxy-compounds which migrated at their normal rate. In each case, between these two spots one or two components could be detected. We believe that these are mono- and di-molybdate complexes of the polyhydroxy-compounds.

The dihydroxy-compounds examined did not migrate during ionophoresis (Table 1) and hence do not form complexes with molybdate. This suggests that at least three hydroxyl groups are required for complex-formation with molybdate, as is the case for sugars and other cyclic polyhydroxy-compounds with six ring-atoms.[2] In agreement with Richardson's results,[4] glycerol did not form a complex.

It can be seen from Fig. 2 and Table 2 that sorbitol and 2-deoxysorbitol will each form a complex containing two molybdenum atoms per molecule. A 3-*O*-substituted sorbitol does not migrate during ionophoresis in molybdate solution (Table 1) and hence does not give a complex. Since the α- and β-D-glucopyranosides and D-galactose do not give a complex,[2] it is evident that each molecule of 4-*O*-substituted sorbitol will form a complex containing one molybdenum atom, and each molecule of 6-*O*-substituted sorbitol a complex containing two molybdenum atoms (Fig. 2). As a mixture of 5-*O*-α-D-glucopyranosylsorbitol and 2-*O*-α-D-glucopyranosyl-D-mannitol, obtained by reduction of leucrose, migrated during ionophoresis at the same rate as 6-*O*-α-D-galactopyranosylsorbitol it is reasonable to assume that a 5-*O*-substituted sorbitol also will form a complex containing two molybdenum atoms.

D-Mannitol and D-arabitol each form a complex containing two molybdenum atoms (Fig. 2 and Table 2). 3-*O*-Substituted D-arabitol does not form a complex (Table 1). Richardson [4] has found by conductometric measurements that erythritol forms a complex containing one molybdenum atom: our chromatographic method (Table 2) has confirmed this.

It is clear that more studies are necessary before the detailed structures of the complexes between molybdate and acyclic polyhydroxy-compounds can be assigned. The problem is more difficult than in the case of cyclic polyhydroxy-compounds: *e.g.*, the number of conformations which can be adopted by an acyclic polyhydroxy-compound is very much greater. It was thought that the application of ionophoresis of polyhydroxy-compounds in molybdate solution was of greater immediate value than the assignment of the structure of the complexes.

In a previous paper [2] we reported the M_S values (defined in Table 1) of sugars and other cyclic polyhydroxy-compounds. It can now be seen that the common sugars may easily be separated from their reduction products. The method has found routine use in checking the complete reduction of sugars during treatment with sodium borohydride.

The compositions of the complexes between molybdate and substituted sorbitols suggested that the M_S values of *O*-glycosylsorbitols obtained by reduction of oligosaccharides with D-glucose as the reducing end-group would fall into three well-defined groups, *i.e.*, of those with 1,3-, 1,4-, and 1,2- or 1,5- or 1,6-glycosidic linkages.[5] This has been shown to be so (Table 1). The M_S values of the three groups of *O*-glycosylsorbitols derived from di- and tri-saccharides are $<0{\cdot}1$, $0{\cdot}4$, and $0{\cdot}7$—$0{\cdot}9$, respectively, thus allowing an efficient separation. Foster [6] has shown that disaccharides of D-glucose with 1,2- or 1,4-glucosidic linkages can be differentiated from those with 1,3- or 1,6-glucosidic linkages by ionophoresis in borate solution. Ionophoresis in molybdate solution is hence complementary to that in borate solution.

Ionophoresis in molybdate solution can be applied also to the reduction products of oligosaccharides containing up to 8 glucose units, as the M_S values are reasonably high, especially if the reducing glucose unit of the original oligosaccharide was linked by a 1,6-linkage. Table 1 shows the M_S values of the reduced oligosaccharides of the isomaltose series.

Table 1 also shows that 3-*O*-glycosyl-D-mannitol and 2-*O*-glycosyl-D-mannitol can be readily separated by ionophoresis in molybdate solution.

Experimental

Effect of pH *on Optical Rotation of Acyclic Polyhydroxy-compounds in Molybdate Solutions.*—Several solutions containing hydrated sodium molybdate and the polyhydroxy-compound (*ca.* 1—3%) in the molar ratio of 3 : 1 were adjusted with sulphuric acid to pH values between 1 and 8. The optical rotations measured were expressed as $[\alpha]_D$ and based on the polyhydroxy-compound. The results are shown graphically in Fig. 1.

Paper Ionophoresis.—Paper ionophoretograms were prepared by applying a voltage of 20—80 V per cm. across 10 cm. wide lengths of Whatman No. 3MM filter-paper for 1—2 hr. in an apparatus similar to that described by Gross,[7] to whom we are grateful for advice. The electrolyte consisted of hydrated sodium molybdate (25 g.) in water (1200 ml.) adjusted to pH 5 with sulphuric acid. Compounds were detected with acetone–silver nitrate–alcoholic sodium hydroxide [8] or by spraying the paper with 0·1N-sulphuric acid and heating it at 120° for 10 min. The latter treatment caused all compounds containing primary hydroxyl groups to appear as bluish-green spots. Migration rates (Table 1) were expressed as M_S values.[2]

Effect of Relative Concentrations of Acyclic Polyhydroxy-compounds and Molybdate on the Optical Rotation of the Former.—Solutions containing the polyhydroxy-compound (*ca.* 1—14%) and hydrated sodium molybdate in varying molar ratios were adjusted to pH 2 with sulphuric acid. The optical rotations measured were expressed as $[\alpha]_D$ and based on the polyhydroxy-compound. The results are shown graphically (Fig. 2) by plotting $[\alpha]_D$ against the molar ratio of molybdate and polyhydroxy-compound. Repetition of the experiments at pH 5 resulted in lower values of $[\alpha]_D$ but no change in the shapes of the curves.

Paper Chromatography of Pre-formed Complexes between Molybdate and Acyclic Polyhydroxy-compounds.—Complexes were formed by dissolving polyhydroxy-compounds (1 mol.) and hydrated sodium molybdate (2 mol.) in water and adjusting the solutions to pH 2 with Amberlite IR-120 (H^+). The solutions were then spotted on to Whatman No. 1 filter-paper and the paper was irrigated with the organic layer of a mixture of butan-1-ol–acetic acid–water (4 : 1 : 5). Acetone–silver nitrate–alcoholic sodium hydroxide [8] was used to locate polyhydroxy-components, and an aqueous solution of catechol [9] (5%) to locate molybdate-containing components. The results are shown in Table 2.

TABLE 2.

Compound	$R_{Glucose}$	$R_{Glucose}$ of components of pre-formed complexes			
Sorbitol	1·0	0·9 *	0·6 *†	0·5 *†	0 †
2-Deoxysorbitol	1·2	1·2 *	0·7 *†	0·4 *†	0 †
D-Mannitol	1·1	0·9 *	0·7 *†	0·5 *†	0 †
Galactitol	1·1	1·1 *	0·7 *†	0·5 *†	0 †
D-Arabitol	1·1	1·1 *	0·7 *†	0·4 *†	0 †
Erythritol	1·7	1·7 *	1·2 *†		0 †
Glycerol	2·2	2·2 *			0 †
Molybdic acid					0 †

* Detected with acetone–silver nitrate–alcoholic sodium hydroxide.
† Detected with aqueous solution of catechol.

The organic phase of a mixture of butanol–ethanol–water (4 : 1 : 5) gave a wider separation of the components but the spots were less discrete.

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COMPLEXES BETWEEN POLYHYDROXY-COMPOUNDS AND INORGANIC OXY-ACIDS. TUNGSTATE COMPLEXES OF SUGARS AND OTHER CYCLIC POLYHYDROXY-COMPOUNDS

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(Received 1 November 1964)

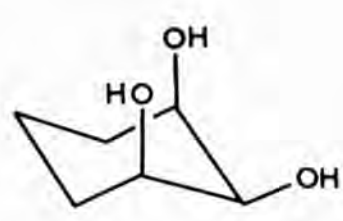
In previous papers[1,2] we have shown that molybdate, in common with ions of other inorganic oxy-acids[3], forms ionic complexes with certain polyhydroxy-compounds. It was already known that tungstate enhances the specific rotation of D-mannitol[4]. The use of tungstate complexes in paper electrophoresis was briefly studied by Frahn and Mills[5]. The present investigation, in which tungstate was used as the inorganic oxy-acid ion, was of interest because of the close resemblance between the chemistry of molybdenum^{VI} and that of tungsten^{VI}, particularly with respect to their oxygen compounds.

The specific rotations of D-mannose and D-ribose in tungstate solutions of pH 6.0 and pH 6.7, respectively, where maximal changes occur, are affected by the relative concentration of the two components. The results are typical of an equilibrium reaction. Measurements with continuous variation of concentration of both components have shown that the complexes formed from D-mannose and D-ribose contain the tungsten atom and the monosaccharide in the ratio 1:1. It is reasonable to assume

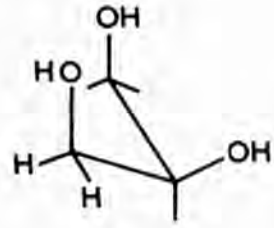
that complexes formed from the compounds discussed below and which contain the same essential structural features as D-mannose and D-ribose are of the same type.

An examination was made of the behaviour of sugars and other cyclic polyhydroxy-compounds during paper electrophoresis in tungstate solution of pH 5. D-Glucitol was used as a standard for comparison of rates of migration, and glycerol or hydroxymethylfurfural as a non-migrating marker for correction of electro-osmosis. Migration rates are thus expressed as M_S(W) or M_S(Mo) values. W and Mo refer to the electrolytes tungstate and molybdate, respectively.

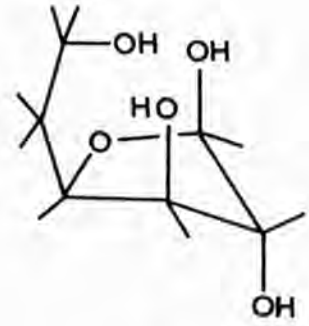
Previously, we established that sugars and other six-membered cyclic polyhydroxy-compounds complex significantly with molybdate only if they possess a cis-cis-1,2,3-triol system and thus, in one of their chair conformations, one equatorial hydroxyl group neighboured by two axial hydroxyl groups [1(ax),2(eq),3(ax)-triol system, (I)].[1] Alternatively, complexing with molybdate occurred when three hydroxyl groups could assume an equivalent spatial disposition, e.g. when one of the hydroxyl groups was not attached to a ring carbon atom (II). The present results show that the same type of "tridentate" complexes are formed with tungstate.



I



II



III

M_S Values of polyhydroxy-compounds

Migrating compounds	$M_S(W)$	$M_S(Mo)$
D-Erythrose	0.2 - 1.1	0.9[1]
L-Threose	0.05	0.6[1]
D-Lyxose	0 - 1.0	1.1[1]
D-Ribose	0.2	0.4[1]
D-Gulose	1.1	1.1[1]
6-Deoxy-D-gulose	1.1	1.1
D-Mannose	0 - 1.1	0 - 0.9[1]
6-Deoxy-L-mannose	0 - 1.1	0 - 0.6[1]
5-Deoxy-D-xylo-hexose	0.3	0.7
Alloinositol	0.1	0.4[1]
Epi-inositol	1.0	1.1[1]

Non-migrating compounds $M_S(W) < 0.05$

Glyceraldehyde	6-Deoxy-D-glucose
L-Arabinose	Methyl α-D-glucopyranoside
Methyl α-D-lyxopyranoside	3,4-Di-O-methyl-D-mannose
Methyl α-D-ribopyranoside	Methyl α-D-mannopyranoside
D-xylose	2-Deoxy-D-arabino-hexose
2-Deoxy-D-erythro-pentose	2-Deoxy-D-lyxo-hexose
D-Galactose	2-Deoxy-D-ribo-hexose
6-Deoxy-D-galactose	2-Deoxy-D-xylo-hexose
D-Glucose	(+) - Inositol
3-O-Methyl-D-glucose	Mucoinositol
4-O-Methyl-D-glucose	Myoinositol
6-O-Methyl-D-glucose	Scylloinositol

Cyclitols.- The rates of migration of cyclitols during electrophoresis in molybdate solution have been related to the instability factors of the conformations which have the 1(ax),2(eq),3(ax)-triol system (I).[1] This influence of instability factors is more pronounced in the case of tungstate.

Aldoses.- The overall pattern of electrophoretic mobilities of aldopentoses and -hexoses in tungstate solution is similar to that in molybdate solution, although the enhanced effect of instability factors described above was again observed (cf. D-ribose and methyl α-D-ribo-pyranoside in molybdate and tungstate). Substitution in, or replacement of, at least one of the hydroxyl groups of the cis-cis-1,2,3-triol system destroys the ability to form a complex with tungstate. On the other hand, substitution in or of hydroxyl groups other than those of the cis-cis-1,2,3-triol system has no effect on complex-formation.

5-Deoxy-D-xylo-hexose is so far the only monosubstituted derivative of D-glucose (or L-idose) which has been shown to complex with tungstate or molybdate. It could complex in its aldehydo form since the spatial disposition of the hydroxyl groups on $C_{(2)}$,$C_{(3)}$, and $C_{(4)}$ of the planar zig-zag conformation is identical with that in (I). The same should apply for D-glucose which, however, shows no tendency to adopt this form. On the other hand, evidence is now accumulating that one of the ring atoms of five-membered ring systems is out of plane[6,7]. The result is that atoms attached to adjacent ring-atoms become slightly staggered. Similarly, $C_{(2)}$ of 5-deoxy-D-xylo-hexofuranose could be out of plane. The hydroxyl groups on $C_{(1)}$,$C_{(3)}$, and $C_{(6)}$ of its β-anomer could then be brought into the same spatial disposition (III) as those of (I). Thus, electrophoresis in tungstate (or molybdate) solution

can be regarded as a method to distinguish between the aldehydo, furanose and pyranose forms of D-glucose; it might well be applied in the future to other appropriate sugars.

Both the aldotetroses, erythrose and threose, migrate during electrophoresis in tungstate (and molybdate) solution. The argument applied to 5-deoxy-D-xylo-hexose can be extended to both compounds. D-Erythrose possesses three hydroxyl groups in a spatial disposition approximating to that of (I) only in its α-furanose form, whereas the hydroxyl groups of the aldehydo form only of L-threose can be brought, without distortion of bond angles, into this arrangement.

2-Amino-2,6-dideoxy-D-talo-hexose (hydrochloride) migrated during electrophoresis in tungstate and molybdate solutions (pH 5) towards the cathode and at approximately the same rate as 2-amino-2-deoxy-D-gluco-hexose hydrochloride. In tungstate solution (pH 7.7) 2-amino-2,6-dideoxy-D-talo-hexose migrated towards the anode with a mobility of ca. 6 - 12 x 10^{-5} cm.^{2} V^{-1} sec.$^{-1}$, whereas 2-amino-2-deoxy-D-gluco-hexose remained immobile. This indicates that at least one hydroxyl group of the triol system (I) can be replaced by a primary amino group.

The authors are indebted to Professors W.G. Overend and T. Reichstein, Drs. J.S. Brimacombe and N.K. Richtmyer, for gifts of chemicals. The financial support by the Sugar Research Foundation and the Department of Scientific and Industrial Research is gratefully acknowledged.

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757. *Complexes between Polyhydroxy-compounds and Inorganic Oxy-acids. Part IV.*[1] *Dimolybdate and Ditungstate Ions as Complexing Agents, and Paper Electrophoresis of Maltodextrinols and Isomaltodextrinols in Molybdate and Tungstate Solutions.*

By H. J. F. Angus and H. Weigel.

Dimolybdate and ditungstate ions have been shown to be agents complexing with polyhydroxy-compounds. The complexes contain either one or two polyol components. Equations for the paper-electrophoretic mobilities of maltodextrinols and isomaltodextrinols are presented.

Molybdate and tungstate have been shown to form anionic complexes with certain polyhydroxy-compounds. In these the M/polyol ratio, where M represents a molybdenum or tungsten atom, is either one [1-3] or two.[2,4] On the basis of evidence previously provided it was not possible to assign detailed structures to these complexes, although the technique of paper electrophoresis in molybdate [2,5] or tungstate [1,4] solutions (pH 5), where widely differing mobilities have been observed, has found much use in the separation and identification of polyhydroxy-compounds. In several instances the affinities of polyhydroxy-compounds for ions of inorganic oxy-acids, *i.e.*, borate, germanate, and stannate, have been related to the known structures of the anions existing in aqueous solutions of the particular pH employed.[6] It was difficult to account for the M/polyol ratios of one [*e.g.*, D-mannose, D-ribose, and 3-*O*-glycopyranosyl-L-gulitols (4-*O*-glycopyranosyl-D-glucitols)] and two [*e.g.*, D-mannitol, D-glucitol, 2-deoxy-D-*arabino*-hexitol and 1-*O*-glycopyranosyl-L-gulitols (6-*O*-glycopyranosyl-D-glucitols)], if the normal ions, MoO_4^{2-} or WO_4^{2-}, or indeed the ions of the isopoly-acids derived therefrom and known to exist in aqueous solutions, were assumed to be the complexing agents. Thus, it was necessary to ascertain the nature of the inorganic complexing agents before any attempt could be made to interpret previous results in relation to the structures of the complexes.

The steps involved in the formation of the ions of isopoly-acids, *e.g.*, isopolytungstates:

$$WO_4^{2-} \xrightarrow{pH\ 6} HW_6O_{21}^{5-} \xrightarrow{pH\ 4} H_3W_6O_{21}^{3-} \xrightarrow{pH<3} H_2W_{12}O_{40}^{6-}$$

have already been deduced from physicochemical studies.[7] We have essentially confirmed these findings. Thus, the potentiometric and conductimetric titrations of sodium tungstate solutions with dilute sulphuric acid gave the results shown in Figs. 1(A) and 2(A). The inflexion points occur at H^+/WO_4^{2-} ratios (Fig. 1A, 1·17 and 1·45; Fig. 2A, 1·13 and 1.50) close to those expected for the formation of $HW_6O_{21}^{5-}$ (1.17) and $H_3W_6O_{21}^{3-}$ (1.50) (or $H_2W_{12}O_{40}^{6-}$) according to equations (1—3).

$$7H^+ + 6WO_4^{2-} \longrightarrow HW_6O_{21}^{5-} + 3H_2O \quad (1)$$

$$2H^+ + HW_6O_{21}^{5-} \longrightarrow H_3W_6O_{21}^{3-} \quad (2)$$

$$2H_3W_6O_{21}^{3-} \longrightarrow H_2W_{12}O_{40}^{6-} + 2H_2O \quad (3)$$

The potentiometric titration of sodium molybdate with sulphuric acid gave a curve (Fig. 1B) with no distinct inflexion point. This is probably not surprising since it is claimed [8] that the condensation gives, in the first instance, three coexisting polymolybdates according to equations (4a—c).

$$8H^+ + 7MoO_4^{2-} \longrightarrow Mo_7O_{24}^{6-} + 4H_2O \quad (4a)$$

$$H^+ + Mo_7O_{24}^{6-} \longrightarrow HMo_7O_{24}^{5-} \quad (4b)$$

$$H^+ + HMo_7O_{24}^{5-} \longrightarrow H_2Mo_7O_{24}^{4-} \quad (4c)$$

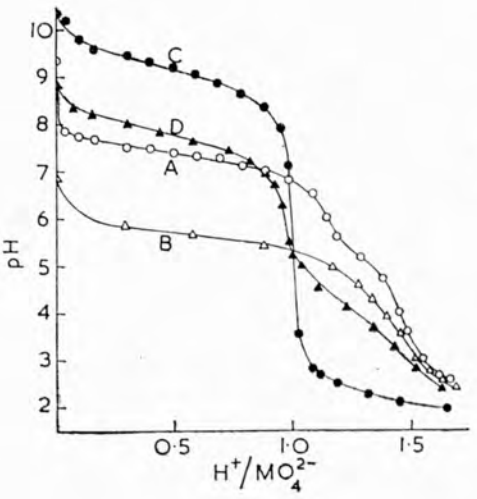


FIG. 1. Potentiometric titrations of tungstate and molybdate solutions. A, Sodium tungstate; B, sodium molybdate; C, D-mannitol and sodium tungstate (molar ratio = 0·2 : 1); D, D-mannitol and sodium molybdate (molar ratio = 1·15 : 1). M represents W or Mo.

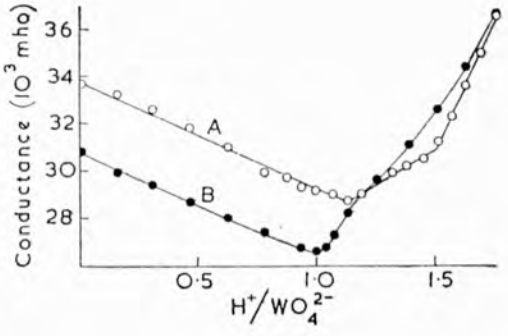


FIG. 2. Conductimetric titration of tungstate solutions. A, Sodium tungstate; B, D-mannitol and sodium tungstate (molar ratio = 1·15 : 1).

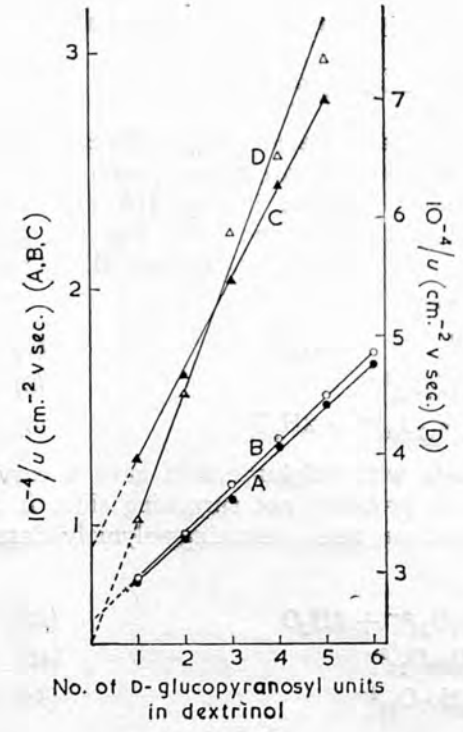


FIG. 3. Reciprocals of paper-electrophoretic mobilities of malto- and isomaltodextrinols in molybdate and tungstate solutions. A, Isomaltodextrinols in molybdate; B, isomaltodextrinols in tungstate; C, maltodextrinols in molybdate; D, maltodextrinols in tungstate.

However, when the titrations were carried out in the presence of excess D-mannitol (*i.e.*, M/polyol <2), inflexion points occurred at H^+/WO_4^{2-} or H^+/MoO_4^{2-} ratios of *ca.* 1·00 (Figs. 1C, 1D, 2B). In a similar manner sodium tungstate was titrated in the presence of other polyhydroxy-compounds. The results (Table 1) show that in all cases, except for

TABLE 1.

Potentiometric titration of tungstate in presence of polyols.

Added polyol	Inflexion points (H^+/WO_4^{2-})		Added polyol	Inflexion points (H^+/WO_4^{2-})
None	1·17	1·45	Erythritol	0·97
D-Glucose	1·17	1·49	Melibiitol	1·04
D-Mannitol	1·00		D-Mannose	1·00
D-Glucitol	0·98		L-Rhamnose	1·03
Galactitol	1·02		D-Fructose	0·99
			L-Sorbose	0·96

D-glucose, inflexion points occurred at H^+/WO_4^{2-} *ca.* 1·0. It is interesting to note that all compounds examined, except D-glucose, migrate during paper electrophoresis in tungstate [1,4] or molybdate solutions.[2,5]

The molybdate and tungstate ions which might formally be produced from a H^+/MO_4^{2-} ratio of 1, where M is a molybdenum or tungsten atom, are HMO_4^-, $M_2O_7^{2-}$, $HM_3O_{11}^{3-}$, $M_4O_{14}^{4-}$, $HM_5O_{18}^{5-}$, and $M_6O_{21}^{6-}$, or their hydrated forms. However, it is reasonable to assume that the complexes of the various polyhydroxy-compounds shown in Table 1 are all produced from the same type of inorganic ions.

With the exception of the ions HMO_4^- and $M_2O_7^{2-}$, all can be eliminated as possible complexing species for the following reasons: the complexing ions are likely to contain either one or an even number of molybdenum or tungsten atoms since polarimetric measurements have shown that in some complexes the M/polyol ratio is 2.[2,4] However, it is difficult to perceive why the ions containing four (or six) Mo or W atoms can also form complexes in which the M/polyol ratio is 1.[1-3] Examination of molecular models of the conceivable structures of an ion containing six molybdenum or tungsten atoms shows that it is impossible to arrange around it three molecules of a hexitol or pentitol known to give complexes in which the M/polyol ratio is 2.

The normal molybdate and tungstate ions (MO_4^{2-}), and presumably the ions corresponding to HMO_4^-, have a tetrahedral symmetry,[9,10] whereas in their known poly-acids Mo and W are 6-co-ordinated.[7] It is also reasonable to assume that the complexes are co-ordination compounds with 6-co-ordinated Mo or W. To complete the 6-co-ordination the ions HMO_4^- would require only two hydroxyl groups. However, no compound possessing fewer than three hydroxyl groups showed any tendency to complex with either molybdate [2] or tungstate.[4]

On the other hand, the M/polyol ratios in the complexes are related to structures of the polyhydroxy-compounds: (*a*) a ratio of 2 is found for complexes formed from acyclic polyhydroxy-compounds possessing at least four adjacent hydroxyl groups; (*b*) compounds possessing a triol system, which can assume a spatial disposition approximating to that of (I), give rise to complexes in which the M/polyol ratio is 1. It is thus likely that the complexing species are the dimolybdate, $Mo_2O_7^{2-}$, and ditungstate, $W_2O_7^{2-}$, ions, or their hydrated forms and that the complexes are formed by reaction between either one molecule (II; M/polyol = 2) or two molecules (III; M/polyol = 1) of a polyhydroxy-compound and one dimolybdate or ditungstate ion. In each case four hydroxyl groups, provided by either one (II) or two (III) molecules of a polyhydroxy-compound, have completed the 6-co-ordination of the two Mo or W atoms. However, in (III) a third hydroxyl group of each molecule of the polyhydroxy-compound has reacted by elimination of water.

In order to substantiate the above conclusions we have examined, by paper electrophoresis, the reduced oligosaccharides of the isomaltose series [isomaltodextrinols; *i.e.*, 1-*O*-α-glycopyranosyl-L-gulitols (6-*O*-α-glycopyranosyl-D-glucitols)] and the maltose series [maltodextrinols; *i.e.*, 3-*O*-α-glycopyranosyl-L-gulitols (4-*O*-α-glycopyranosyl-D-glucitols)]

in molybdate and tungstate solutions. These were chosen because (*a*) in each the hexitol portion of the molecule acquires a negative charge by complexing, and (*b*) 1- and 3-*O*-glycopyranosyl-L-gulitols form, at least with molybdate, complexes in which the M/polyol ratios are 2 and 1, respectively.

The plot of the reciprocals of the electrophoretic mobilities ($1/u$) against the number of D-glucopyranosyl units, n, present in the dextrinols gives a straight line (Fig. 3). It is unlikely that the charge acquired by the hexitol portion is affected by the molecular size of the dextrinols. Thus, the frictional resistance to motion increases linearly with the number of D-glucopyranosyl units. Extrapolation to $n = 0$ gives the reciprocal of the paper-electrophoretic mobility of the unsubstituted hexitol portion (D-glucitol, $5{\cdot}88 \times 10^3$ cm.$^{-2}$ v sec.) only in the series of isomaltodextrinols (Fig. 3, plots A and B). Thus D-glucitol behaves as the first member in the isomaltodextrinol series, whereas maltitol behaves as the first maltodextrinol. For practical use (*e.g.*, determination of molecular size of dextrinols) the $M_s(Mo$ and $W)$ values (M_s = mobility relative to D-glucitol; *Mo* and *W* refer to the electrolytes molybdate and tungstate, respectively) of isomaltodextrinols can be expressed by equations (5) and (6), respectively:

$$1/M_s(Mo) = 1 + 0{\cdot}31n, \quad (5)$$

$$1/M_s(W) = 1 + 0{\cdot}32n, \quad (6)$$

whereas those of the maltodextrinols are given by equations (7) and (8), respectively:

$$1/M_s(Mo) = 1{\cdot}52 + 0{\cdot}65n, \quad (7)$$

$$1/M_s(W) = 4{\cdot}07 + 1{\cdot}81n. \quad (8)$$

It is interesting to note that the M_{BG} values (mobilities relative to *N*-benzyl-D-glucosyl ammonium ion) of the *N*-benzyl-glycosyl ammonium ions of maltose, maltotriose, maltotetraose, and maltopentaose (n = 1,2,3, and 4, respectively) reported by Barker, Bourne, Grant, and Stacey,[11] obey a similar relation [equation (9)]:

$$1/M_{BG} = 1 + 0{\cdot}34n. \quad (9)$$

These compounds, of course, migrate as cations.

The plots of $1/u$ against n (Fig. 3) can be affected by (*a*) the size of the complexing agents; (*b*) the number of dextrinol molecules, P, combining with the complexing agents; (*c*) the ionic charge, z, of the complexes; and (*d*) the stability of the complexes. It is reasonable to assume that the complexing agents are of the same type and, since the interatomic distances in MoO_4^{2-} and WO_4^{2-} (and presumably in the lower poly-anions) are comparable,[9,10] it is not surprising that D-glucitol has the same mobility in both electrolytes. It has also been shown that, under the conditions of the electrophoresis, 1- and 3-*O*-glycopyranosyl-L-gulitols are completely converted into their molybdate complexes.[2,4] Thus, variation of P and z for two series of complexes (S_1 and S_2) should give the relations between the plots of $1/u$ *vs.* n shown in Table 2. The results (Fig. 3)

TABLE 2.

Effect of variations of P and z on plots of $1/u$ against n of two series of complexes (S_1 and S_2).

P (in S_1 and S_2)	z (in S_1 and S_2)	Relation between plots Theor.*	Exp. (series)
Identical	Identical	$u_0(S_1)/u_0(S_2) = \Delta(S_2)/\Delta(S_1) = 1$	A, B
Identical	Not identical	$u_0(S_1)/u_0(S_2) = \Delta(S_2)/\Delta(S_1)$	(A, B); C, D
$P(S_2) > P(S_1)$	Identical	$\Delta(S_2)/\Delta(S_1) = 2, 3 \ldots$	A, C; B, C
Not identical	Not identical	$u_0(S_1)/u_0(S_2) \neq \Delta(S_2)/\Delta(S_1)$	A, D; B, D

* u_0 = mobility when $n = 0$; Δ = slope of plot $1/u$ *vs.* n.

show that the complexes of series A (molybdate–isomaltodextrinols), B (tungstate–isomaltodextrinols), and C (molybdate–maltodextrinols) have identical charges, but those

of C contain twice as many dextrinol molecules as are contained in the complexes of series A or B. Since the complexes of series C and D (tungstate–maltodextrinols) contain the same number of dextrinol molecules, it is concluded that those of D also contain twice as many dextrinol molecules as are contained in the complexes of series A or B, but are less stable. These results are in agreement with our conclusions drawn from potentiometric and conductimetric measurements.

It would now be expected that three distinct complexes are formed from mixtures of two maltodextrinols (M_1 and M_2) containing (*a*) only M_1, (*b*) only M_2, and (*c*) M_1 and M_2. In order to test this hypothesis mixtures of two maltodextrinols (M_1 and M_2) were subjected to paper electrophoresis in molybdate solution. In all cases (Table 3) a third component

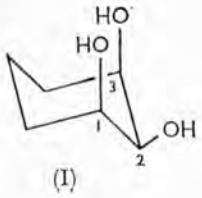
TABLE 3.

Electrophoresis of mixtures of two maltodextrinols (M_1 and M_2) in molybdate solution.

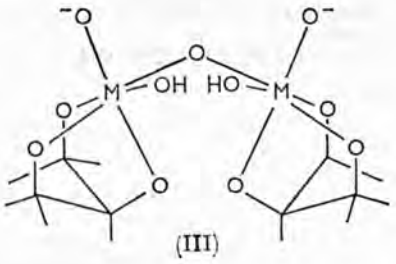
No. of glucopyranosyl units in maltodextrinol (*n*)		$M_s(Mo)$ of components of mixture			$M_s(Mo)$ of $M_{1,2}$ (calc.)
M_1	M_2	M_1	M_2	$M_{1,2}$	
1	4	0·46	0·24	0·33	0·32
1	5	0·46	0·21	0·29	0·29
3	4	0·29	0·24	0·27	0·26

($M_{1,2}$), in addition to those corresponding to the added maltodextrinols (M_1 and M_2) was detected. The mobilities of the components $M_{1,2}$ are in good agreement with those calculated from equation (7) and $n = (n_{M1} + n_{M2})/2$.

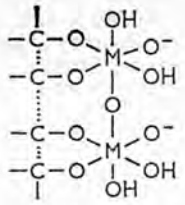
Since 3-deoxy-D-*ribo*-hexitol, 3-deoxy-D-*arabino*-hexitol, and 2-deoxy-D-*erythro*-pentitol do not form complexes [4] with molybdate or tungstate it now becomes clear that an acyclic compound, not possessing four adjacent hydroxyl groups, will form a complex only if it contains a 1,2,3(α, αT)-triol system (IV) (Barker and Bourne's nomenclature [12]) or, presumably a 1,2,3(αT, αT)-triol system (V). The hydroxyl groups of (V), when the carbon chain is in the planar zig-zag conformation, are in the same relative disposition as those in (I), whereas those of (IV) can be brought, without distortion of the conformation of the carbon chain, into this spatial arrangement. Considerable distortion of the planar zig-zag conformation of the carbon chain would be required to bring other acyclic triol systems [*e.g.*, 1,2,3(α, αC)-, in 3-deoxy-D-*ribo*-hexitol] into a spatial disposition approximating to that of (I).



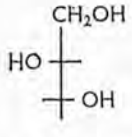
(I)



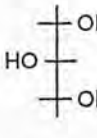
(III)



(II)



(IV)



(V)

If the M–O (M = Mo or W) distances in the co-ordination compounds do not differ greatly from those in Ag_2MoO_4 [10] and $BaWO_4$ [9] the O–O distances in octahedral molybdate and tungstate can be estimated to be 2·59 and 2·56 Å, respectively. These values are close to the O–O distances in (I) ($O_{(1)}$–$O_{(2)}$, 2·82 Å; $O_{(1)}$–$O_{(3)}$, 2·51 Å). Thus (III) is suggested as the structure (partial) of molybdate and tungstate complexes of five- or six-membered ring compounds possessing a *cis-cis*-1,2,3-triol system, and acyclic compounds which contain a 1,2,3(α, αT)- or 1,2,3(αT, αT)-triol system.

Experimental

Materials.—These were obtained from our Departmental Collection.

Potentiometric Titrations.—Several solutions containing sodium molybdate or sodium tungstate and the polyhydroxy-compound (*ca.* 1%) in the molar ratio of *ca.* 0·1 : 1—0·2 : 1 were titrated with 0·1N-sulphuric acid. The pH of the solutions was measured initially and after each addition of acid. The results are shown in Fig. 1 and Table 1. In control experiments, solutions containing only sodium molybdate or sodium tungstate were titrated with 0·1N-sulphuric acid.

Conductimetric Titrations.—A solution containing sodium tungstate and D-mannitol (1·2%) in the molar ratio of 1·15 : 1 was titrated with 0·1N-sulphuric acid. The conductance of the solution was measured initially and after each addition of acid. In a control experiment a solution containing sodium tungstate only was titrated with 0·1N-sulphuric acid. The results are shown in Fig. 2.

Paper Electrophoresis.—The apparatus used was capable of delivering up to 10,000 V at 100 mA. Electrophoresis was carried out on sheets of Whatman No. 3MM filter paper 10 cm. wide. The electrolytes consisted of 2% aqueous sodium molybdate or sodium tungstate solutions, adjusted to pH 5 with concentrated sulphuric acid. Electrophoretograms were prepared by applying a voltage of *ca.* 70 V/cm. for *ca.* 1 hr. Compounds were detected by spraying with acetone–silver nitrate–ethanolic sodium hydroxide.[13] Under the conditions used D-glucitol had a mobility (u) of $1{\cdot}7 \times 10^{-4}$ cm.^{2} V^{-1} sec.$^{-1}$ in both electrolytes. Glycerol or hydroxymethylfurfural were used as non-migrating markers for correction of electro-osmosis. The results are shown in Fig. 3 and Table 3.

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4. *Complexes between Polyhydroxy-compounds and Inorganic Oxy-acids. Part V.*[1] *Tungstate Complexes of Acyclic Polyhydroxy-compounds.*

By H. J. F. Angus, E. J. Bourne, and H. Weigel.

The paper-electrophoretic behaviour of acyclic polyhydroxy-compounds in tungstate solution is described. The method can be used to determine the position of the glycosidic linkage in oligosaccharides. Structures have been suggested for the complexes formed between the ditungstate ion (and dimolybdate ion) and acyclic vicinal tetritols.

In Part IV [1] we have shown that ionic complexes are formed by reaction between one or two molecules of a polyhydroxy-compound, depending on its structure, and one dimolybdate or ditungstate ion. We now report studies on the complexes formed between tungstate and simple polyhydroxy-compounds.

Fig. 1 shows the effect of pH on the specific rotation of D-mannitol in tungstate solutions. Curve A–B was obtained when (*a*) solutions containing D-mannitol and sodium tungstate were acidified to pH 2—10 by addition of sulphuric acid, and (*b*) D-mannitol was added to sodium tungstate solutions previously adjusted to pH 6—9, the optical rotation being measured immediately. On addition of D-mannitol in case (*b*) the pH increased when initially $>ca.$ 7 and decreased when initially $<ca.$ 7. On storage of the mixture, the rotation, which, after addition of sulphuric acid or D-mannitol, had pH $>ca.$ 6, increased and the final rotations corresponded to curve A′—B. pH values in Fig. 1 were recorded at the time of the polarimetric measurements: the exact reproducibility of curve A depends, of course, on the time required for measurements of optical rotations. Similar results have been obtained with D-glucitol. Although paper electrophoresis in tungstate solution has shown that 1-*O*-α-D-galactopyranosyl- and 3-*O*-β-D-glucopyranosyl-L-gulitol form complexes with tungstate (see below), their specific rotations were not markedly affected by the presence of tungstate.

The composition of the tungstate complexes of D-mannitol and D-glucitol formed at pH 5·5, *i.e.*, where immediate formation of stable complexes occurs, was determined as for molybdate complexes.[2] The rotations became constant (Fig. 2) when 2 mol. of tungstate had been added. Since the ditungstate ion, $W_2O_7^{2-}$, is the complex-forming agent the complexes are derived from one molecule of D-mannitol or D-glucitol and one ditungstate ion. The same composition, *i.e.*, W/D-mannitol = 2, has been found for the D-mannitol complex, which shows a negative rotation and is formed by the addition of the hexitol to a sodium tungstate solution of pH 7·15. The conversion of the (−)-complex into the (+)-complex is a first-order reaction with a rate constant $k = 4{\cdot}31 \times 10^{-3}$ min.$^{-1}$; possibly an intramolecular reaction.

Paper electrophoresis in tungstate solution was carried out at *ca.* pH 5, which allows the immediate formation of stable complexes and moderate ionisation. The mobilities of polyhydroxy-compounds relative to D-glucitol in tungstate [$M_s(W)$] and molybdate solutions [$M_s(Mo)$] [2] are shown in Tables 1—3. Since methyl D-gluco-, D-manno-, and D-galactopyranoside do not form complexes with tungstate [3] (or molybdate [4]), studies on the effect of substitution in hexitols on complex formation were also possible by the use of glycopyranosyl-hexitols.

TABLE 1.

Mobilities and structures of tungstate and molybdate complexes of acyclic polyols possessing a vicinal tetritol system.

Polyol	$M_s(W)$	$M_s(Mo)$	Carbon atoms in polyol corresponding with positions 1—4 in (II—V): 1,2,3,4 R^2	1,2,3,4 R^3 *	2,3,4,5 R^2 *	2,3,4,5 R^3 *	3,4,5,6 R^2 *	3,4,5,6 R^3
Tetritols								
Erythritol	0·81—0·94	1·0[2]	II H	H				
L-Theitol	0·24	0·5[2]	IV H	H				
Pentitols								
D-Arabinitol	1·04	1·1[2]	V H	5	III H	H		
(D-)Ribitol †	1·03	1·1[2]	III H	5				
(D-)Xylitol †	1·04	1·1[2]	IV H	H				
Deoxy-pentitols								
1-Deoxy-D-arabinitol	1·09	1·03			III H	H		
1-Deoxy-D-lyxitol	0·58—0·65	0·95			V 1	H		
1-Deoxy-D-xylitol	0·82	0·96			V H	H		
Hexitols								
(D-)Allitol †	0·86—0·97	0·94	III H	5,6	III 1	6		
D-Altritol	0·83—0·97	0·99	V H	5,6	III H	6	III 2,1	H
(D-)Galactitol †	1·00	1·0[2]	IV H	5,6	II H	H		
D-Glucitol	1·00	1·0[2]	IV H	H	V H	6	III H	H
D-Mannitol	1·00	1·0[2]	II H	H	V 1	6		
L-Iditol	1·00		IV H	H	V H	H		
Monodeoxy-hexitols								
1-Deoxy-D-altritol	0·88—1·00	0·98			III H	6	III 2,1	H
1-Deoxy-L-galactitol	1·03	1·0[2]			III H	H	IV 2,1	H
1-Deoxy-D-glucitol	0·98	0·98			V H	6	III H	H
1-Deoxy-L-gulitol	0·98	0·94			V 1	H	IV H	H
1-Deoxy-D-mannitol	1·00	1·0[2]			V 1	6	III H	H
1-Deoxy-D-talitol	0·83—1·04	1·04			II 1	H	V 2,1	H
2-Deoxy-D-*arabino*-hexitol	1·00	1·0[2]					III H	H
2-Deoxy-D-*lyxo*-hexitol	0·42—0·61	0·80					V 2,1	H
2-Deoxy-D-*ribo*-hexitol	0·17—0·57	0·13—0·57					III 2,1	H
2-Deoxy-L-*xylo*-hexitol	1·09	1·07					IV H	H
Dideoxy-hexitols								
1,6-Dideoxy-D-altritol	1·05	0·90			III H	6		
1,6-Dideoxy-galactitol	1·09	0·98			II H	H		
1,6-Dideoxy-L-mannitol	0·87—0·95	1·00			IV 1	6		
O-Methylhexitols								
1-*O*-Methyl-L-gulitol	0·89	0·93			V 1	H	IV H	H
2-*O*-Methyl-D-mannitol	0·98	1·0[2]					III H	H
1,2-Di-*O*-methyl-D-mannitol	0·95	1·0[2]					III H	H

* The numbers indicate the carbon atoms of the polyol present in the substituents R^2 or R^3.
† The prefix D indicates that the D-isomer only of the 1,2,3,4-complex is illustrated.

TABLE 2.

Electrophoresis in tungstate and molybdate solutions of acyclic polyols possessing a vicinal triol system.

Polyol	$M_s(W)$	$M_s(Mo)$
3-*O*-Methyl-L-gulitol	0·09	0·47
3-Deoxy-L-*xylo*-hexitol	0	0·21

Not complex-forming [$M_s(W$ or $Mo) < 0·05$]
Glycerol
2-Deoxy-D-*erythro*-pentitol
2,3-Di-*O*-methyl-D-glucitol
3-*O*-Methyl-D-glucitol
3-Deoxy-D-*arabino*-hexitol
3-Deoxy-D-*ribo*-hexitol

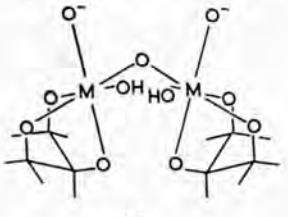
Acyclic diols do not migrate during electrophoresis in molybdate solution.[2] The same compounds have now been shown not to form complexes with tungstate. Also, 1-deoxy-2,5-di-*O*-methyl-L-mannitol did not migrate in either electrolyte. Of the compounds examined that possess only three adjacent hydroxyl groups (in the acyclic polyol portion), only 3-substituted L-gulitols formed complexes, *i.e.*, those with a 1,2,3(α,αT)-triol system (Barker and Bourne's nomenclature [5]). We have already shown [1] that the complexes of compounds containing such structural features are formed from two molecules of the

TABLE 3.

Mobilities of glycopyranosyl-hexitols.

Compound	$M_s(Mo)$	$M_s(W)$	Compound	$M_s(Mo)$	$M_s(W)$
Derivatives of D-*glucitol*			*Derivatives of* L-*gulitol*		
2-*O*-α-D-Glucopyranosyl-	0·69	0·76	1-*O*-α-D-Galactopyranosyl- ...	0·80	0·68
2-*O*-β-D-Glucopyranosyl-	0·70	0·73	1-*O*-α-D-Glucopyranosyl-	0·78	0·76
3-*O*-α-D-Glucopyranosyl-	0	0	1-*O*-β-D-Glucopyranosyl-	0·69	0·66
3-*O*-β-D-Glucopyranosyl-	0	0	2-*O*-α-D-Glucopyranosyl-	0·72	0·76
			3-*O*-β-D-Galactopyranosyl- ...	0·40	0·10
Derivatives of D-*mannitol*			3-*O*-α-D-Glucopyranosyl-	0·46	0·17
2-*O*-α-D-Glucopyranosyl-	0·72	0·70	3-*O*-β-D-Glucopyranosyl-	0·37	0·10
2-*O*-α-D-Mannopyranosyl- ...	0·80				
3-*O*-α-D-Mannopyranosyl-.....	0	0			

polyol and one ditungstate (or dimolybdate) ion (I, partial structure; M = W or Mo). It would be expected that glycerol and 3-deoxy-L-*xylo*-hexitol also form complexes with tungstate, though this is less likely with glycerol owing to the greater freedom of rotation about C–C bonds. The Tables show that the smaller the 3-substituent of L-gulitol the smaller the mobility (cf. 3-*O*-glycopyranosyl-L-gulitols, 3-*O*-methyl-L-gulitol, 3-deoxy-L-*xylo*-hexitol). On the other hand, 3-deoxy-L-*xylo*-hexitol does form a complex with molybdate, although its $M_s(Mo)$ value is lower than would be expected. These results



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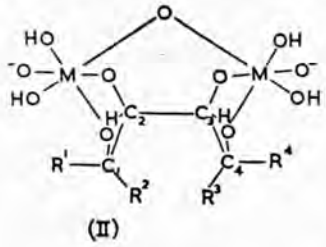

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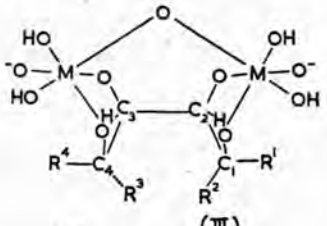

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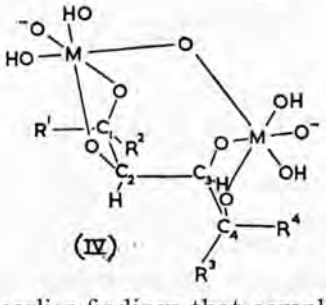

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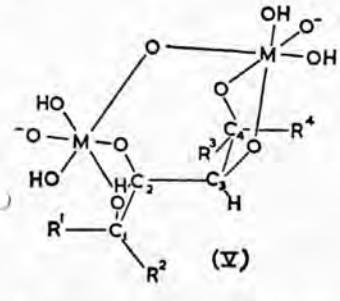

(V)

agree with our earlier findings that complex formation is less affected by conformational factors with molybdate than with tungstate.[3]

All compounds with at least four adjacent hydroxyl groups (in the acyclic polyol portion) form complexes with tungstate (or molybdate), presumably with the same composition as those produced from D-mannitol and D-glucitol (cf. molybdate complex of 2-deoxy-D-*arabino*-hexitol [2]). There are four possible structures (II—V) for such complexes, the 2,3-diol groups having *erythro* (II and III) and *threo* (IV and V) configurations. Table 1 shows that the electrophoretic mobilities, and thus the stabilities of the complexes, are influenced by the sizes of the substituents R^2 and R^3 in (II—V). The

mobilities of the polyols with only four adjacent hydroxyl groups (1-deoxypentitols, and 2-deoxy- and 1,6-dideoxy-hexitols) that form a complex in which R^2 and/or R^3 contain carbon atoms are smaller than those of compounds of the same molecular size that form the same type of complex (*i.e.*, type II and III, or IV and V), the substituents R^2 and/or R^3 of which, however, are hydrogen atoms (*i.e.*, 1-deoxy-D-lyxitol, 1-deoxy-D-xylitol; 2-deoxy-D-*ribo*-hexitol, 2-deoxy-D-*arabino*-hexitol; 2-deoxy-D-*lyxo*-hexitol, 2-deoxy-L-*xylo*-hexitol; 1,6-dideoxy-D-altritol, 1,6-dideoxy-galactitol).

On the other hand, pentitols, hexitols, and 1-deoxy-hexitols might form two or three complexes involving their various vicinal tetritol systems. The facts that galactitol, D-glucitol, D-mannitol, and L-iditol have identical mobilities and that those of 1-deoxy-L-galactitol, -D-glucitol, -L-gulitol, and -D-mannitol are comparable indicate preferential formation of complexes in which R^2 and R^3 are hydrogen. The substituents R^2 and/or R^3 of all possible complexes of allitol, D-altritol, 1-deoxy-D-altritol, and 1-deoxy-D-talitol contain carbon atoms. Consequently, these compounds streak during electrophoresis in tungstate solution (less so for molybdate; see above).

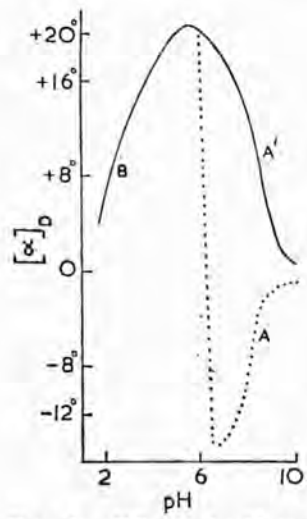

FIG. 1. Effect of pH on $[\alpha]_D$ of D-mannitol in tungstate solution.

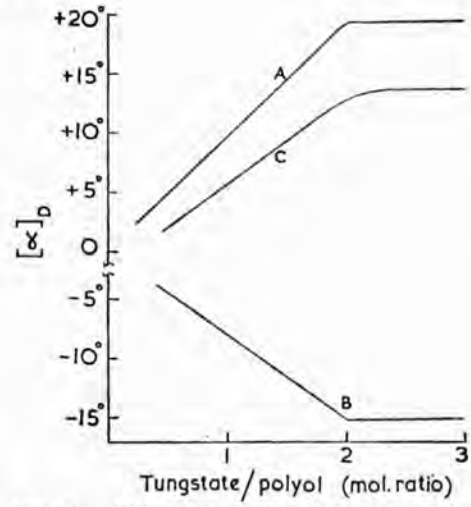

FIG. 2. Effect of relative concentrations of polyols and tungstate on $[\alpha]_D$ of polyol. A, D-mannitol, method (i); B, D-mannitol, method (ii); C, D-glucitol, method (i).

The mobilities of erythritol and L-threitol suggest that complexes of types (II) and (III) are generally more stable than those of types (IV) and (V).

Table 3 shows that during electrophoresis in tungstate solution, glycopyranosyl-hexitols fall into the same three classes, as in molybdate:[2] (*a*) 3-*O*-glycopyranosyl-D-glucitols; (*b*) 2-*O*-glycopyranosyl-D-glucitols, 1- and 2-glycopyranosyl-L-gulitols; and (*c*) 3-*O*-glycopyranosyl-L-gulitols. In addition, the α-isomers of D-glucopyranosyl-D-glucitols and -L-gulitols have slightly higher mobilities than their corresponding β-isomers. Thus, electrophoresis of D-glucopyranosyl-D-glucoses[6] and their reduction products in borate and tungstate or molybdate[2] solutions, respectively, can indicate the position of the glycosidic linkage to the reducing moiety in the original disaccharide.

Further evidence for preferential formation of complexes with the smallest interactions between R^2 and R^3 (II—V) was provided by periodate oxidation of hexitol–tungstate complexes. Solutions of complexes were prepared by adjusting solutions containing sodium tungstate and the polyols in the molar ratio of 2·1—3·0 : 1 to *ca.* pH 5·2 at which immediate formation of stable complexes occurs, and then to pH 7·8, in order to carry out the periodate oxidations in more neutral solutions.

The tungstate complexes of D-glucitol, D-mannitol, galactitol, allitol, D-altritol, and 2-deoxy-D-*arabino*-hexitol were treated with 2 mol. (per polyol) of sodium periodate. The components of the reaction mixtures, disclosed by paper-chromatographic analysis, both before and after adjustment of the solution to pH 2, are shown in Table 4. (We had already found that the R_F values of complex-forming polyols are affected by the presence of tungstate at pH 7—8, but are unaffected when the solutions are adjusted to pH 2.) The materials obtained from the complexes of D-glucitol, D-mannitol, and galactitol were fractionated on charcoal columns, further purified by paper chromatography, and converted into derivatives.

TABLE 4.

Periodate oxidation of polyol–tungstate complexes.

	Component of reaction mixture			Component of reaction mixture
	Paper-chromatographic	Derivative		Paper-chromatographic
Polyol	identity	(m. p.)	Polyol	identity
D-Glucitol	L-Xylose	155—156° *	Allitol	(DL-)Ribose
	D-Arabinose	182—183 †	D-Altritol	D-Altritol
	D-Glucitol	128—129 ‡	2-Deoxy-D-*arabino*-	2-Deoxy-D-*arabino*-
D-Mannitol	D-Arabinose	182—183 †	hexitol	hexitol
	D-Mannitol (trace)			
Galactitol	Galactitol	169—170 §		

* L-Xylose *p*-nitrophenylhydrazone. † D-Arabinose *p*-nitrophenylhydrazone. ‡ Hexa-*O*-benzoyl-D-glucitol. § Hexa-*O*-acetylgalactitol.

The results shown in Table 4 are not those expected from the partial periodate oxidation of the polyols themselves [7,8] and should thus indicate the vicinal tetritol system of the polyols involved in complex formation. It is evident that tungstate forms complexes with the tetritol systems on C-1, C-2, C-3, and C-4 of allitol and D-mannitol, and those on C-2, C-3, C-4, and C-5 of D-altritol and galactitol. Table 1 shows that the interaction between R^2 and R^3 of the complexes formed with these tetritol systems is the smallest. The materials obtained from D-glucitol arise from its three possible complexes of comparable stability. As expected, the tungstate complex of 2-deoxy-D-*arabino*-hexitol was unaffected by periodate.

The results of the periodate oxidation of the polyol–tungstate complexes are thus in agreement with the above correlation between their structures and mobilities during paper electrophoresis.

EXPERIMENTAL

Effect of pH *on Optical Rotations of Acyclic Polyhydroxy-compounds in Tungstate Solution.*—(i) Several solutions containing sodium tungstate and D-mannitol or D-glucitol (*ca.* 0·6—1%) in the molar ratio of 3 : 1 were adjusted with sulphuric acid to pH values between 2 and 10. The optical rotations, expressed as $[\alpha]_D$ and based on the polyhydroxy-compound, were measured immediately. The results obtained with D-mannitol are shown graphically (Fig. 1, curve A—B). The rotation of solutions adjusted to pH $>$*ca.* 6 increased on storage, the final rotations after 2 days corresponding to curve A′—B. D-Glucitol exhibited max. $[\alpha]_D$ 13·5°. Addition of alkali to solutions at pH 4 resulted in changes in optical rotation represented essentially by curve A′—B (Fig. 1, for D-mannitol).

(ii) D-Mannitol (0·12 g.) or D-glucitol (0·12 g.) in water (1 ml.) was added to each of several solutions of anhydrous sodium tungstate (0·4 g.) in water (15 ml.) previously adjusted to pH values between 6 and 9 by treatment with Amberlite IR-120(H^+). The pH and the optical rotation, expressed as $[\alpha]_D$ and based on the hexitols, of the solutions were measured immediately (Fig. 1, curve A—B) and again after *ca.* 18 hr. (Fig. 1, curve A′—B). The results obtained with D-mannitol and D-glucitol were essentially identical, except for the magnitude of rotational changes (D-glucitol: min. −9·3°, max. +14·0°). On addition of hexitols to the tungstate solutions the pH increased when initially >7 and decreased when initially <7.

Kinetic Measurements.—D-Mannitol (0·22 g.) in water (5 ml.) was added to a solution of anhydrous sodium tungstate (0·8 g.) in water (10 ml.) adjusted to pH 7·15 by treatment with Amberlite IR-120(H^+), and the whole was made up to 25 ml. and kept at room temperature.

The optical rotatory power of the solution was measured (4 dm. tube, sodium light) initially (α_0), at time intervals (α) and after *ca.* 18 hr. (α_∞). The plot of $\log [(\alpha_\infty - \alpha_0)/(\alpha_\infty - \alpha)]$ against time gave a straight line. From this the rate constant $k = 4{\cdot}31 \times 10^{-3}$ min.$^{-1}$ was calculated.

Effect of Relative Concentrations of Acyclic Polyhydroxy-compounds and Tungstate on Optical Rotation.—(i) The same method was used as for molybdate complexes,[2] but with pH *ca.* 5·5. Optical rotations were expressed as $[\alpha]_D$, based on the polyhydroxy-compound. The results are shown graphically in Fig. 2.

(ii) To each of several solutions of hydrated sodium tungstate (0·8 g.) in water (10 ml.) adjusted to *ca.* pH 7 was added 4·42% D-mannitol solution of such volume to give molar ratio of tungstate to D-mannitol between 0·5 : 1 and 3 : 1. The whole was made up to 25 ml. The optical rotation, expressed as $[\alpha]_D$ and based on D-mannitol, was measured immediately. The results are shown graphically in Fig. 2.

Paper Electrophoresis.—The methods used were those described previously.[3,4]

Paper Chromatography.—The solvent system was butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase).

Periodate Oxidation of Polyol–Tungstate Complexes.—(i) Separate solutions containing sodium tungstate and D-glucitol (1·8%), D-mannitol (1·8%), galactitol (1·8%), allitol (0·2%), D-altritol (0·2%), or 2-deoxy-D-*arabino*-hexitol (0·4%) in the molar ratio of 2·1—3·0 : 1 were first adjusted with 2N-sulphuric acid to pH 5·2 and then, after *ca.* 1 hr., with N-sodium hydroxide to pH 7·8. To each was added standard sodium periodate solution (2 mol., relative to polyol) and the whole set aside overnight. The mixtures were analysed by paper chromatography. Small portions were adjusted with 2N-hydrochloric acid to pH 2 and, next morning, were analysed by paper chromatography. The results are shown in Table 4.

(ii) *Characterisation of products from D-glucitol–, D-mannitol–, and galactitol–tungstate complexes.* The components of the reaction mixtures were fractionated on a column (50 × 3·5 cm.) of Ultrasorb S.C. 120/140 (activated charcoal) (supplied by British Carbo Norit Union, Ltd.). Water (*ca.* 500 ml.) removed inorganic materials. Aqueous ethanol (3 : 1) eluted (*a*) L-xylose, D-arabinose, and D-glucitol (from the D-glucitol complex and in that order), (*b*) D-arabinose (from the D-mannitol complex), and (*c*) galactitol (from the galactitol complex). These materials were further purified by paper chromatography. The pentoses were converted into their *p*-nitrophenyl hydrazones, D-glucitolinto its hexabenzoate, and galactitol into its hexa-acetate (m. p.s unchanged when mixed with authentic specimens).

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COMPLEXES BETWEEN POLYHYDROXY-COMPOUNDS AND INORGANIC OXY-ACIDS

VI. PAPER ELECTROPHORESIS IN STANNATE SOLUTION*

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Polyols are known to form complexes with the anions of several inorganic oxy-acids, *e.g.* borate[2,3], arsenite[3], germanate[4,5], antimonate[6], molybdate[7], and tungstate[7]. Such complexes form the basis for paper electrophoresis of carbohydrates and related compounds. In many cases their electrophoretic mobilities have been correlated with the structures of both the inorganic complexing agents and the polyols. We now report the paper electrophoretic behaviour of polyols in stannate solution.

EXPERIMENTAL

Electrophoresis was carried out on 10 cm wide sheets of Whatman No. 3MM filter paper. The electrolyte was a solution of sodium stannate in water (2 %, pH 11.5). Compounds were detected with acetone–silver nitrate–ethanolic sodium hydroxide[8]. D-Glucitol was used as a standard for comparison of rates of migration, and hydroxymethylfurfural as a non-migrating marker for correction of electro-osmosis. Migration rates in stannate solution are thus expressed as $M_g(Sn)$ values. Under the conditions used, D-glucitol had a mobility (u) of 14.3×10^{-5} cm^2 V^{-1} sec^{-1}.

RESULTS AND DISCUSSION

In all cases examined the mobilities (u) of polyols in stannate solution are appreciably higher than in 0.1 *N* sodium hydroxide[3]. Moreover, the sequence of mobilities of the polyols in stannate solution is markedly different from that in 0.1 *N* sodium hydroxide. Thus, migration in stannate solution of the compounds examined is due primarily to complex formation rather than ionisation of hydroxyl groups, and the method can be regarded as complementary to those in other electrolytes[7].

Table I shows that only two hydroxyl groups are required for complex formation. It is interesting that the mobility of *erythro*-2,3-butanediol is about half that of the *threo*-isomer. On the other hand, the mobility of *erythro*-2,3-butanediol in borate[3], arsenite[3], and sulpho-benzeneboronic acid[9] is *ca.* 25, 18, and 0 %, respectively, of that of *threo*-2,3-butanediol. Neither of these isomers migrates in germanate solution[10]. From the Sn–O distances[11] in sodium stannate, $Na_2Sn(OH)_6$, and potassium stannate,

* For Part V see ref. 1.

TABLE I

$M_g(Sn)$ VALUES OF POLYHYDROXY COMPOUNDS

Polyhydroxy compounds	$M_g(Sn) \times 10^2$
Acyclic compounds	
1,2-Ethanediol	1
1,2-Propanediol	1
erythro-2,3-Butanediol	2
threo-2,3-Butanediol	4
Glycerol	23
Erythritol	57
L-Threitol	62
D-Arabinitol	95
1-deoxy-	58
D-Lyxitol, 1-deoxy-	78
Ribitol	72
Xylitol	100
1-deoxy-D-	88
Allitol	88
D-Altritol	95
1-deoxy-	80
1,6-dideoxy-	45
Galactitol	99
1-deoxy-L-	87
1,6-dideoxy-	72
D-Glucitol	100
1-deoxy-	89
3-O-methyl-	30
L-Gulitol	
1-deoxy-	94
3-O-methyl-	85
D-*arabino*-Hexitol	
2-deoxy-	48
3-deoxy-	24
D-*ribo*-Hexitol, 3-deoxy-	24
D-Mannitol	93
1-deoxy-	94
1,2-di-O-methyl-	66
2-O-methyl-	88
L-Mannitol, 1,6-dideoxy-	67
Cyclitols	
*Allo*inositol	100
(+)-Inositol	55
*Epi*inositol	101
*Muco*inositol	67
*Myo*inositol	42
*Scyllo*inositol	50
Aldoses and derivatives	
DL-Glyceraldehyde	94
D-Erythrose	107
L-Threose	103
D-Arabinose	84
methyl α-pyranoside	48
D-Lyxose	115
methyl α-pyranoside	53
D-Ribose	104
methyl β-pyranoside	104

(continued on p. 362)

TABLE I (*continued*)

Polyhydroxy compounds	$M_R(Sn) \times 10^2$
D-*erythro*-Pentose	
2-deoxy-	24
1,2-dideoxy-	19
D-Xylose	81
methyl α-furanoside	3
D-Altrose, 1,6-anhydro-β-pyranose	80
D-Galactose	78
1,6-anhydro-β-pyranose	81
methyl β-pyranoside	43
L-Galactose, 6-deoxy- (L-fucose)	69
D-Glucose	63
1,6-anhydro-β-pyranose	0
6-deoxy-	63
3-O-methyl-	78
4-O-methyl-	44
methyl α-pyranoside	28
D-Gulose	107
1,6-anhydro-β-pyranose	77
6-deoxy-	105
D-*arabino*-Hexose, 2-deoxy-	31
D-*lyxo*-Hexose, 2-deoxy-	23
D-*ribo*-Hexose, 2-deoxy-	52
D-Mannose	100
1,6-anhydro-β-pyranose	96
3,4-di-O-methyl-	71
methyl α-pyranoside	41
L-Mannose, 6-deoxy- (L-rhamnose)	100
Ketoses and derivatives	
D-Fructose	91
1-O-methyl-	80
D-*erythro*-Hexulose, 3-deoxy-	60
L-Sorbose	94
Disaccharides	
α,α-Trehalose	11
Sophorose	57
Nigerose	57
Laminaribiose	75
Maltose	65
Cellobiose	62
Isomaltose	58
Gentiobiose	65

$K_2Sn(OH)_6$, an average O–O distance in $Sn(OH)_6^{2-}$ ions of 2.77 Å can be calculated. This is probably great enough to allow the formation of a non-planar 5-membered ring (I, from *threo*-2,3-butanediol). In this event, the complex of *erythro*-2,3-butanediol (II) will be more stable (relative to that of the *threo*-isomer) than those formed from the other oxy-acid anions.

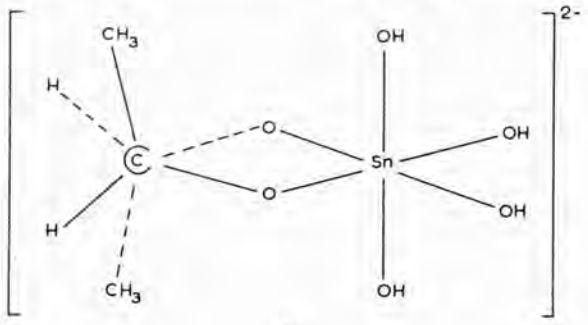
The order of mobility of acyclic polyols of identical molecular size is, with the exception of 1-deoxy-D-mannitol, related to the number of *threo*-1,2-diol groupings in each. This is not unexpected as, in the planar zig-zag conformation of these compounds, the O–O distance in *threo*-1,2-diol groups (2.82 Å) is close to that in the

$Sn(OH)_6^{2-}$ ion. Thus, the contribution to mobility arising from *threo*-disposed adjacent hydroxyl groups is larger than that from *erythro*-1,2-diol groupings.

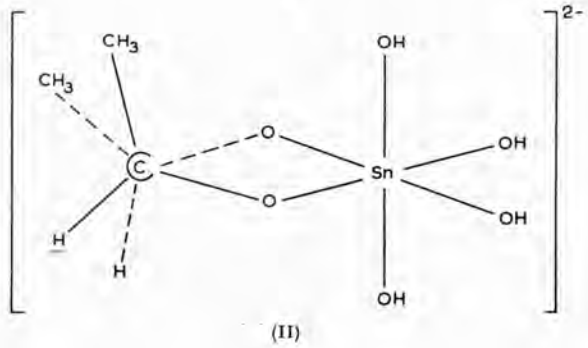
In the series of aldopentoses and -hexoses the largest contribution to mobility seems to arise from *cis*-1,2-diol groupings of their pyranose forms, although the O–O distances in the chair conformation of *cis*- and *trans*-1,2-diols of six-membered ring compounds are identical (2.82 Å). This is in agreement with the differences in reactivity of 1,2-cyclohexanediols observed in other cyclisation reactions[12]. Substitution in or of one of the *cis*-disposed adjacent hydroxyl groups reduces the mobility (*e.g.* D-mannose, 2-deoxy-D-*arabino*-hexose, D-ribose; 2-deoxy-D-*erythro*-pentose). By virtue of the α,β-equilibrium all pyranoses can possess a *cis*-1,2-diol grouping. Thus, in all cases examined, except D-ribose, glycoside formation also reduces the mobility. It is noteworthy that D-ribose is thought to exist, in aqueous solution, almost entirely in its β-pyranose form[13], which possesses the same number of *cis*-1,2-diol groupings as its methyl pyranoside.

The complex-forming 1,6-anhydro-β-pyranoses have higher $M_s(Sn)$ values than expected (*cf.* 1,6-anhydro-β-D-galactopyranose, methyl β-D-galactopyranoside; 1,6-anhydro-β-D-mannopyranose, methyl α-D-mannopyranoside). It is probable that, owing to the formation of the 1,6-anhydro-ring, their *cis*-related, adjacent hydroxyl groups have moved into a spatial disposition even more favourable for complex formation.

Of the 1,6-anhydro-β-pyranoses examined the glucose derivative is the only compound which did not migrate during electrophoresis in stannate solution. Its pyranose ring can, theoretically, adopt the 1C and 3B conformations (REEVES' nomenclature[14]), possessing, respectively, axially and equatorially disposed hydroxyl



(I)



(II)

Fig. 1.

groups. The fact that a 1(*eq*),2(*eq*)-diol grouping can complex with stannate (*cf.* methyl α-D-glucopyranoside, D-glucose, 6-deoxy-D-glucose) suggests that the pyranose ring in 1,6-anhydro-β-D-glucopyranose exists in the 1C conformation.

The cyclitols possessing four *cis*-1,2-diol groupings (*epi*inositol and *allo*inositol) have higher mobilities than those possessing only two (*muco*-, *dextro*-, and *myo*inositol). However, *scyllo*inositol, which possesses only *trans*-arranged hydroxyl groups, migrates faster than *myo*inositol. A tridentate structure, as proposed for borate complexes of certain cyclitols[15], would not account for this effect unless two stannate ions could combine with *scyllo*inositol, when all hydroxyl groups are axially disposed.

Tin and germanium are members of the same group in the periodic table of elements and form the same type of anion, *i.e.* $Sn(OH)_6^{2-}$ and $Ge(OH)_6^{2-}$. The sequences of electrophoretic mobilities of polyols in stannate and germanate[4] solutions show certain similarities, *e.g.* in the series of aldohexoses, methyl pyranosides of aldohexoses, and cyclitols. However, the same similarities are not observed in the series of aldopentoses and acyclic polyols. The investigation has also shown that stannate forms complexes with a greater range of polyols than do molybdate and tungstate[7], which require, for complex formation, very specific structural features.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. R. KUHN, Prof. T. REICHSTEIN and Dr. N. K. RICHTMYER for kindly providing some of the specimens, to the Department of Scientific and Industrial Research for the award of a Scholarship (to E.M.L.), and to Prof. E. J. BOURNE for helpful discussions.

SUMMARY

Paper electrophoresis in stannate solution has shown that stannate forms complexes with several acyclic and cyclic polyols. *Threo*-1,2-diol groups in acyclic compounds complex more strongly than the corresponding *erythro* groupings. With six-membered ring compounds the largest contribution to mobility arises from *cis*-1,2-diol groups, although *trans*-1,2-diol groups can complex. The paper electrophoretic mobilities of the polyols are discussed from the view-point of the conformations of the polyols and the structure of the stannate ion.

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35.

Angew. Chem., 1961, 73, 766

HELMUT WEIGEL, London: *Komplexe von Polyhydroxyl-Verbindungen mit anorganischen Sauerstoffsäuren.*

Die optische Drehung mehrerer Polyhydroxyl-Verbindungen ist in Gegenwart einiger anorganischer Sauerstoffsäuren erhöht. In Gegenwart von Molybdat erreicht die optische Drehung ein Maximum bei p_H ca. 2. Mit Wolframat tritt dieses Maximum bei p_H 5,5 auf. Bei p_H 5,5 gebildete Wolframat-Komplexe sind noch bei p_H 8 bis 9 beständig.

Die Komplexe sind Anionen und können zur Papierelektrophorese von Kohlehydraten und verwandten Verbindungen benutzt werden. Cyclische Polyhydroxyl-Verbindungen mit 6 Ringatomen bilden Komplexe mit Molybdat oder Wolframat, wenn sie ein cis-cis-1.2.3-Triol-System besitzen. Solche Verbindungen haben in mindestens einer ihrer Konstellationen ein 1(ax),2(eq),3(ax)-Triol-System. Die M_s-Werte (Wanderungsstrecke während der Elektrophorese, relativ zu der von Sorbit) sind von der relativen Beständigkeit der entsprechenden Konstellation beeinflußt. Myo-inosit hat M_s(Mo in Molybdat) 0,2, M_s(W) (in Wolframat) 0; Epi-inosit hat M_s(Mo) 1,1, M_s(W) 1,0.

Die Zusammensetzung der Komplexe, sowie die M_s-Werte von substituierten acyclischen Polyhydroxyl-Verbindungen sind von der Stelle der Substitution abhängig: 2-Glucosyl-sorbit, M_s(Mo) 0,9, M_s(W) 0,7; 3-Glucosyl-sorbit, M_s(Mo) und M_s(W) 0; 4-Glucosyl-sorbit, M_s(Mo) 0,4, M_s(W) 0,2; 5- und 6-Glucosyl-sorbit, M_s(Mo) 0,8, M_s(W) 0,7.

Oxydation von D-Sorbit mit Perjodat in Gegenwart von Wolframat liefert D-Sorbit, D-Arabinose und L-Xylose zu gleichen Teilen. D-Mannit liefert D-Arabinose. Dulcit wird nicht abgebaut. Demnach ist ein 1.2.4(αT, βT)- oder 1.2.4(αT, β)-Triol-System an der Komplexbildung beteiligt.

Durch Elektrophorese in Molybdat- oder Wolframat-Lösung wurden Verbindungen wie 3.6-Di-α-glucosyl-sorbit, M_s(Mo) 0, und 6-α-Nigerosyl-sorbit, M_s(Mo) 0,65, getrennt, die durch Reduktion von enzymatischen Abbauprodukten eines verzweigten Dextran (3¹-α-Glucosyl-isomaltose, bzw. 3²-α-Glucosyl-isomaltose) erhalten wurden.

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PAPER ELECTROPHORESIS OF CARBOHYDRATES

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I. Introduction

The technique of electrophoresis[1] involves the migration of charged substances in a conducting solution under the influence of an applied electrical field. Boundary electrophoresis refers to migration in free solution, whilst the term zone electrophoresis is applied to the process of migration in supported electrolytes. In the field of carbohydrate chemistry, the most commonly used support for the electrolyte is filter paper, although others have been used when this seemed desirable.[1a–3]

The development of paper electrophoresis of neutral carbohydrates[4] stems from the observation that certain polyhydroxy compounds react

(1) The distinction between the terms electrophoresis and ionophoresis is arbitrary. Some authors use the former for the migration of large molecules and particles, and reserve the latter for the movement of small ions.
(1a) S. Gardell, A. H. Gordon, and S. Åqvist, *Acta Chem. Scand.*, **4**, 907 (1950).
(2) E. J. Bourne, A. B. Foster, and P. M. Grant, *J. Chem. Soc.*, 4311 (1956).
(3) D. R. Briggs, E. F. Garner, and F. Smith, *Nature*, **178**, 154 (1956).
(4) R. Consden and W. M. Stanier, *Nature*, **169**, 783 (1952).

with borate to give anionic complexes.[5] Electrophoresis of carbohydrates in the presence of borate has been excellently reviewed in this Series by Foster.[6] It was then stated that "whilst chromatography has reached its majority, zone electrophoresis is currently adolescent, although its potentialities have been fully recognized." The technique has since undergone considerable expansion, the most important development being the exploration of electrolytes other than borate solution. All these methods can be regarded as complementary tools in the analysis of carbohydrates, and it now becomes increasingly possible to separate and identify components of hitherto unresolvable mixtures. The present review is to be regarded as a continuation of Foster's article.[6] Its aim is to describe the behavior of carbohydrates during electrophoresis in the various electrolytes, to discuss the modes and positions of attachment of the complexing agents to the polyhydroxy compounds, and to indicate the use of these methods in current problems. The facts described in Foster's article[6] will not be repeated here, except where completeness seems desirable. For this reason, mobility values of carbohydrates during electrophoresis in borate solution have been included in the Tables (see pp. 84–87).

II. Specificity of Electrolytes

Molecules which possess a net charge, or which may be given one by simply controlling the pH of their environment (for example, carboxylic acids and amines), will migrate in an applied electrical field as anions or cations according to the properties of their functional groups. A number of formally neutral polyhydroxy compounds also migrate toward the anode during electrophoresis in sodium hydroxide solution. This is probably due to the ionization of hydroxyl groups. However, in electrolytes containing a variety of inorganic oxy acids, anionic complexes are formed by reactions between the acid ions and the formally neutral polyhydroxy compounds. On the other hand, basic lead acetate and cations of the alkali metals and alkaline-earth metals afford cationic complexes. Greatly differing electrophoretic mobilities of polyhydroxy compounds have been observed in these electrolytes. Clearly, the structures of the inorganic complexing agents and of the polyhydroxy compounds will, together, decide their point or points of attachment, if any.

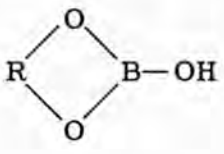
The index of electrophoretic mobility as used in this article is defined in Section IV (p. 82).

(5) J. Böeseken, *Advan. Carbohydrate Chem.*, **4**, 189 (1949).
(6) A. B. Foster, *Advan. Carbohydrate Chem.*, **12**, 81 (1957).

1. *Borate*

The Raman spectrum of the aqueous borate ion[7] and x-ray studies on boron minerals[8] have shown that the borate ion has tetrahedral symmetry and is, in all likelihood, $B(OH)_4^{\ominus}$. In 3 *M* sodium perchlorate solution at 25° and with a total concentration of boron of $B<0.025$ *M*, essentially the mononuclear species boric acid, $B(OH)_3$, and the anion $B(OH)_4^{\ominus}$ seem to be present, whilst, for $B>0.025$ *M*, two polynuclear species, $B_3O_3(OH)_4^{\ominus}$ and $B_3O_3(OH)_5^{2\ominus}$, can also be detected.[9] This means that boric acid does not act as a proton donor but as a Lewis acid, accepting the electron pair of the base (for example, $OH^{\ominus}$) to form the tetrahedral anion $B(OH)_4^{\ominus}$.

Most esters and complexes between boric acid and borate ions, respectively, and polyhydroxy compounds can be formulated as follows:



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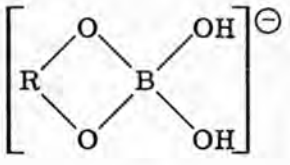

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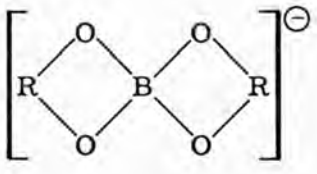

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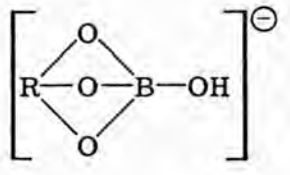

(4)

The ionic species (**2**), (**3**), and (**4**) migrate during electrophoresis.[6]

The O–O distances for trigonal and tetrahedral boron are 2.36 to 2.39 Å. and 2.40 to 2.44 Å., respectively.[10] It thus appears that the borate ion would form complexes with those polyhydroxy compounds in which the oxygen atoms of at least two hydroxyl groups are separated by, or can easily approach each other to, a distance of approximately 2.4 Å. Such a distance is found in the *cis*-1,2-diols of five-membered ring compounds

(7) J. O. Edwards, G. C. Morrison, V. F. Ross, and J. W. Schultz, *J. Am. Chem. Soc.*, **77**, 266 (1955).

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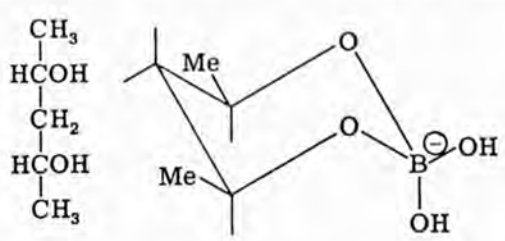
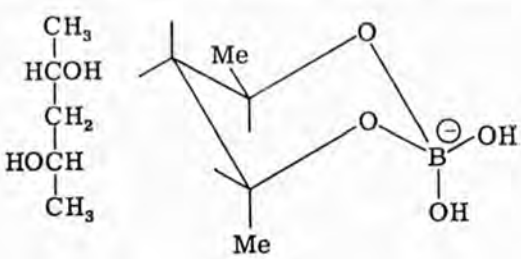
(10) C. L. Christ, J. R. Clark, and H. T. Evans, Jr., *Acta Cryst.*, **11**, 761 (1958).

(2.49 Å.)[11]; these react more strongly with borate ions than do their *trans* isomers[5] (O–O, 3.40 Å.). In the planar, zigzag conformation of acyclic *cis*- and *trans*-1,2-diols,[11a] the oxygen atoms are separated by a distance of 3.65 and 2.82 Å., respectively, but can, in an eclipsed conformation, approach each other to a distance of 2.49 Å. The greater electrophoretic mobility of the acyclic *trans*-1,2-diol, as compared with that of the *cis* isomer, has been related to the interaction energy of the nonbonded atoms in the essentially planar diol–borate complex.[6,12]

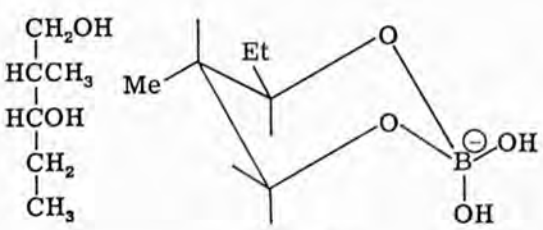
The O–O distances in the planar, zigzag conformation of 1,3-propanediol and acyclic *trans*- and *cis*-1,3-diols are 4.84, 3.43, and 2.51 Å., respectively. By rotation about C–C bonds, these can be reduced to 1.74 Å., when C-1, C-2, C-3, and the oxygen atoms are coplanar. Thus, with such compounds, the borate ion, $B(OH)_4^{\ominus}$, would fit well, to form puckered, six-membered rings. The resolution of *cis*-(**5a**)[M_G(B) 0.18] and *trans*-2,4-pentanediol (**6a**)[M_G(B)0][13] shows that the mobility is decreased when the six-membered ring [(**5b**) and (**6b**)] contains an axial alkyl group. 2-Methyl-1,3-pentanediol yielded, when subjected to electrophoresis, two components [M_G(B) 0.08 and 0.24].[13] Presumably, the sub-



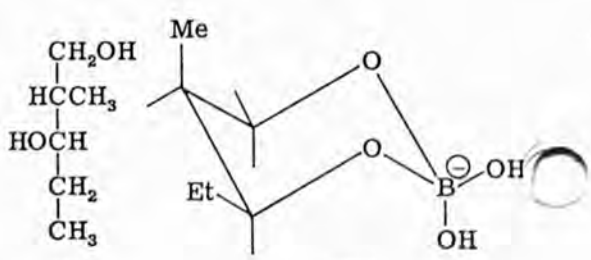
(5a) (5b)



(6a) (6b)



(7a) (7b)



(8a) (8b)

(11) In calculating O–O distances in diol groupings, the bond angles at all carbon atoms were taken as being the tetrahedral angle (109°28′) and the bond lengths as C–C 1.54 and C–O 1.42 Å. In the case of cyclic compounds, the calculations have been based on the cyclopentane and cyclohexane ring-systems.

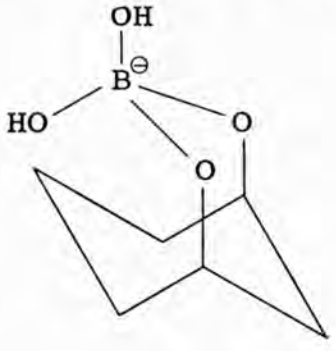
(11a) In this article, the terms *cis* and *trans* refer to Fischer projection formulas.

(12) D. H. Barton and R. C. Cookson, *Quart. Rev.* (London), **10**, 44 (1956).

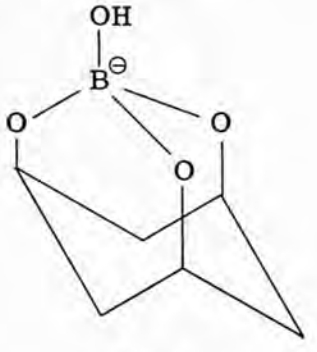
(13) J. L. Frahn and J. A. Mills, *Australian J. Chem.*, **12**, 65 (1959).

stance was a mixture of two diols [(**7a**) and (**8a**)], and, based on the mobilities of (**5b**) and (**6b**), the faster migrating diol is, in all likelihood, (**7a**). Their borate complexes are (**7b**) and (**8b**). In agreement with other observations on six-membered ring compounds,[14] two axial groups on the same side of the ring (**6b**) will decrease the electrophoretic mobility more than do two axial groups on opposite sides (**8b**). The relative stabilities of crystalline sodium borate complexes of acyclic 1,3-diols have been interpreted in a similar manner.[15]

cis-1,3-Cyclohexanediol does not migrate during electrophoresis in borate solution,[16] although the O–O distance in one of its conformations is the same as that in the planar, zigzag conformation[17] of acyclic *cis*-1,3-diols (2.51 Å.). This is to be expected, as the axial interactions in the complex (**9**) would be too great to allow its formation. On the other hand, the cyclic boric ester of *cis*-1,3-cyclohexanediol (**1**; $R=C_6H_{10}$), in which the axial interactions are diminished, has been obtained.[15]



(**9**)



(**10**)

When the six-membered ring compound contains a *cis-cis*-1,3,5-triol system, a cage structure (**10**) can readily be formed. Cyclitols containing such a system exhibit marked electrophoretic mobilities. Angyal and McHugh[16] have correlated these with the disposition of the remaining hydroxyl groups. The migration during electrophoresis of pentaerythritol is presumably due to the formation of a complex having a similar cage structure.

The O–O distances in the chair conformation of *cis*- and *trans*-1,2-cyclohexanediol are identical (2.82 Å.). However, only in the former can this distance readily[12] be diminished to a value (2.49 Å. in a boat or half-chair conformation) small enough for complexing with the borate ion. Consequently, only the *cis* isomer exhibits electrophoretic mobility.[18]

(14) W. Klyne, *Progr. Stereochem.*, **1**, 36 (1954).
(15) J. Dale, *J. Chem. Soc.*, 922 (1961).
(16) S. J. Angyal and D. J. McHugh, *J. Chem. Soc.*, 1423 (1957).
(17) S. A. Barker, E. J. Bourne, and D. H. Whiffen, *J. Chem. Soc.*, 3865 (1952)

2. *Sulfonated Benzeneboronic Acid*

Benzeneboronic acid, $PhB(OH)_2$, is three times as strong as boric acid.[18] Its ion, $PhB(OH)_3^{\ominus}$, might be expected to have a tetrahedral structure similar to that of the borate ion, $B(OH)_4^{\ominus}$. Diols or triols having the stereochemical modifications discussed in the preceding Section should thus have similar affinities for benzeneboronate and borate ions. This view is confirmed by the results of Garegg and Lindberg,[19] who found, at pH 7, no significant increase in the relative electrophoretic mobility of sugars in solutions containing benzeneboronic acid instead of boric acid. However, sulfonated benzeneboronic acid gave, at neutral pH values, much more selective reactions than is the case with borate at pH 10. With reducing sugars and glycosides, by far the largest contribution to the mobilities was found to arise from *cis*-1,2-diols of five-membered ring compounds, but, in contrast to borate at pH 10, none arises from *cis*-1,2-diols of six-membered ring compounds or from such a diol group as that on C-4 and C-6 in D-glucopyranose.

The effect of the sulfonic acid group on the boronic acid group is to decrease its ionization, thus leading to trigonal boron. Because of this circumstance, the products formed under the conditions of the electrophoresis are, in all likelihood, esters of boronic acid, their migration being attributable to the ionization of the sulfonic acid group.

A comparison of the absolute mobilities during electrophoresis in these two electrolytes, respectively, shows that, in all cases except those of *epi*-inositol and, probably, methyl α-D-mannofuranoside, the values are appreciably smaller in the solutions containing sulfonated benzeneboronic acid. The extent of the decrease is probably too great to be due solely to the difference in ionic radii of pairs of the migrating substances. Although different degrees of ionization of such pairs will contribute to this effect, it also seems that sulfonated benzeneboronic acid has, in general, a lower affinity for diols than has the borate ion.

The different affinities of sulfonated benzeneboronic acid and of the borate ion for *cis*-1,2-diols of cyclic compounds might be explained by the relative stabilities of five-membered rings containing trigonal and tetrahedral boron. Hubert, Hargitay, and Dale[20] have shown that the five-membered ring is strained if it contains trigonal boron but not if it contains tetrahedral boron. Thus, whilst the oxygen atoms of true *cis*-1,2-diols (as in furanoid compounds) are in such a position that the difference

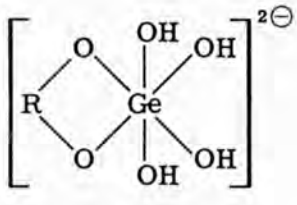
(18) W. Gerrad, "The Organic Chemistry of Boron," Academic Press Inc., London, Engl. and New York, N. Y., 1961, p. 67.
(19) P. J. Garegg and B. Lindberg, *Acta Chem. Scand.*, **15**, 1913 (1961).
(20) A. J. Hubert, B. Hargitay, and J. Dale, *J. Chem. Soc.*, 931 (1961).

between the free-energy changes involved in the two reactions is approximately that for the interconversion of structures containing trigonal and tetrahedral boron, only a fraction, f, of the molecules of *cis*-1,2-diols of six-membered ring compounds have the oxygen atoms in that position. Thus, an entropy term, $RT \ln 1/f$, has to be added to the free-energy changes involved in the reactions. This term could reduce the affinity of sulfonated benzeneboronic acid for the latter diols to such an extent that, under the conditions of electrophoresis, no reaction would occur.

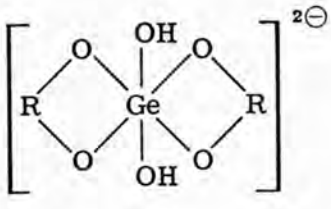
The lower affinity of sulfonated benzeneboronic acid for diols, together with this entropy effect, probably also explains the observation that, in contrast to borate solution, no contribution to mobility arises from such a diol grouping as that on C-4 and C-6 in D-glucopyranose (shortest O–O distance, 2.51 Å.). However, there is an indication that sulfonated benzeneboronic acid reacts with *cis*-1,3-diol groupings of six-membered ring compounds when, in the most stable conformation, the two hydroxyl groups are axially disposed [as in *epi*-inositol, M_M(PhB) 1.8].

3. *Germanate*

The complexes formed between polyhydroxy compounds and germanic acid have been found[21,22] to contain the former and germanium in the ratios 1:1, 2:1, or 3:1. Germanic acid readily undergoes polymerization reactions. At pH 9.4, the pentagermanate ion, $Ge_5O_{11}^{2\ominus}$, is present in solution. Decrease or increase in pH results in gradual depolymerization, and, at pH 11, the ion $Ge(OH)_6^{2\ominus}$ becomes the major component in a germanate solution. Lindberg and Swan[23] have investigated the behavior of carbohydrates and related compounds during paper electrophoresis in sodium germanate solution of pH 10.7 at 40°. It is probable that, under the conditions of the electrophoresis, complexes of structures (**11**) or (**12**) are formed.



(**11**)



(**12**)

(21) P. J. Antikainen, *Acta Chem. Scand.*, **13**, 312 (1959).
(22) D. A. Everest and J. C. Harrison, *J. Chem. Soc.*, 1745 (1960).
(23) B. Lindberg and B. Swan, *Acta Chem. Scand.*, **14**, 1043 (1960). Mobilities of selected compounds at lower temperatures and at pH 10 are given by W. J. Popiel, *Chem. Ind.* (London), 434 (1961).

The Ge–O distance in $Ge(OH)_6^{2\ominus}$ has not yet been determined. However, if it does not differ very greatly from that in tetrahedral germanium compounds,[24,25] the O–O distance can be calculated to be[23] approximately 2.64 Å. This value falls between those of the O–O distances in *cis*-1,2-diols of five- (2.49 Å.) and six-membered ring compounds (2.82 Å.; a chair conformation). Thus, Lindberg and Swan[23] have shown that compounds possessing such structural features will form ionic complexes during electrophoresis in germanate solution. The value of 2.49 Å. is considerably smaller than that calculated for the O–O distance in $Ge(OH)_6^{2\ominus}$. On the other hand, Beevers and Crochran[26] have shown that C-4 of the D-fructofuranose moiety of sucrose is out of the plane by about 0.5 Å., affording a nonplanar conformation. The result is that atoms attached to adjacent ring-carbon atoms become slightly staggered, increasing thereby the distance of their separation. The O–O distance in *cis*-1,2-diols of furanosides could thus approach that in the $Ge(OH)_6^{2\ominus}$ ion. Indeed, Lindberg and Swan[23] have noted that the germanate complexes of such diols are particularly strong.

The mobilities of the methyl D-glucopyranosides and D-xylofuranosides during electrophoresis in borate solution have been attributed to complex-formation involving O-4 and O-6, and O-3 and O-5, respectively.[6] The complex-formation of such 1,3-diols seems to be very limited with germanate.[23] However, it is interesting to note that the order of mobility of substituted D-glucoses (for example, *O*-methyl-D-glucoses and disaccharides of D-glucose and of deoxy derivatives thereof) is the same in borate and in germanate. The sequence of mobilities in borate solution has been rationalized by postulating that the aldehydo form of the sugar is the principal form involved in complex-formation and that the pair of hydroxyl groups sterically most favorable for complex-formation are those[6] on C-2 and C-4. Thus, the same could be true for complex-formation with germanate. On the other hand, the sequence of mobilities in germanate solution of monosubstituted D-glucitols differs from that of the corresponding D-glucose derivatives; for example, the M_G(Ge) values of 2-*O*-methyl-D-glucitol and 2-*O*-methyl-D-glucose are 1.5 and 0, respectively. This indicates that complexes of germanate with reducing sugars are formed exclusively by 1,2-diol groupings in a cyclic form of the sugar.[23] The same authors suggested that this is also true for borate complexes with sugars. The mobilities in borate solution of monosubstituted D-glucitols have not yet been determined. However, periodate oxidation of the D-glucitol–

(24) L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 2nd Edition, 1948, p. 179.
(25) Y. Ginetti, *Bull. soc. chim. Belges*, **63**, 209, 460 (1954).
(26) C. A. Beevers and W. Crochran, *Proc. Roy. Soc.* (London), *Ser. A*, **190**, 257 (1947).

borate complex yielded L-xylose as the major product.[27] This result indicated that D-glucitol forms a complex with borate similar to that suggested for D-glucose,[6] through participation of the hydroxyl groups on C-2 and C-4.

Lindberg and Swan[23] suggested that, in the case of cyclitols, *cis*-1,2-diol groupings and *cis*-related hydroxyl groups at C-1, C-3, and C-5 are involved in complex-formation with germanate. Indeed, the sequence of electrophoretic mobilities in germanate solution of cyclitols is again parallel to that in borate solution, thus lending substance to this suggestion.

For the reason discussed in the case of borate, it is improbable that germanate forms complexes involving *cis* related hydroxyl groups on C-1 and C-3 of six-membered ring compounds.

4. *Stannate*

The alkali stannates (for example, $Na_2O \cdot SnO_2 \cdot 3\ H_2O$) derive from the anion $Sn(OH)_6^{2\ominus}$, in which the 6-coordination shell of tin is filled. From the Sn–O distances[28] in sodium stannate, $Na_2Sn(OH)_6$, and in potassium stannate, $K_2Sn(OH)_6$, an average O–O distance in $Sn(OH)_6^{2\ominus}$ ions of 2.77 Å. can be calculated.

Electrophoresis in sodium stannate solution[29] has shown that polyhydroxy compounds form anionic complexes with the stannate ion. Only two hydroxyl groups are required for their formation and, from the O–O distance in the $Sn(OH)_6^{2\ominus}$ ion, it was to be expected that complexes would be formed from acyclic 1,2-diols. It is interesting that the mobility of *cis*-2,3-butanediol is about half that of the *trans* isomer. This result is in contrast to that for other electrolytes discussed in this review, particularly for sulfonated benzeneboronic acid, although the absolute mobilities of these isomers are much lower in stannate solution. The O–O distance in $Sn(OH)_6^{2\ominus}$ is probably great enough to allow the formation of a nonplanar 5-membered ring. In this event also, the complex of *cis*-2,3-butanediol should be relatively stable.

The order of mobility of pentitols and hexitols is related to the number of *trans*-1,2-diol groupings in each. This is not unexpected as, in the planar, zigzag conformation of these compounds, the O–O distance between *trans* arranged hydroxyl groups (2.82 Å.) is close to that in the $Sn(OH)_6^{2\ominus}$ ion. Thus, although the *cis*-1,2-diol grouping can complex with stannate, by far the largest contribution to mobility comes from *trans* disposed hydroxyl groups.

It is not certain whether equatorially disposed *trans*-1,2-diol groupings

(27) D. H. Hutson and H. Weigel, *J. Chem. Soc.*, 1546 (1961).
(28) C. O. Björling, *Arkiv Kemi, Mineral. Geol.*, **15**, No. 2 (1941).
(29) E. M. Lees and H. Weigel, unpublished results.

of 6-membered ring compounds can form complexes with stannate. Certainly, of those cyclitols whose mobilities have been measured, those having four *cis*-1,2-diol groupings exhibit greater mobility than those having only two. However, *scyllo*-inositol, which possesses only *trans* arranged hydroxyl groups, migrates faster than *myo*-inositol. A tridentate structure, as proposed for borate complexes of certain cyclitols,[16] would *not* account for this effect unless two stannate ions could combine with *scyllo*-inositol when all its hydroxyl groups are axially disposed.

In the series of pentoses and hexoses, by far the largest contribution seems to arise from *cis* arranged 1,2-diol groups. Substitution in or of one of the *cis* disposed hydroxyl groups reduces the mobility. However, it is possible that vicinal hydroxyl and hydroxymethyl groups, present in some glycosides, also complex with stannate ions.

5. *Arsenite*

Arsenious acid, $As(OH)_3$, behaves as a weak acid with a dissociation constant[30] of about 8×10^{-10} at 25°. As is the case with boric acid,[5] the addition of D-mannitol to aqueous solutions of arsenious acid increases the acidity of the solution.[31] The formation constants for complexes between polyhydroxy compounds and the arsenite ion have been found to be considerably smaller than those for the corresponding borate complexes.[32] This is also reflected in the absolute mobilities of such compounds during electrophoresis in arsenite solution.

Little information is available about the structure of the arsenite ion that complexes with polyhydroxy compounds. Most of the salts of arsenious acid are not derived from the *ortho* but from the *meta* acid, $HAsO_2$. Roy, Laferriere, and Edwards[32] found that the logarithms of formation constants for arsenite and borate complexes form a good straight line on the linear, free-energy plot. This result indicates that the two ions have similar steric properties. As the borate ion is known to be tetrahedral,[7] these authors suspected the arsenite ion to be $As(OH)_4^{\ominus}$ and to have a distorted, tetrahedral structure. However, as they found no evidence for the existence of complexes containing a polyhydroxy compound and arsenic in the ratio of 2:1, as is the case with borate, the alternative structure, $AsO(OH)_2^{\ominus}$ was not excluded. Arsenic trioxide, As_4O_6, has a tetrahedral structure.[33] It might be expected that the same would be true for the ion $AsO(OH)_2^{\ominus}$. In this event, the O–O distance in $AsO(OH)_2^{\ominus}$ might

(30) F. Ishikawa and I. Aoki, *Bull. Inst. Phys. Chem. Research* (Tokyo), **19,** 136 (1940).
(31) B. Englund, *Rec. trav. chim.*, **51,** 135 (1932).
(32) G. L. Roy, A. L. Laferriere, and J. O. Edwards, *J. Inorg. Nucl. Chem.*, **4,** 106 (1957).
(33) G. C. Hampson and A. J. Stosick, *J. Am. Chem. Soc.*, **60,** 1814 (1938).

not differ very greatly from that in As_4O_6, which is 2.76 Å. Hence, the arsenite ion should readily form complexes with six-membered ring compounds containing *cis*-1,2-diol groupings and with acyclic *trans*-1,2-diols. In forming such complexes, very little distortion of the chair or planar zigzag conformation, respectively, would be required. Frahn and Mills[13] have shown that the mobilities of inositols and acyclic polyhydroxy compounds bear a relationship to the number of such groupings present in the molecule. This is also exemplified in the behavior of some glycopyranosides. However, when both hydroxyl groups of the *trans*-1,2-diol grouping of six-membered ring compounds are in equatorial positions, the O–O distance is the same as that in the *cis*-1,2-diol grouping. Thus, some contribution to the mobilities should be expected from such structural features, as is indeed shown by the mobility of *trans*-1,2-cyclohexanediol.

Frahn and Mills[13] expected that, for such reagents as arsenite, which preferentially afford five-membered cyclic complexes, the most favorable site for complexing would be provided by the *cis*-1,2-diol grouping of five-membered ring compounds, and that reducing sugars which have a high content of furanose form (because of conformational instability of the pyranose form) should show high mobilities in arsenite solution, provided that two adjacent *cis*-hydroxyl groups are free. Although such a generalization neglects the steric properties of the complexing ion (see stannate), this expectation is realized fairly completely among the aldoses. As is pointed out by the same authors, the mobilities of reducing sugars in 0.1 *N* sodium hydroxide also show a trend toward highest rates for compounds possessing, in their chair conformations, a considerable degree of conformational instability. Although the absolute mobilities of reducing sugars in sodium arsenite solution (pH 9.6) are appreciably smaller than those in 0.1 *N* sodium hydroxide, no appreciable migration was observed in 0.1 *M* sodium carbonate at pH 10. This suggested that the observed migrations of formally neutral polyhydroxy compounds in sodium arsenite solution are due only to the formation of complexes, and not in part to the alkalinity of the electrolyte. Clearly, for a more satisfactory explanation of the behavior of reducing sugars during electrophoresis in arsenite solution, more knowledge about the composition of the electrolyte is needed, as it might be possible that uncharged products are formed which would compete with the charged complexes.

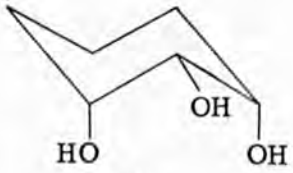
6. *Molybdate and Wolframate*

Molybdates and wolframates (tungstates) in alkaline solution are represented by the formulas M_2MoO_4 and M_2WO_4, respectively. Each tends to

condense in acidic solution to form anions which can be formulated to contain several molecules of the acid anhydride, as, for example, $M_2O \cdot n\ MoO_3 \cdot aq$. Several isopoly acids (that is, those containing only one type of acid anhydride) and heteropoly acids are known. Much confusion has existed, and to some extent still exists, about the structure of the isopoly acids and the mechanism of their formation.

It has long been known that molybdate[34,35] and wolframate[36–38] form complexes with polyhydroxy compounds. Whereas the reasonably well-known structures of the anions described in the preceding Sections could readily be correlated to their modes and positions of attachment to polyhydroxy compounds, the complexity of acid solutions of molybdate and wolframate did not allow logical predictions to be made. For this reason, the behavior of polyhydroxy compounds in the present of molybdate and wolframate will be discussed first. It will be seen later that these investigations have yielded information about the nature of the complexing, condensed acids. With certain exceptions, the results obtained with molybdate and wolframate are identical or similar. Hence, the two anions will be discussed simultaneously.

Paper electrophoresis in molybdate[39] (pH 5.0) and wolframate[40] (pH 5.0) solutions has revealed that only those aldohexoses and aldopentoses will migrate which possess in their cyclic forms, most probably their pyranose form, a *cis-cis*-1,2,3-triol system and, thus, in at least one of their chair conformations, one equatorial hydroxyl group neighbored by two axial hydroxyl groups (**13**). The suggestion that the pyranose form



(13)

is involved in complex-formation is based on the fact that methyl α-D-ribopyranoside, which only possesses the 1*a*,2*e*,3*a*-triol system (**13**) in the

(34) A. Honnelâitre, *Ann. chim.* (Paris), **3,** 5 (1925).
(35) N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **73,** 2249 (1951).
(36) J. Lefort, *Compt. rend.*, **82,** 1182 (1876). A. Rosenheim, *Ber.*, **26,** 1191 (1893). H. Grossmann and H. Krämer, *Z. anorg. Chem.*, **41,** 43 (1904). A. Rosenheim and H. Itzig, *Ber.*, **33,** 707 (1900).
(37) G. G. Henderson, T. W. Orr, and R. J. G. Whitehead, *J. Chem. Soc.*, **75,** 542 (1899).
(38) D. Klein, *Compt. rend.*, **89,** 484 (1880).
(39) E. J. Bourne, D. H. Hutson, and H. Weigel, *J. Chem. Soc.*, 4252 (1960).
(40) H. J. F. Angus and H. Weigel, unpublished results.

1C conformation, exhibits appreciable mobility in (at least) molybdate solution. Migration of D-*glycero*-aldoheptoses which, in their pyranose form, do not possess a *cis-cis*-1,2,3-triol system could be due to complex-formation involving the hydroxyl groups on C-4, C-6, and C-7. These can be brought, without distortion of bond angles, into the same relative positions as those of the 1*a*,2*e*,3*a*-triol system.[40a]

Similar suggestions have been made for ketoses.[39] By virtue of the $\alpha \rightleftharpoons \beta$ equilibrium and the free rotation around the C-1 to C-2 bond, unsubstituted hydroxyl groups on C-1 and C-2 and an equatorial hydroxyl group on C-3 of the pyranose form of 2-ketoses can form structures approximating that of (**13**). The same applies for the furanose form of any 2-ketose having unsubstituted hydroxyl groups on C-1, C-2, and C-3. Thus, compounds not possessing such structural features show little or no tendency to form complexes with molybdate and wolframate.

Less ambiguous results have been obtained by examination of the electrophoretic behavior of cyclitols. With these, the possibility of complex-formation with open-chain and with five-membered ring structures, as in the case of aldoses and ketoses, does not arise. The complex-forming compounds of this group all possess a *cis-cis*-1,2,3-triol system. Their rates of migration have been related to the instability factors[12] of their conformations possessing this triol system as in (**13**).

Of the 1,6-anhydro-β-D-aldopyranoses, only 1,6-anhydro-β-D-mannopyranose has been found to migrate in molybdate solution. A unique feature of this compound is the *a*,*e*,*a* relation of the hydroxyl groups on C-2 and C-3 and the anhydro-ring oxygen atom.

Molybdate and wolframate differ from the complexing agents discussed in the preceding Sections as they only form relatively strong complexes with sugars and other six-membered cyclic polyhydroxy compounds when three or more hydroxyl groups are available in the correct relative position. However, compounds containing the enediol grouping, as in L-ascorbic acid and pyrocatechol (*o*-dihydroxybenzene), form stable complexes with molybdate at pH 5.0. These complexes have been used for the electrophoresis and chromatography of phenolic compounds[41,42] and their intense color indicates a conjugated system. In this case, a diester structure is probably resonance-stabilized. A similar complex with 1,6-anhydro-β-D-mannopyranose could thus be stabilized by hydrogen bonding to the anhydro-ring oxygen atom.

(40a) Recent work in collaboration with Professor W. G. Overend suggests that derivatives of D-glucofuranose having unsubstituted hydroxyl groups on C-1, C-3, and C-6 complex with molybdate and wolframate.

(41) J. B. Pridham, *J. Chromatog.*, **2**, 605 (1959).

(42) J. Halmekoski, *Suomen Kemistilehti*, **B32**, 170 (1959).

Complexes between certain acyclic polyhydroxy compounds and molybdate or wolframate seem to be more stable than the corresponding ones of sugars, where, in some cases, oxidation of the reducing group occurs. Consequently, more attention has been focused on the former. Since D-gluco-, D-manno-, and D-galacto-pyranosides do not complex with either reagent,[39,40] use has also been made of reduced disaccharides for studying the effect (on complex-formation) of substitution in a hexitol.

Changes in optical rotation of solutions (adjusted to pH 2, where maximal changes occur) containing substituted D-glucitols (L-gulitols) and molybdate in various relative concentrations show that the substituted D-glucitols (L-gulitols) fall into three classes: (*a*) 3-substituted D-glucitols do not form a complex; (*b*) 2-substituted D-glucitols and 1-substituted L-gulitols form complexes containing the substituted hexitol molecule and the molybdenum atom in the ratio of 1:2; and (*c*) 3-substituted L-gulitols form complexes containing the substituted hexitol and the molybdenum atom in the ratio[43] of 1:1. Similar deductions have been made for the corresponding complexes with wolframate, where maximal changes in optical rotation occur[40] at pH 5.5, although the 3-substituted L-gulitols form a much weaker complex with wolframate than with molybdate. These effects can be extremely useful in deducing the point of linkage in disaccharides containing a D-glucose residue as the reducing moiety.

As regards electrophoresis in molybdate[43] and wolframate[40] solutions, the substituted D-glucitols fall into the same classes: (*a*) 3-substituted D-glucitols; (*b*) 2-substituted D-glucitols and 1- and 2-substituted L-gulitols; and (*c*) 3-substituted L-gulitols.

No acyclic compound containing fewer than four hydroxyl groups migrates during electrophoresis in either electrolyte. When these hydroxyl groups are not on adjacent carbon atoms, the mobility is diminished (as with 3-*O*-α-D-glucopyranosyl-L-gulitol) or the compound does not migrate at all (for example, 3-*O*-α-D-glucopyranosyl-D-glucitol and 2-deoxy-D-*erythro*-pentitol).

The wolframate complexes are much less readily decomposed by alkali than are the corresponding molybdate complexes, and they can thus be used for selective reactions. For example, oxidation of the D-glucitol–wolframate complex with 2 moles of periodate[40] per mole furnished D-arabinose and L-xylose in the ratio of 1:0.76, representing a 40% oxidation of the D-glucitol. In the absence of wolframate, DL-glycerose would have been the major product.[27] Similar investigations on wolframate complexes of other acyclic polyhydroxy compounds, notably galactitol and 2-deoxy-D-*arabino*-hexitol ("2-deoxy-D-glucitol") which are not oxidized, have

(43) E. J. Bourne, D. H. Hutson, and H. Weigel, *J. Chem. Soc.*, 35 (1961).

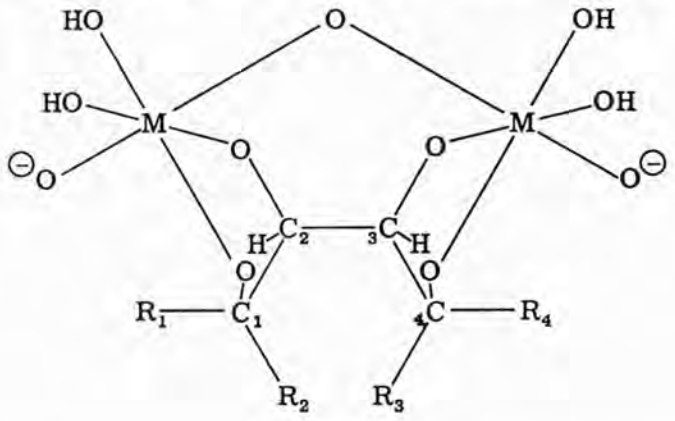
shown that, in complexes which contain the polyhydroxy compound and the wolfram (tungsten) atom in the ratio of 1:2, four adjacent hydroxyl groups are involved in complex-formation.

The steps involved in the formation of the ions of isopoly acids (for example, isopolywolframates)

$$WO_4^{2\ominus} \xrightarrow{\text{pH 6}} HW_6O_{21}^{5\ominus} \xrightarrow{\text{pH 4}} H_2W_6O_{21}^{3\ominus} \xrightarrow{\text{pH 3}} H_2W_{12}O_{40}^{6\ominus}$$

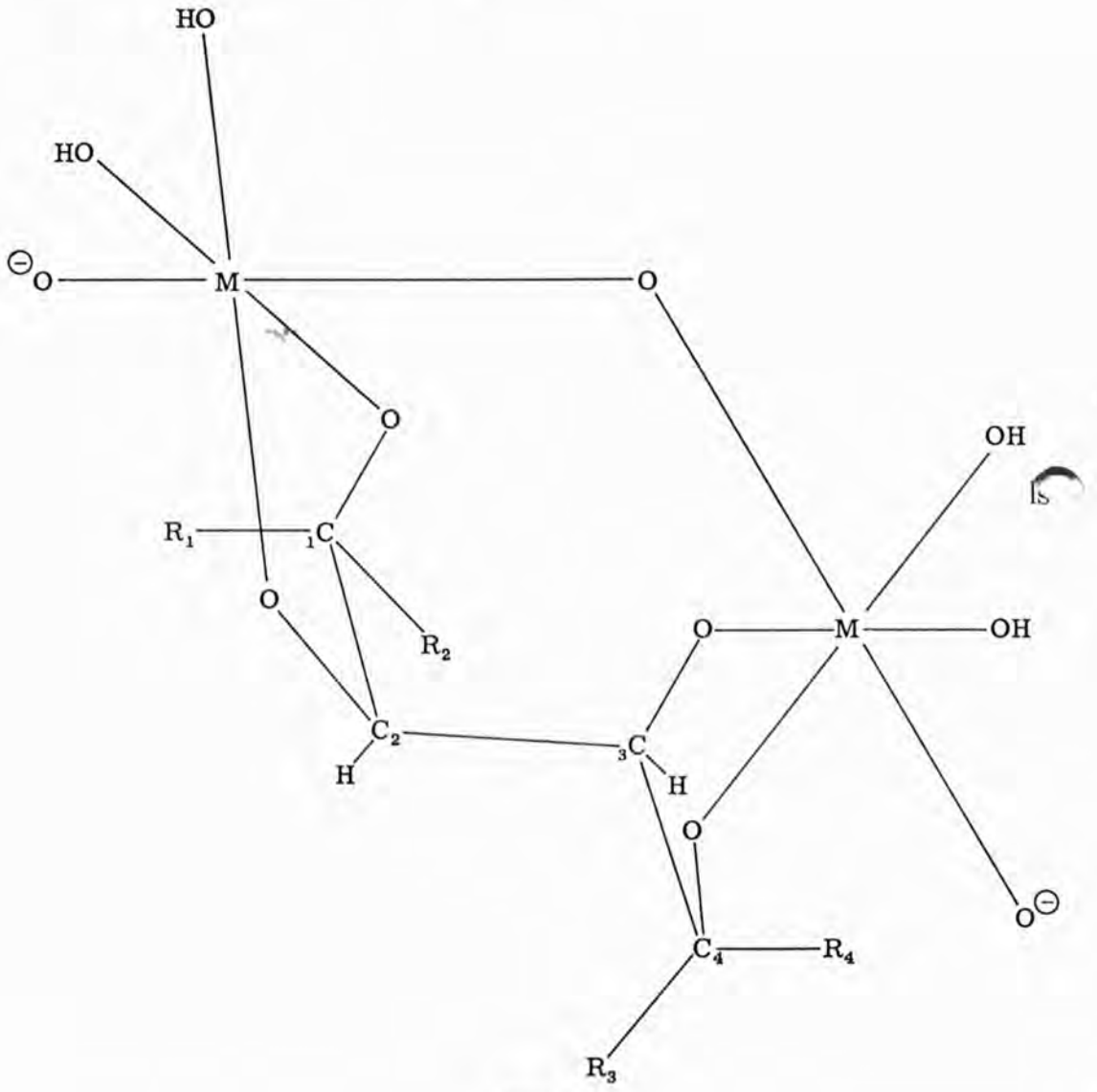
have been deduced from physicochemical studies.[44] None of the described isopolymolybdates or isopolywolframates seem to be of the species which complexes with polyhydroxy compounds, except for the dimolybdate, $Mo_2O_7^{2\ominus}$, but it is doubtful whether this ion exists at all in simple aqueous solution.[45] However, 1.0 $H^{\oplus}$ ion per $WO_4^{2\ominus}$ ion is consumed when sodium wolframate, Na_2WO_4, is potentiometrically titrated in the presence of D-mannitol.[40] This result, together with the results of electrophoresis in wolframate solution, shows that a diwolframate ion, $W_2O_7^{2\ominus}$, is the complexing species. It is likely that, in the case of molybdate, the dimolybdate ion, $Mo_2O_7^{2\ominus}$, is the complexing species, since the interatomic distances in $WO_4^{2\ominus}$ and $MoO_4^{2\ominus}$ are almost identical[46,47] and D-mannitol has the same absolute mobility in wolframate and in molybdate solutions.

The structures (**14**) and (**15**) have thus been postulated for molybdate or wolframate complexes of acyclic polyhydroxy compounds containing four adjacent hydroxyl groups, (**14**) being formed from such a tetritol system where the hydroxyl groups on C-2 and C-3 are in *cis* arrangement,



(**14**)

(44) H. J. Emeléus and J. S. Anderson, "Modern Aspects of Inorganic Chemistry," Routledge and Kegan Paul, Ltd., London, Engl., 2nd Edition, 1952, p. 210.
(45) I. Lindqvist, *Nova Acta Regiae Soc. Sci. Upsaliensis*, **15**, No. 1 (1950).
(46) L. G. Sillén and A. Nylander, *Arkiv Kemi, Mineral. Geol.*, **16B**, No. 7 (1943).
(47) J. Donohue and W. Shand, *J. Am. Chem. Soc.*, **69**, 222 (1947).



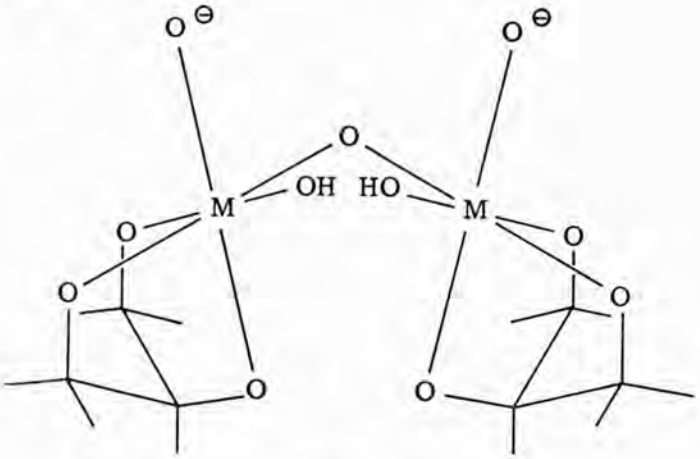
(15)

whereas those in (15) are in a *trans* arrangement. M is either an octahedral molybdenum or wolfram atom. It is apparent that the sizes of the substituents R_2 and R_3 should influence the stability of the complex, and, hence, the migration during electrophoresis. Indeed, it was found that pentitols and hexitols (or their derivatives) which have M_s(W) values of less than 0.95 and which streak on migration bear carbon substituents at positions R_2 or R_3.

On the basis of the effective radii of the complexes between 3-*O*-D-glycopyranosyl-L-gulitols and molybdate,[48] it is suggested that, in these, two molecules of the polyhydroxy compound combine with the dimolybdate

(48) H. Weigel, unpublished results.

ion. This conclusion is in agreement with the results of polarimetric studies[43] and the fact[48] that electrophoresis of mixtures of two 3-*O*-glycopyranosyl-L-gulitols (for example, A and B) in molybdate solution reveals the presence of three components which contain (*a*) A; (*b*) B; and (*c*) A and B. Similar results have been obtained with a mixture of a 3-*O*-glycopyranosyl-L-gulitol and D-gulose. The structure [partial (**16**)] has been suggested[48] for complexes containing the molybdenum or wolfram atom and the polyhydroxy compound in the ratio 1:1. These are produced from compounds which can easily possess three hydroxyl groups in a spatial arrangement approximating to that of (**13**).



(16)

7. *Sodium Hydroxide*

Migration of polyhydroxy compounds during electrophoresis in sodium hydroxide solution is probably due to ionization of hydroxyl groups rather than to formation of cyclic complexes.[13] Although it is difficult to perceive a common steric principle which would account for the wide differences in acidity displayed for stereoisomers, Frahn and Mills[13] have observed certain patterns in the electrophoretic mobilities. Thus, the mobilities show a trend toward highest rates for those reducing sugars and cyclitols for which *both* chair conformations possess great elements of instability. However, the relative high rates of migration of D-glucose and D-mannose show that such a structural feature alone is not the only factor determining electrophoretic mobility.

The behavior of (acyclic) pentitols and hexitols in sodium hydroxide solution shows a good correlation between mobility and the number of *cis*-1,2-diol groupings. This effect is in contrast to the mobilities in stannate,[29] arsenite,[13] and basic lead acetate[13] solutions. Since the group $RO^{\ominus}$ is more strongly solvated, and thus has a greater effective volume than the group *R*OH, it is probably to be expected that the acidity (and hence

mobility) is the greater, the greater the O–O distances in the polyhydroxy compounds. The flexibility of the conformations of these acyclic compounds makes a quantitative treatment of this aspect difficult. However, on the basis of the planar zigzag conformation of pairs of acyclic hexitols which possess the same types of 1,3-diol grouping, it can be seen that the step allitol→L-iditol involves a total diminution (Δ) in O–O distance (in the 1,2-diol groupings) of 2.49 Å., whereas that involved in the steps D-altritol→D-glucitol and D-mannitol→galactitol is 0.83 Å. The difference between electrophoretic mobilities follows a similar pattern; thus ΔM_{Ri}(Na) allitol–L-iditol, D-altritol–D-glucitol, and D-mannitol–galactitol are 0.16, 0.05, and 0.04, respectively.

8. *Acetates of Alkali Metals and Alkaline-Earth Metals*

Complexing of polyhydroxy compounds with alkali-metal hydroxides and acetates in nonaqueous media is a fairly general phenomenon.[49] In addition, several carbohydrates have long been known to form crystalline addition compounds with sodium and calcium salts. Recently, Mills[50] has provided evidence for the existence, in dilute aqueous solutions, of complexes of neutral polyhydroxy compounds with cations of the alkali metals and alkaline-earth metals. When subjected to paper electrophoresis in solutions containing the metal acetates, many compounds migrated toward the cathode (for examples, see Table I). Except for *cis*-inositol,

TABLE I

Relative Migrations of Polyhydroxy Compounds in the Presence of Metallic Ions[50]

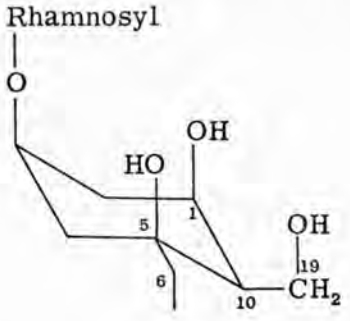
	Cationic mobility[a]			
Compound	$Ba^{\oplus\oplus}$	$Mg^{\oplus\oplus}$	$Na^{\oplus}$	$K^{\oplus}$
cis-Inositol	82	20	10	6
epi-Inositol	25	2	3	3
L-Iditol	13	1	1	1
Allitol	5	1	1	1
D-Talose	18	0	2	2

[a] Cationic mobilities are given as percentages of the anionic migration (about 10 cm.hr.$^{-1}$ at 20 V. cm.$^{-1}$) of *p*-nitrobenzenesulfonic acid,[13] with 2,3,6-tri-*O*-methyl-D-glucose as the nonmigrating marker. The electrolyte was a 0.1 *M* solution of metal acetate in 0.2 *M* aqueous acetic acid.

(49) J. A. Rendleman and J. E. Hodge, *Abstracts Papers Am. Chem. Soc.*, **140**, 13D (1961).

(50) J. A. Mills, *Biochem. Biophys. Res. Commun.*, **6**, 418 (1961/62).

the movement of compounds showed a nearly linear dependence on the concentration of the metal ions. Of the reducing sugars, only D-talose and D-ribose showed appreciable migration in solutions containing the acetates of calcium, strontium, and barium, respectively. The migration of acyclic polyhydroxy compounds in these solutions could be related to the number of *trans*-1,2-diol groupings in each. Mills[50] suggested that the presence of several, closely adjacent hydroxyl groups suitably placed for simultaneous close approach to a cation (as in *cis*-inositol and β-D-talopyranose) results in strong complexing power. He further drew attention to the fact that ouabain, which is physiologically very active and which selectively influences the transport of sodium and potassium, has a conformation [partial structure (**17**)] in which the hydroxyl groups at C-1, C-5 ,and



(**17**)

C-19 can have the same spatial disposition as the three axial hydroxyl groups in *cis*-inositol.

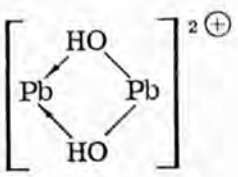
9. *Basic Lead Acetate*

The presence of basic lead acetate has been shown to affect the optical rotation of aqueous solutions of sucrose, D-glucose, and D-fructose.[51,52] Frahn and Mills[13] found that, during electrophoresis in basic lead acetate solution (pH 6.8), polyhydroxy compounds migrate as cationic complexes.

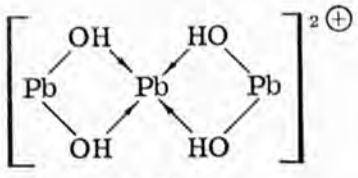
Lead forms two series of basic salts, frequently formulated as $Pb(OH)X$ and $PbX_2 \cdot 2Pb(OH)_2$. Weinland and coworkers[53,54] showed, by measurements of electrical conductivity, that, in solutions of salts of both series, a bivalent cation [(**18**) and (**19**), respectively] was present. It is thus possible that, in the cationic complexes formed from a polyhydroxy compound and basic lead acetate, a diol grouping of the former co-ordinates

(51) F. Bates and J. C. Blake, *Z. Ver. deut. Zucker-Ind.*, **614,** 314 (1907).
(52) J. T. N. Gaskin and R. J. Mesley, *Intern. Sugar J.*, **60,** 65 (1958).
(53) R. Weinland and R. Stroh, *Ber.*, **55,** 2706 (1922).
(54) R. Weinland and F. Paul, *Z. anorg. Chem.*, **129,** 243 (1923).

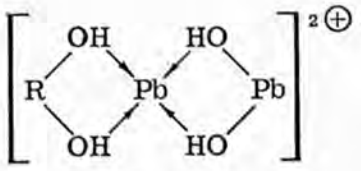
with the lead atom [as in (20)]. Such complexes have not, to the best of



(18)

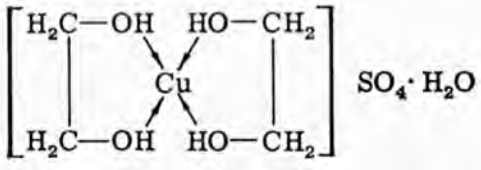


(19)

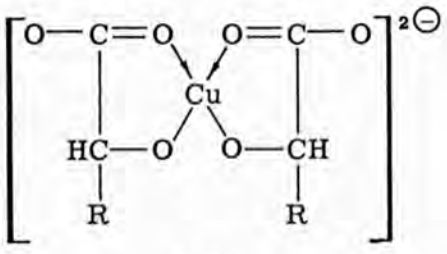


(20)

the author's knowledge, been characterized. However, a compound of ethylene glycol (1,2-ethanediol) and copper sulfate, having the structure (21), has been isolated.[55] On the other hand, Pfeiffer and coworkers[56] regard the copper complexes present in Fehling solution as having structure (22). The existence of complexes of this type has been demonstrated



(21)



(22)

by paper electrophoresis of cupric, plumbous, and ferric salts in D-mannitol solution (pH 12), when the metal ions migrated toward the anode.[57]

(55) A. Werner, "Neuere Anschauungen auf dem Gebiete der anorganischen Chemie," revised by P. Pfeiffer, Vieweg, Braunschweig, Ger., 1923, p. 202.
(56) P. Pfeiffer, H. Simons, and E. Schmitz, *Z. anorg. Chem.*, **256**, 318 (1948).
(57) A. J. F. Angus and H. Weigel, unpublished observation.

It is not yet possible to correlate the structure of the cation in basic lead acetate with the stereochemistry of the site of attachment in the polyhydroxy compounds. Frahn and Mills[13] found that no compound containing a single pair of complexable hydroxyl groups migrated during electrophoresis in basic lead acetate solution. There was also no evidence for the formation of six-membered ring complexes. Indeed, the behavior of acyclic polyhydroxy compounds showed that the formation of five-membered cyclic complexes only is the dominant reaction. As in stannate and arsenite, the mobility of hexitols in basic lead acetate solution increases with an increase in the number of *trans*-1,2-diol groupings. Similarly, the mobilities of the cyclitols can be fairly well correlated with the number of *cis* related hydroxyl groups on adjacent carbon atoms. However, the same correlation cannot be made for the glycopyranosides. The authors also found it difficult to perceive a stereochemical principle which would account for the rates of migration of reducing sugars.

10. *Vanadate*

Preliminary studies have shown that reducing sugars and their reduction products migrate, during electrophoresis in sodium metavanadate solution, as anionic complexes.[13,58] However, this electrolyte has not been examined further.

11. *Tellurate*

The acidity of telluric acid is enhanced by the presence of a number of polyhydroxy compounds,[32,59] and, during electrophoresis in tellurate solution (pH 10), the polyhydroxy compounds migrate as anions.[60] The following order of migration has been observed: D-mannitol, D-glucitol, galactitol, D-ribose > D-mannose, D-fructose > D-galactose, glycerol, *myo*-inositol, D-arabinose > 1,2-propanediol > D-glucose, D-xylose, ethylene glycol (1,2-ethanediol), cellobiose > sucrose. With the exception of the hexitols, all compounds studied gave rather elongated spots.

12. *Hydrogen Sulfite*

Paper electrophoresis of reducing sugars and carbonyl derivatives of carbohydrates in solutions containing hydrogen sulfite has been reviewed in this Series by Foster[6] and Theander.[61]

(58) J. L. Frahn and J. A. Mills, *Chem. Ind.* (London), 578 (1956).
(59) A. Rosenheim and M. Weinheber, *Z. anorg. Chem.*, **69**, 266 (1911).
(60) W. J. Popiel, *Chem. Ind.* (London), 434 (1961).
(61) O. Theander, *Advan. Carbohydrate Chem.*, **17**, 223 (1962).

III. Electrolytes Not Affording Ionic Complexes

Electrolytes which do not afford ionic complexes with common hexitols and reducing sugars are aqueous solutions of lead acetate, copper sulfate, zinc sulfate, ferrous ammonium sulfate, calcium chloride, potassium dichromate, ferric chloride (pH 3), aluminum sulfate, magnesium sulfate, sodium sulfate, potassium antimonyl tartrate, sodium arsenate or arsenic acid, sodium phosphate, and hydrochloric acid.[13] It is not certain whether sodium aluminate (in 0.1 *N* sodium hydroxide) affords ionic complexes with carbohydrates, as aqueous alkali, alone, permits their migration during electrophoresis.

Electrolytes in which polyhydroxy compounds do not migrate during electrophoresis, such as phosphate solution (pH 7.2), can be used for the separation of acidic from neutral compounds.[62]

IV. Description of Tables

Table II gives the absolute mobilities during paper electrophoresis of reference compounds which have been used for measurements of relative mobilities, $M_R(E)$, where R and E refer to the reference compound and electrolyte, respectively, and[6]

$$M_R = \frac{\text{true distance of migration of substance}}{\text{true distance of migration of reference compound}}.$$

Suitable nonmigrating markers are also given. The absolute mobilities of the reference compounds provide an indication of the time required for the resolution of a particular mixture of compounds. It should be borne in mind that the absolute and relative mobilities may vary with pH and concentration of the electrolyte.

Tables III to XV give the relative mobilities ($10^2 \times M_R$) of carbohydrates and related compounds. The symbols used for the various electrolytes are given in Table II, column 1. The references are given in th heading to each column, unless otherwise stated. The letters e and s indicate elongated spots and extensive streaking, respectively. It must be appreciated that not all measurements have been made under standard conditions (for example, with precision electrophoresis equipment). However, the values given provide a general guide to the behavior of these compounds during paper electrophoresis.

Two reference compounds have been used for electrophoresis in solu-

(62) E. J. Bourne, D. H. Hutson, and H. Weigel, *J. Chem. Soc.*, 5153 (1960).

tions containing sulfonated benzeneboronic acid. D-Mannitol is the reference compound for values quoted in Tables X, XI, XII, XIV, and XV. Also, the values given for 2-*O*-methyl-L-arabinitol and 2-*O*-methyl-L-lyxitol in germanate solution (see Table XI) were made with reference to D-mannitol. All other rates of migration in solutions containing sulfonated benzeneboronic acid have been measured with D-glucose as the reference compound.

The mobility values, mainly in borate solution, of many di- and oligosaccharides (other than those quoted in Tables VIII and IX) have been reported, but these have not been included here, since many have been measured under different conditions. They are to be found in the literature (chiefly under the compound concerned).

The different problems encountered in the resolution of mixtures and the characterization of carbohydrates make it impracticable to discuss applications of paper electrophoresis. For individual problems, reference should, therefore, be made to the Tables; for example. Tables VIII and XIV together show that the electrophoresis of disaccharides and reduced disaccharides in borate and molybdate or wolframate solutions, respectively, can be used for determining the position of the glycosidic linkage to the reducing moiety of the original saccharide.

TABLE II

Reference Compounds

Electrolyte (E)	*Reference Compound (R)*	*Mobility 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$*	*Nonmigrating marker*[a]	*References*
Borate (*B*)	D-glucose (*G*)	14.8 (pH 9.2)	a, b	13
		12.2 (pH 10)		2
Sulfonated benzeneboronic acid (*PhB*)	D-glucose (*G*)	1.1 (pH 6.5)	c	19
	D-mannitol (*M*)	9.4 (pH 6.5)	c	19
Germanate (*Ge*)	D-glucose (*G*)	6.2–8.1 (pH 10.7; 40°)	a	23
Stannate (*Sn*)	D-glucitol (*S*)	14.3 (pH 11.5)	c	29
Arsenite (*As*)	D-ribose (*Ri*)	5.9 (pH 9.6)	a, b	13
Molybdate (*Mo*)	D-glucitol (*S*)	17.0 (pH 5)	c, d	40
Wolframate (*W*)	D-glucitol (*S*)	17.0 (pH 5)	c, d	40
Sodium hydroxide (*Na*)	D-ribose (*Ri*)	9.6 (0.1 *N*)	b, e	13
Basic lead acetate (*Pb*)	D-ribose (*Ri*)	8.1 (pH 6.8)	a, b	13

[a] Nonmigrating markers: a, 2,3,4,6-tetra-*O*-methyl-D-glucose; b, caffeine; c, 5-(hydroxymethyl)-2-furaldehyde; d, glycerol; e, 1,4-dideoxy-L-threitol (L-*threo*-2,3-butanediol).

TABLE III

Trioses and Tetroses

Triose or tetrose	*B*[2]	*Sn*[29]	*Mo*[39]	*W*[40]
1,3-Dihydroxy-2-propanone	78			
D-Erythrose		107	90	110[a]
DL-Glyceraldehyde	79	94	0	0
L-Threose		103	60	5

TABLE IV

Aldopentoses and Derivatives

Aldopentose or derivative	*B*[6]	*PhB*[19]	*Ge*[23]	*Sn*[29]	*Pb*[13]	*As*[13]	*Mo*[39]	*W*[40]	*Na*[13]
D-Arabinose				84			0	0	
methyl α-D-furanoside	4								
β anomer	4								
methyl α-D-pyranoside	38			48			0		
β anomer	38						0		
L-Arabinose	96	240	150		7	30		0	79
methyl α-L-furanoside			0						
methyl α-L-pyranoside			70						
β anomer			60						
2-Deoxy-D-*erythro*-pentose	33[2]			24			0	0	
1,2-Dideoxy-D-*erythro*-pentose				19			0		
D-Lyxose	71[13]	230	190	115	30	42	110	104[8]	97
methyl α-D-pyranoside	45			53			0	0	
β anomer	27						0		
D-Ribose	77	470	210	104	100	100	40	20	100
methyl α-D-pyranoside							10	0	
β anomer	53			104					
D-Xylose	100	180	140	81	8	17	0	0	93
3,5-di-*O*-methyl-		930							
2-*O*-methyl-	39[19]	0	0						
3-*O*-methyl-	66[19]	290	170						
4-*O*-methyl-	21[19]	0	30						
5-*O*-methyl-		1300							
methyl α-D-furanoside	56	230		3			0		
β anomer	33		5						
methyl α-D-pyranoside	0		0						
β anomer	0	0	0						

TABLE V

Aldohexoses and Derivatives

Aldohexose or derivative	*B*[6]	*PhB*[19]	*Ge*[23]	*Sn*[29]	*Pb*[13]	*As*[13]	*Mo*[39]	*W*[40]	*Na*[13]
D-Allose	83[13]		180		33	75			68
D-Altrose	97[13]	580			10	77	0		96
1,6-anhydro-, β-D-pyranose				80				0	
methyl α-D-pyranoside	58[13]				1				7
2-Deoxy-D-*arabino*-hexose	29			31			0	0	
2-Deoxy-D-*lyxo*-hexose	37			23			0[40]	0	
2-Deoxy-D-*ribo*-hexose				52			0	0	
3-Deoxy-D-*ribo*-hexose	85[23]		160						
2-Deoxy-D-*xylo*-hexose								0	
D-Galactose	93	180	130	78	10	28	0	0	65
2-acetamido-2-deoxy-	35								
1,6-anhydro-, β-D-pyranose				81			0	0	
2,3-di-*O*-methyl-			10						
2,4-di-*O*-methyl-			0						
2,6-di-*O*-methyl-			50						
2-*O*-methyl-	43[23]		60						
3-*O*-methyl-	63[23]		140						
4-*O*-methyl-	30[23]		40						
6-*O*-methyl-	86[23]		120						
methyl α-D-furanoside	41								
β anomer	31	40	20[19]						
methyl α-D-pyranoside	38		60		5	19			6
β anomer	38	0	50	43	5	19			7
L-Galactose									
6-deoxy- (L-fucose)	89			69	6	22	0[40]	0	60

D-Glucose	100	100	100	63	6	16	0	0	80
2-acetamido-2-deoxy-	23		10						
1,6-anhydro-, β-D-pyranose	0[13]		0	0	8	0	0	0	5
3-*O*-benzyl-			120						
4-*O*-benzyl-	17[23]		20						
catechol β-D-pyranoside							0		
6-deoxy-				63			0[40]	0	
2,3-di-*O*-methyl-	12		0						
2,4-di-*O*-methyl-	<5								
3,4-di-*O*-methyl-	31								
1,2-*O*-isopropylidene-, α-D-furanose	73				0	25			31
2-*O*-methyl-	23	0	0						
3-*O*-methyl-	80	130	140	78	0	13	0	0	77
4-*O*-methyl-	24	0	30	44			0	0	
5-*O*-methyl-	65[23]		160						
6-*O*-methyl-	80	50	96				0[40]	0	
methyl α-D-furanoside	73								
β anomer		200							
methyl α-D-pyranoside	11	0	0	28	1	9	0	0	9
β anomer	19		0		3	6			9
phenyl β-D-pyranoside	15[13]				4	12	0		9
2,3,4,6-tetra-*O*-methyl-	0				0	0	0		74
2,3,4-tri-*O*-methyl-	0						0		
2,3,6-tri-*O*-methyl-	0[13]				0	0			71
2,4,6-tri-*O*-methyl-	0[13]				0	0			66
3,5,6-tri-*O*-methyl-	71				8	134			
D-Gulose	82[13]			107	31	53	110	110	70
1,6-anhydro-, β-D-pyranose				77			0	0	
6-deoxy-				105			110[40]	107	
methyl α-D-pyranoside	59								
β anomer	72								

TABLE V—*Continued*

Aldohexose or derivative	*B*[6]	*PhB*[19]	*Ge*[23]	*Sn*[29]	*Pb*[13]	*As*[13]	*Mo*[39]	*W*[40]	*Na*[13]
L-Idose	102[13]				42	115			96
D-Mannose	72	110	140	100	41	35	90[a]	110[a]	84
1,6-anhydro-, β-D-pyranose				96			50	0	
3,4-di-*O*-methyl-				71			0	0	
3-*O*-methyl-			80						
methyl α-D-furanoside		1600	140						
methyl α-D-pyranoside	42		50	41	0	17	0	0	9
β anomer	31	0	40				0		
L-Mannose									
6-deoxy- (L-rhamnose)	52	50	130	100	28	32	60[a]	110[a]	88
2,3-di-*O*-methyl-	<5								
2,4-di-*O*-methyl-	<5								
3,4-di-*O*-methyl-	36								
D-Talose	87[13]				110	119	70		103

TABLE VI

Aldoheptoses

Heptose	*Sn*[29]	*Mo*[39]	*W*[40]
D-*glycero*-D-*allo*-	120	90	48[e]
D-*glycero*-D-*galacto*-		40	94[a]
D-*glycero*-L-*galacto*-	41	40	21[e]
D-*glycero*-L-*gluco*-	30	20	0
D-*glycero*-D-*gulo*-		110	98[a]
D-*glycero*-D-*ido*-		100	73[e]
D-*glycero*-L-*manno*-		80	100[a]

TABLE VII

Pentuloses, Hexuloses, and Heptuloses

Compound	*B*[6]	*PhB*[19]	*Ge*[23]	*Sn*[29]	*Pb*[13]	*As*[13]	*Mo*[39]	*W*[40]	*Na*[13]
3-Deoxy-D-*erythro*-hexulose				60			0[40]	0	
L-*galacto*-Heptulose	89								
D-*gluco*-Heptulose				90			100		
D-*manno*-Heptulose	87			103			40		
D-*arabino*-Hexulose (D-fructose)	90	930	210	91	22	75	50	25[8]	89
6-deoxy-							70[63]		
1-*O*-methyl-				80			30[63]	0	
D-*lyxo*-Hexulose (D-tagatose)	95[13]	860	240		65	103	105[40]	110[8]	82
D-*ribo*-Hexulose (D-psicose)	76[13]				91[8]	188			125
L-*xylo*-Hexulose (L-sorbose)	95	850	200	94	16	73	30	20[8]	88
D-*erythro*-Pentulose	90[13]				73	209			
D-*threo*-Pentulose	75[13]				41	194			

(63) E. J. Bourne, D. H. Hutson, and H. Weigel, *Chem. Ind.* (London), 1111 (1960).

TABLE VIII

O-D-*Glucopyranosyl*-D-*glucoses*

Disaccharide	*Linkage*	B^{6}	Ge^{23}	Sn^{29}	Pb^{13}	As^{13}	Mo^{39}	W^{40}	Na^{13}
Trehalose, α, α-	α-D-(1↔1)-α-D	19		11			0^{40}	0	
α, β-	α-D-(1↔1)-β-D	23							
β, β-	β-D-(1↔1)-β-D	19							
Kojibiose	α-D-(1→2)	32^{64}					0^{40}	0	
Sophorose	β-D-(1→2)	24		57			0	0	
Nigerose	α-D-(1→3)	69	130	57			0	0	
Laminaribiose	β-D-(1→3)	69	110	75			0	0	
Maltose	α-D-(1→4)	32	40	65	7	15	0	0	68
Cellobiose	β-D-(1→4)	23	30	62	10	15	0	0	68
Isomaltose	α-D-(1→6)	69	90	58			0	0	
Gentiobiose	β-D-(1→6)	75	100	65			0	0	

(64) S. Haq and W. J. Whelan, *Nature*, **178,** 1221 (1956).

TABLE IX

O-D-*Glucopyranosyl*-D-*fructoses*

Disaccharide	*Linkage G–Fru*	B^{63}	As^{63}	Mo^{63}	W^{40}	Na^{63}
1-*O*-β-D-Glucopyranosyl-D-fructose	β-D-(1→1)	74	78	25	0	35
Sucrose	α-D-(1↔2)-β	10	25	0	0	15
Turanose	α-D-(1→3)	69	80	10	0	28
Maltulose	α-D-(1→4)	63	76	15	0	60
Leucrose	α-D-(1→5)	56	62	35	4	28
Isomaltulose	α-D-(1→6)	60	72	64	70[8]	73

TABLE X

Acyclic Diols, Triols, and Tetritols

Compound	B^{13}	PhB^{19}	Ge^{23}	Sn^{29}	Pb^{13}	As^{13}	Mo^{43}	W^{40}	Na^{13}
Ethylene glycol (1,2-ethanediol)	11	0	0	<1	0	3	0	0	
1,2-Propanediol	16			<1	0	5	0	0	0
1,3-Propanediol	5			2		0	0	0	
1,3-Butanediol	10			0		0	0	0	
1,4-Butanediol	0					0	0	0	
1,4-Dideoxyerythritol (*cis*-2,3-butanediol)	13	0		2	0	6	0[40]	0	0
1,4-Dideoxythreitol (*trans*-2,3-butanediol)	51	30		4	0	33	0[40]	0	0
1,5-Pentanediol	0					0	0	0	
erythro-(*cis*)-2,4-Pentanediol	18				0	0			0
threo-(*trans*)-2,4-Pentanediol	0					0			
2-Methyl-1,3-pentanediol	8; 24					0			
1,6-Hexanediol							0	0	
Glycerol	49	0	40	23	3	24	0	0	0
Erythritol	75	10	100	57	3	53	100	90[e]	3
L-Threitol	75	30		62	11	96	50	24	3
Pentaerythritol	85			10	0	21	0	0	12

TABLE XI

Acyclic Pentitols and Derivatives

Acyclic pentitol or derivative	*B*[13]	*PhB*[19]	*Ge*[23]	*Sn*[29]	*Pb*[13]	*As*[13]	*Mo*[43]	*W*[40]	*Na*[13]
D-Arabinitol	87			95	14	124	110	104	7
1-deoxy-				58			103[40]	109	
L-Arabinitol		60	180						
2-*O*-methyl-		10	10[19]						
D-Lyxitol, 1-deoxy-				78			95[40]	65[e]	
L-Lyxitol, 2-*O*-methyl-		50	50[19]						
D-*erythro*-Pentitol, 2-deoxy-							0[40]	0	
Ribitol	85	30	120	72	4	76	110	103	10
Xylitol	79	90	170	100	25	155	110	104	3
1-deoxy-D-				88			96[40]	82	
1-*O*-methyl-L		60							
2-*O*-methyl-D-		40	120						
3-*O*-methyl-		10	50						

TABLE XII

Acyclic Hexitols and Derivatives

Hexitol or derivative	*B*[13]	*PhB*[19]	*Ge*[23]	*Sn*[29]	*Pb*[13]	*As*[13]	*Mo*[40]	*W*[40]	*Na*[13]
Allitol	90			88	9	92	94	97[e]	23
D-Altritol	89			95	17	138	99	97[e]	16
1-deoxy-				80			98	100[e]	
1,6-dideoxy-				45			90	105	
Galactitol	97	100	210	99	32	145	100[43]	100	8
1-deoxy-L-				87			100[43]	103	
1,6-dideoxy-				72			98	109	
1-*O*-methyl-L-		80	160						
2-*O*-methyl-D		50	170						
3-*O*-methyl-D-		100	140						
D-Glucitol	83	130	190	100	47	161	100[43]	100	11
1-deoxy-		90		89			98	98	
2,3-di-*O*-methyl-							0[43]	0	
2-*O*-methyl-		120	150						
3-*O*-methyl-		10	80	30		36[40]	0[43]	0	
L-Gulitol									
1-deoxy-				94			94	98	
1-*O*-methyl-		60					93	89	
3-*O*-methyl-		130	140	85			47	9	
D-*arabino*-Hexitol									
2-deoxy-				48			100[43]	100	
3-deoxy-				24			5	0	
D-*lyxo*-Hexitol, 2-deoxy-							80	61[s]	
D-*ribo*-Hexitol									
2-deoxy-							57[e]	57[s]	
3-deoxy-				24			5	0	

L-*xylo*-Hexitol, 2-deoxy-							107	109	
L-Iditol	81				57	173			7
D-Mannitol	91	100	190	93	23	130	100[43]	100	12
1-deoxy-				94			100[43]	100	
1,2-di-*O*-methyl-				66			100[43]	95	
2-*O*-methyl-				88			100[43]	98	
L-Mannitol									
1-deoxy- (L-Rhamnitol)		80							
1-deoxy-2,3-di-*O*-methyl-								0	
1,6-dideoxy-				67			100	95[e]	
D-Talitol, 1-deoxy-							104	94[e]	

TABLE XIII

Heptitols

Heptitol	B^{13}	Pb^{13}	As^{13}	Na^{13}
(*meso*)-*glycero-allo*-	95	11	100	54
D-*glycero*-D-*altro*-	92	27	144	44
D-*glycero*-D-*galacto*-	98	51[e]	140	11
D-*glycero*-D-*gluco*-	88	53[e]	171	30
D-*glycero*-L-*gluco*-	95	59[e]	176	17
(*meso*)-*glycero-gulo*-	85	72[e]	160	27
D-*glycero*-D-*ido*-	85	71[e]	168	20
(*meso*)-*glycero-ido*-	78	79[e]	182	17
D-*glycero*-D-*talo*-	93	34	140	24

TABLE XIV

Reduced Disaccharides

Reduced disaccharide	PhB^{19}	Sn^{29}	As^{40}	$Mo^{39,40}$	W^{40}
D-Glucitol					
2-*O*-*α*-D-glucopyranosyl-		81		69	76
2-*O*-*β*-D-glucopyranosyl-	120	85		70	73
3-*O*-*α*-D-glucopyranosyl-			39	0	0
3-*O*-*β*-D-glucopyranosyl-	10	59		0	0
L-Gulitol					
1-*O*-*α*-D-galactopyranosyl-			100		68
3-*O*-*β*-D-galactopyranosyl-		82	63		10
1-*O*-*α*-D-glucopyranosyl-				78	76
1-*O*-*β*-D-glucopyranosyl-	60	89		69	66
2-*O*-*α*-D-glucopyranosyl-				72	70
3-*O*-*α*-D-glucopyranosyl-		81	66	46	17
3-*O*-*β*-D-glucopyranosyl-	110	82	63	37	10
D-Mannitol					
2-*O*-*α*-D-glucopyranosyl-				72	70
2-*O*-*α*-D-mannopyranosyl-				80	
3-*O*-*α*-D-mannopyranosyl-				0	0

TABLE XV

Cyclitols

Compound	B^{13}	PhB^{19}	Ge^{23}	Sn^{29}	Pb^{13}	As^{13}	Mo^{39}	W^{40}	Na^{13}
cis-1,2-Cyclohexanediol	6				0	9			0
trans-1,2-Cyclohexanediol	0				0	2			
allo-Inositol	85			100	62	50	40	8	9
cis-Inositol	79				116	40			9
(+)-Inositol		0	100	55			0[40]	0	
3-*O*-methyl- (pinitol)	59		100		2	34	0		
epi-Inositol	76	180	180	101	74[s]	29	110	100	5
(−)-Inositol	59				20	23			3
2-*O*-methyl-	24				14	27			
muco-Inositol	97			67	41	36	0	0	15
myo-Inositol	49[e]	0	70	42	75[e]	16	20	0	2
1-*O*-methyl-	11				26	20			
5-*O*-methyl- (sequoyitol)	15		50		22	25			
neo-Inositol	59				64	43			1
scyllo-Inositol	2		20	50	ads.	7	0	0	3
C-methyl- (mytilitol)			20				0		

37.

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1964

CHAPTER 8

SPECIFICITY OF INORGANIC OXY-ACIDS IN PAPER ELECTROPHORESIS OF CARBOHYDRATES AND RELATED COMPOUNDS

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INCREASED interest in the naturally occurring phenolic glycosides has paralleled the development of new analytical techniques. A great deal is now known about the carbohydrate components of these compounds and much of this knowledge has come to light using paper chromatography and paper electrophoresis. Both the techniques make it increasingly possible to identify carbohydrates and related compounds and to separate hitherto unresolvable mixtures. The following discussion describes the types of ions formed from these compounds and used in paper electrophoresis and outlines applications of this technique.

Electrophoresis is the movement of charged substances in a conducting solution under the influence of an applied electrical field. Boundary electrophoresis refers to migration in free solution, whilst the term zone electrophoresis is applied to the process of migration in supported electrolytes. The most commonly used support for the electrolyte is filter paper, although others have been used when this seemed desirable.[1–3] Figure 1 illustrates schematically an apparatus for paper electrophoresis.

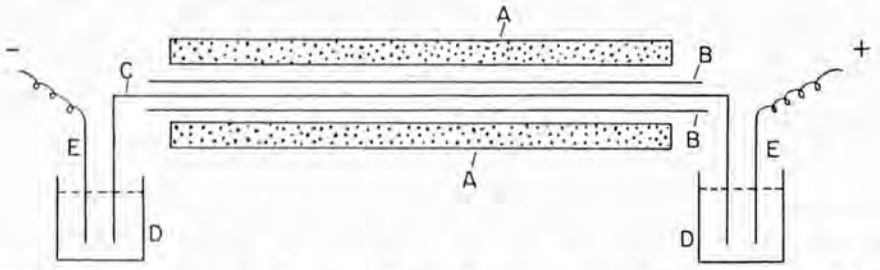


FIG. 1. Schematic illustration of paper electrophoresis apparatus: A, Cooling blocks with inlets and outlets for water. B, Polythene insulators. C, Filter paper. D, Electrode vessels. E, Electrodes.

Molecules possessing a net charge, or those which may be given one by controlling the pH of their environment, e.g. carboxylic acids and amines, will migrate in an applied electrical field as anions or cations according to

the properties of their functional groups. On the other hand, formally neutral polyhydroxy compounds react with a variety of inorganic oxy-acids and basic salts of lead and copper to give anionic and cationic complexes, respectively. These, in addition to the anions produced from polyhydroxy compounds in strongly alkaline solutions, form the basis for paper electrophoresis of carbohydrates and related compounds.

A convenient index of the relative mobility of a compound during paper electrophoresis is the $M_R(E)$ value, where R and E refer to the reference compound and electrolyte, respectively, and

$$M_R = \frac{\text{true distance of migration of compound}}{\text{true distance of migration of reference compound}}$$

The true distance of migration takes into account the effect of electro-osmosis on the migration. This is found by measuring the displacement of substances which either do not ionize, form no ionic complexes or have an electrophoretic mobility sufficiently low for it to be taken as zero. The true distance of migration is thus

$$D = D_e \pm D_o,$$

where D_e is the observed movement of the compound and D_o the displacement due to electro-osmosis. Table 1 gives the absolute mobilities during paper electrophoresis of reference compounds which have been used for measurements of relative mobilities.

TABLE 1. REFERENCE COMPOUNDS

Electrolyte (*E*)	Reference compound (*R*)	Mobility 10^5 cm^2 V^{-1} sec^{-1}	Non-migrating marker	Reference
Sodium hydroxide (*Na*)	D-Ribose (*Ri*)	9·6 (0·1 N)	a, b	4
Borate (*B*)	D-Glucose (*G*)	14·8 (pH 9·2)	a, c	4
		12·2 (pH 10·0)		2
Sulpho-benzeneboronic acid (*PhB*)	D-Glucose (*G*)	1·1 (pH 6·5)	d	10
	D-Mannitol (*M*)	9·4 (pH 6·5)	d	10
Germanate (*Ge*)	D-Glucose (*G*)	6·2–8·1 (pH 10·7; 40°)	c	11
Stannate (*Sn*)	D-Glucitol (*S*)	14·3 (pH 11·5)	d	12
Arsenite (*As*)	D-Ribose (*Ri*)	5·9 (pH 9·6)	a, c	4
Molybdate (*Mo*)	D-Glucitol (*S*)	17·0 (pH 5·0)	d, e	17
Tungstate (*W*)	D-Glucitol (*S*)	17·0 (pH 5·0)	d, e	17

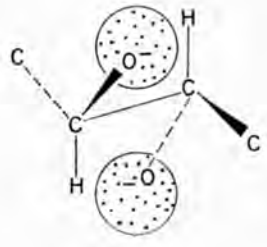
Non-migrating makers: a, caffeine; b, L-*threo*-2,3-butanediol; c, 2,3,4,6-tetra-O-methyl-D-glucose; d, 5-hydroxymethylfurfural; e, glyceritol.

SPECIFICITY OF ELECTROLYTES

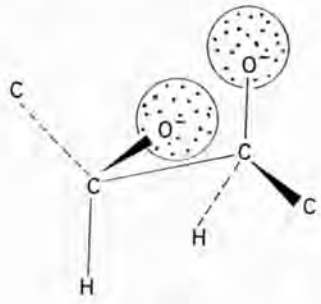
Greatly differing electrophoretic mobilities of polyhydroxy compounds have been observed in the various electrolytes. Clearly, the structures of the inorganic complexing agents and the polyhydroxy compounds will together decide their point of attachment, if any. Thus, paper electrophoresis of polyhydroxy compounds is not only a tool for their separation, but can also be used to detect certain spatial arrangements of hydroxyl groups in organic compounds. In the following sections only the specificities of the electrolytes containing inorganic oxy-acids are discussed. For paper electrophoretic mobilities of the majority of compounds in all electrolytes reference should be made to the original literature.

1. *Sodium Hydroxide*

Migration of polyhydroxy compounds during electrophoresis in sodium hydroxide solution is due to ionization of hydroxyl groups.[4] Since the group RO^- is more strongly solvated and hence has a greater effective volume than the group ROH it is to be expected that the acidity (and hence mobility) is the greater, the greater the O—O distances in the polyhydroxy compound. Thus, the hydroxyl groups of *erythro*-1,2-diols, when in their planar zigzag conformation (I) (O—O, 3·65 Å), should ionize more readily than those of the *threo*-isomers (II) (O—O, 2·82 Å).* The behaviour of acyclic pentitols



I



II

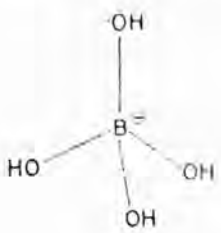
and hexitols in sodium hydroxide solution shows a good correlation between mobility and the number of *erythro*-1,2-diol groupings.

2. *Borate*

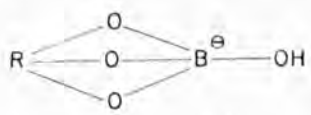
The products of the reactions between borate ions, $B(OH)_4$ (III), and polyhydroxy compounds have structures of the types IV–VI.[5] The borate ion (III) has a tetrahedral symmetry and its oxygen atoms are separated by

* In calculating O—O distances in diol groupings the bond angles at all carbon atoms were taken as the tetrahedral angle (109° 28′) and the bond lengths as C—C, 1·54, and C—O, 1·42 Å. In the case of cyclic compounds the calculations have been based on the cyclopentane and cyclohexane rings systems.

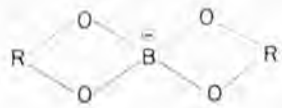
a distance of 2·40–2·44 Å.[6] It thus appears that the borate ion can form complexes with those polyhydroxy compounds in which the oxygen atoms of at least two hydroxyl groups are separated by, or can easily approach each other to a distance of approximately 2·4 Å.



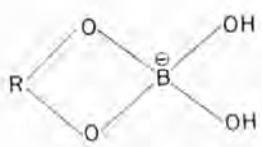
III



VI

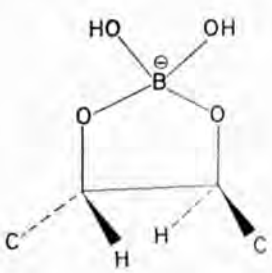


V

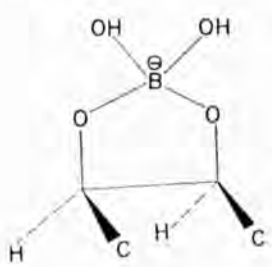


IV

The oxygen atoms of acyclic *erythro*- and *threo*-1,2-diols can, in an eclipsed conformation, approach each other to a distance of 2·49 Å. The greater electrophoretic mobility of the borate complex of acyclic *threo*-1,2-diols (VII), as compared with that of the *erthro*-isomers (VIII), has been related to the interaction energy of the non-bonded atoms.[5]

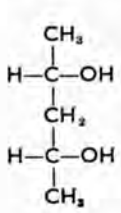


VII

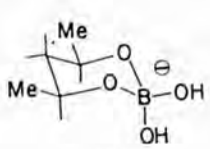


VIII

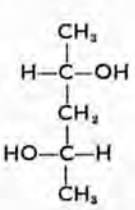
The borate ion, $B(OH)_4^-$, also fits well to form puckered six-membered rings with acyclic 1,3-diols. The stability of the complexes formed, and hence their rate of migration during electrophoresis, is decreased by the presence of axial alkyl groups[7] [e.g. *erythro*-2,4-pentanediol (IX a and IX b), M_G (*B*) 0·18; *threo*-2,4-pentanediol (X a and X b), M_G (*B*) O].



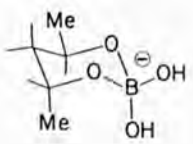
IXa



IXb



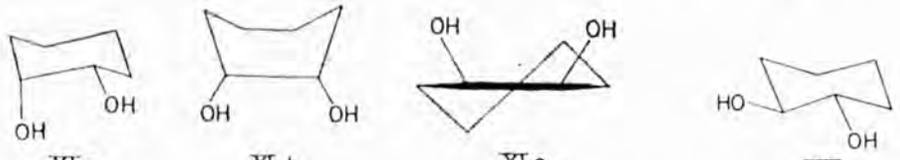
X a



Xb

Cis-1,2-diols of five-membered ring compounds (O—O, 2·49 Å) react more strongly with borate ions than their *trans*-isomers[8] (O—O, 3·40 Å).

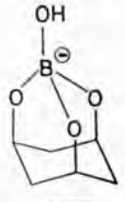
The O—O distances in the chair conformations of *cis*- (XI a) and *trans*-1,2-cyclohexanediol (XII) are identical (2·82 Å). However, only in the former can this distance easily be reduced to a value [2·49 in a boat (XI b) or "half-chair" (XI c) conformation] small enough for complex formation. Consequently, only the *cis*-isomer exhibits electrophoretic mobility.[4]



XI a XI b XI c XII

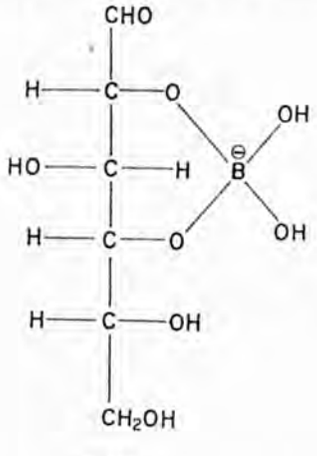
Cis-1,3-cyclohexanediol does not migrate during electrophoresis in borate solution. It is probable that the interactions of non-bonded atoms in the complex would be too great to allow its formation.

Inositols possessing a *cis-cis*-1,3,5-triol system yield a "tridentate" complex (XIII). Their electrophoretic mobilities have been correlated with the dispositions of the remaining hydroxyl groups.[9]



XIII

The sequence of mobilities of substituted D-glucoses (i.e. O-methyl- and deoxy-D-glucoses, and reducing disaccharides of D-glucose) has been rationalized by postulating that the aldehydo form of the sugar is the principal one involved in complex formation, and that the hydroxyl groups sterically most favourable for complex formation are those on C2 and C4 (XIV).[5]

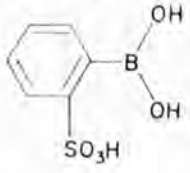


XIV

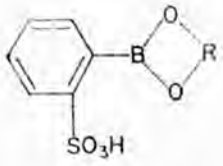
The mobilities of methyl pyranosides of aldohexoses are attributable to complex formation with *cis*-1,2-diol groupings and/or the hydroxyl groups on C_4 and C_6, the *cis*-1,2-diol grouping having, however, a greater affinity for the borate ion.

3. *Sulpho-benzeneboronic Acid*

The products formed from *o*-sulpho-benzeneboronic acid (XV) and polyhydroxy-compounds under the conditions of the electrophoresis (pH 6·5)[10] are in all likelihood esters of the boronic acid (XVI), their migration being due to the ionization of the sulphonic acid group. With reducing sugars and glycosides, by far the largest contribution to the mobilities was



XV

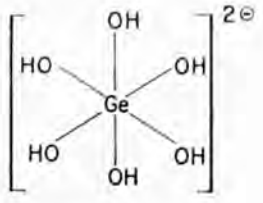


XVI

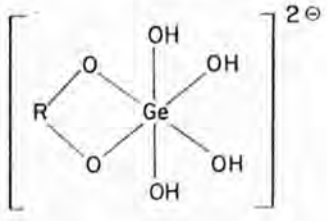
found to arise from *cis*-1,2-diols of five-membered ring compounds, but in contrast to borate at pH 10, no contribution arises from *cis*-1,2-diols of six-membered ring compounds or from a diol group such as that on C4 and C6 in glucopyranose. The different affinities of sulpho-benzeneboric acid and the borate ion for *cis*-1,2-diols of six-membered ring compounds are probably due to the relative stabilities of five-membered rings containing trigonal and tetrahedral boron.[7]

4. *Germanate*

Germanic acid readily undergoes polymerization reactions, the degree of polymerization being dependent on the pH of the solution. At pH 11 the ion $Ge(OH)_6^{2-}$ (XVII) is the major component. The O—O distance in this ion has been estimated to be approximately 2·64 Å.[11] This value falls in



XVII

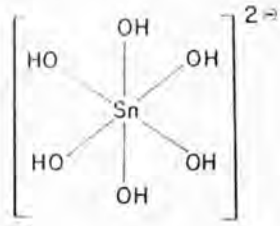


XVIII

between those of the O—O distances in *cis*-1,2-diols of five- (2·49 Å) and six-membered ring compounds (2·82 Å; chair conformation). Compounds possessing such structural features will form ionic complexes during electrophoresis in germanate solution (XVIII).[11] The sequence of mobilities of substituted D-glucoses is the same as that in borate solution.

5. *Stannate*

Electrophoresis in sodium stannate solution has shown that polyhydroxy compounds form anionic complexes with the stannate ion, $Sn(OH)_6^{2-}$ (XIX).[12] Only two hydroxyl groups are required for their formation.

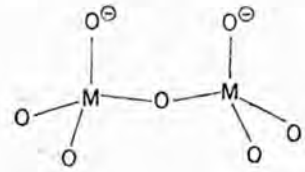


XIX

The order of mobility of pentitols and hexitols is related to their number of *threo*-1,2-diol groupings. This is expected, as in the planar zigzag conformation of these compounds the distance between the oxygen atoms of the *threo*-1,2-diol grouping (2·82 Å) is close to that in the $Sn(OH)_6^{2-}$ ion (2·77 Å). Although *erythro*-1,2-diols can complex with stannate, by far the largest contribution to mobility comes from *threo*- disposed hydroxyl groups.

6. *Arsenite*

Little information is available about the structure of the arsenite ion complexing with polyhydroxy compounds, although the ions $As(OH)_4^-$ and $AsO(OH)_2^-$ have been suggested.[13] If the O—O distance in the complexing ion does not differ greatly from that in As_4O_6 (2·76 Å) arsenite complexes should easily be formed from six-membered ring compounds containing *cis*-1,2-diol groupings (O—O, 2·82 Å; chair conformation) and acyclic *threo*-1,2-diols (O—O, 2·82 Å; planar zigzag conformation).[7] This is in agreement with the electrophoretic behaviour of inositols, glycopyranosides, pentitols, and hexitols.[4] However, contribution to electrophoretic mobility should also be expected from *trans*-1,2-diol groupings of six-membered ring compounds if both hydroxyl groups are in an equatorial position (O—O, 2·82 Å).



XX

7. *Molybdate and Tungstate*

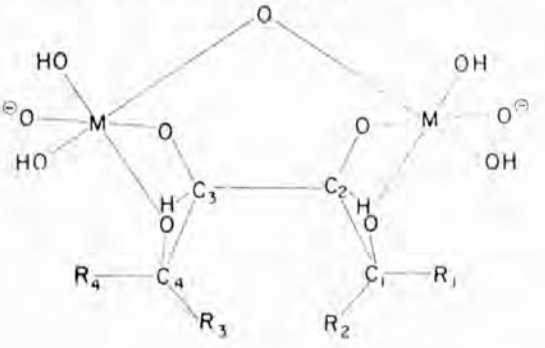
The specific rotation of several polyhydroxy compounds is markedly affected by the presence of molybdate[14–16] and tungstate[17] at acidic pH

values. The complexes formed are anionic and migrate during electrophoresis. The complexing species are the dimolybdate, $Mo_2O_7^{2-}$, and ditungstate ions, $W_2O_7^{2-}$ (XX; in this and the following structures M represents Mo or W), which, in complex formation, expand from tetrahedral to octahedral structures.[17]

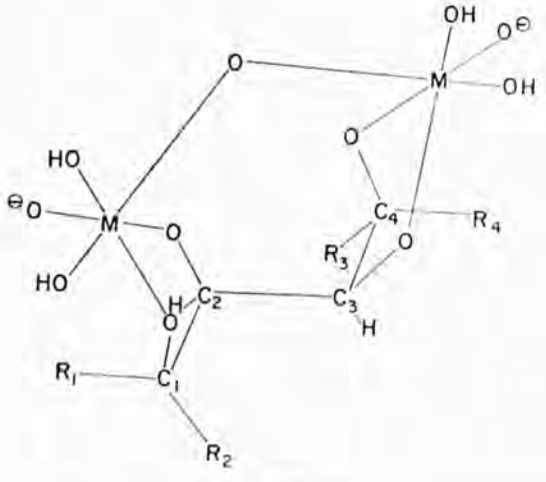
Acyclic polyhydroxy compounds possessing at least four adjacent hydroxyl groups afford complexes containing two of the metal atoms per molecule of polyhydroxy compound, thus:

$$R(OH)_4 + M_2O_7^{2-} \rightarrow [R \cdot M_2O_{11}H_4]^{2-} \qquad (1)$$

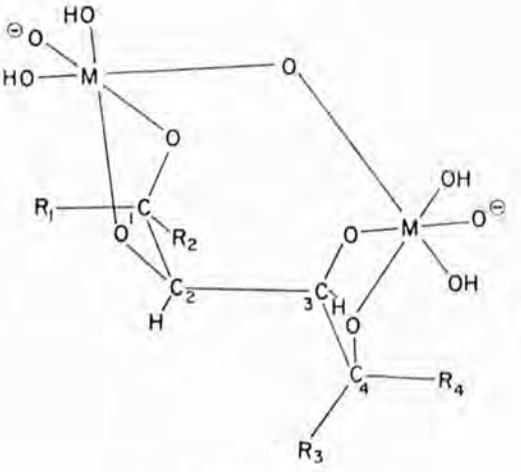
The structures XXI–XXIII have been postulated for these complexes,[17] XXI being formed from such a tetritol system where the hydroxyl groups on



XXI



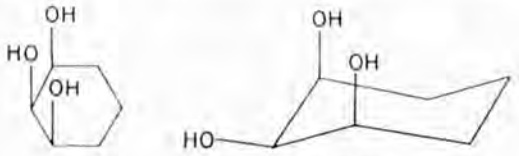
XXII



XXIII

C2 and C3 are in an *erythro* arrangement, whereas those in XXII and XXIII are in D-*threo* and L-*threo* arrangements, respectively. It is apparent that the sizes of the substituents R_2 and R_3 should influence the stability of the complex. It has indeed been found that the rate of migration during electrophoresis of acyclic polyhydroxy compounds of equal molecular size is influenced in this way.

Six-membered ring compounds complex with molybdate[18] and tungstate[17] only if they contain a *cis-cis*-1,2,3-triol system (XXIV a). Such compounds possess in at least one of their conformations one equatorial hydroxyl group neighboured by two axial hydroxyl groups (XXIV b). The

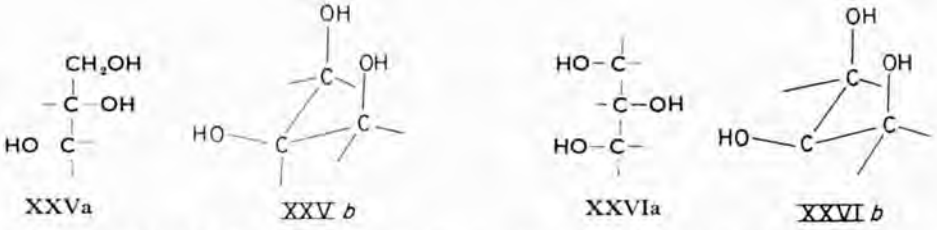


XXIV *a* XXIV *b*

complexes formed contain one molybdenum or tungsten atom per molecule of the polyhydroxy compound, that is:

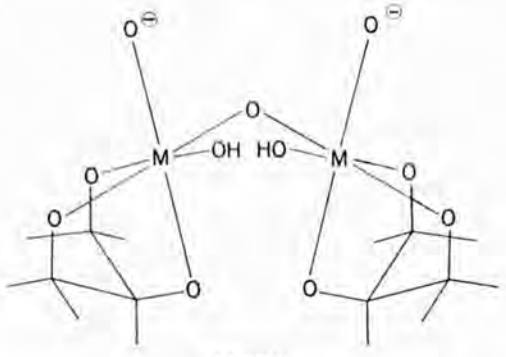
$$2R(OH)_3 + M_2O_7^{2-} \rightarrow [R_2 \cdot M_2 O_{11} H_2]^{2-} + 2H_2O \qquad (2)$$

The same type of complex is also produced from compounds possessing a triol system which can assume a spatial arrangement approximating to that of XXIV b, e.g. the hydroxyl groups on C1, C2, and C3 of a 4-substituted D-glucitol (XXV), and presumably those of a *xylo*-1,2,3-triol system



XXVa XXV *b* XXVIa XXVI *b*

(XXVI). The O—O distances in the octahedral molybdate and tungstate have been estimated to be *ca.* 2·59 and 2·57 Å, respectively, whereas those in the triol system XXIV b are 2·82 Å (O1–O2) and 2·51 Å (O1–O3). Thus



XXVII

the dimolybdate and ditungstate ions would fit well to give XXVII. Since XXVII contains two polyol components care has to be exercised in the interpretation of the elephoretic analysis of mixtures of polyhydroxy compounds which can give rise to this type of complex.

D-Gluco-, D-manno-, and D-galactopyranosides do not complex with either reagent[17, 18] as they lack the triol system XXIV. Thus the reduction products of disaccharides with D-glucose as the reducing end group will fall, during electrophoresis in molybdate or tungstate solutions, into three classes[15, 17] according to the position of the glycosidic linkage to the D-glucitol moiety: (a) 3-substituted D-glucitols, which do not complex; (b) 4-substituted D-glucitols, which form complexes according to eqn. (2); (c) 2-, 5- and 6-substituted D-glucitols, which form complexes according to eqn. (1). Table 5 shows that measurements of rates of migration of reducing disaccharides of D-glucose in borate solution[5, 19] and of their reduction products in molybdate solution[15, 17] will jointly determine the nature of the glucosidic linkage in the original disaccharide.

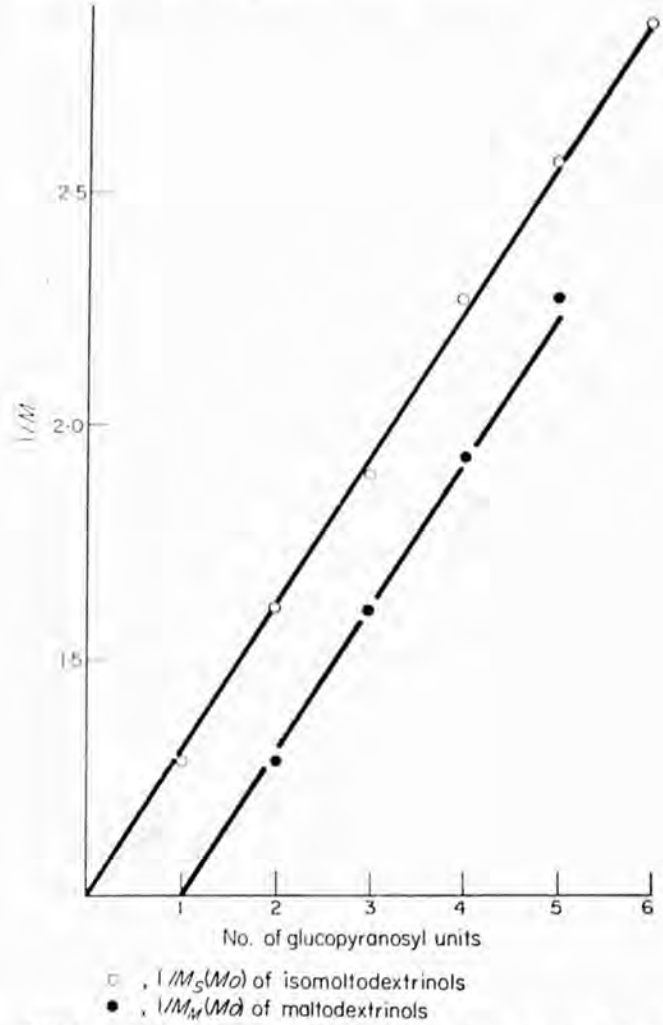


FIG. 2. Relative mobilities of isomaltodextrinol and maltodextrinol molybdate solutions.

Members of the isomaltodextrinol (reduced oligosaccharides of the isomaltose series) and maltodextrinol series (reduced oligosaccharides of the maltose series) can be effectively separated by paper electrophoresis in molybdate solution (Fig. 2). The method can be used for determination

of molecular size.[20] The paper electrophoretic mobilities of isomaltodextrinols can be expressed by

$$1/M_S\,(Mo) = 1 + 0{\cdot}3\,n, \tag{3}$$

where M_S is the mobility with respect to D-glucitol and n is the number of glucopyranosyl units present in the molecule. The mobilities of maltodextrinols are given by

$$1/M_M\,(Mo) = 1 + 0{\cdot}3\,(n - 1), \tag{4}$$

where maltitol is used as the reference compound.

ELECTROPHORETIC MOBILITIES

Table 2. Aldoses

Compound	*Na*[4]	*B*[5]	*PhB*[10]	*Ge*[11]	*Sn*[12]	*As*[4]	*Mo*[18]	*W*[17]
DL-Glycerose		79[2]			94		0	0
D-Erythrose					107		90	110[8]
L-Threose					103		60	5
D-Arabinose					84		0	0
L-Arabinose	79	76	240	150		30		
D-Lyxose	97	71[4]	230	190	115	42	110	104[8]
D-Ribose	100	77	470	210	104	100	40	20
D-Xylose	93	100	180	140	81	17	0	0
D-Allose	68	83[4]		180		75		
D-Altrose	96	97[4]	580			77	0	
D-Galactose	65	93	180	130	78	28	0	0
D-Glucose	80	100	100	100	63	16	0	0
D-Gulose	70	82[4]			107	53	110	110
L-Idose	96	102[4]				115		
D-Mannose	84	72	110	140	100	35	90[8]	110[8]
D-Talose	103	87[4]				119	70	

Table 3. Ketoses

Compound	*Na*[4]	*B*[5]	*PhB*[10]	*Ge*[11]	*Sn*[12]	*As*[4]	*Mo*[18]	*W*[17]
D-Fructose	89	90	930	210	91	75	50	25[8]
D-Psicose	125	76[4]				188		
L-Sorbose	88	95	850	200	94	73	30	20[8]
D-Tagatose	82	95[4]	860	240		103	105[17]	110[8]

The absolute mobilities of reference compounds (Table 1) will give an indication of the time required for the resolution of a particular mixture of compounds.

Tables 2 to 5 give the relative mobilities ($10^2 \times M_R\,(E)$) of carbohydrates and acyclic polyhydroxy compounds. The symbols used for the various

TABLE 4. ACYYLIC POLYHYDROXY COMPOUNDS

Compound	$Na^{(4)}$	$B^{(4)}$	$PhB^{(10)}$	$Ge^{(11)}$	$Sn^{(12)}$	$As^{(4)}$	$Mo^{(15)}$	$W^{(17)}$
Glyceritol	0	49	0	40	23	24	0	0
Erythritol	3	75	10	100	57	53	100	90^{E}
L-Threitol	3	75	30		62	96	50	24
D-Arabinitol	7	87			95	124	110	104
Ribitol	10	85	30	120	72	76	110	103
Xylitol	3	79	90	170	100	155	110	104
Allitol	23	90			88	92	94^{17}	97^{E}
D-Altritol	16	89			95	138	99^{17}	97^{E}
Galactitol	8	97	100	210	99	145	100	100
D-Glucitol	11	83	130	190	100	161	100	100
L-Iditol	7	81	173					
D-Mannitol	12	81	100	190	93	130	100	100

TABLE 5. D-GLUCOPYRANOSYL-D-GLUCOSES AND D-GLUCOPYRANOSYL-D-GLUCITOLS

D-Glucopyranosyl-D-glucose		D-Glucopyranosyl-D-glucitol	
Linkage	$10^2 \times M_G$ $(B)^{(5)}$	Linkage	$10^2 \times M_S$ $(Mo)^{(17, 18)}$
β—1 → 6	75	α—1 → 6	78
α—1 → 6	69	β—1 → 6	69
α—1 → 3	69	α—1 → 5	72
β—1 → 3	69	α—1 → 2	69
		β—1 → 2	70
α—1 → 4	32		
β—1 → 4	23	α—1 → 4	46
α—1 → 2	32^{19}	β—1 → 4	37
β—1 → 2	24		
		α—1 → 3	0
		β—1 → 3	0

electrolytes are found in Table 1. The references are given in the heading to each column, unless otherwise stated. The letters *E* and *S* indicate elongated spots and extensive streaking, respectively.

Two reference compounds have been used for paper electrophoresis in solutions containing sulpho-benzeneboronic acid. The mobility values quoted in Tables 2 and 3 are with reference to D-glucose and those in Table 4 are with reference to D-mannitol.

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303. *Partial Periodate Oxidation of* D-*Glucitol and its Borate Complex.*

By D. H. Hutson and H. Weigel.

The products of oxidation of D-glucitol with a limited quantity of periodate have been characterised and determined. The results show that the order of susceptibility of the C–C bonds in D-glucitol to cleavage by periodate is: 3,4 (αT-glycol) > 2,3 (αT-glycol) > 4,5 (αC-glycol) > 5,6 (α-glycol) > 1,2 (α-glycol). In borate buffer the favoured complex between D-glucitol and borate involves the participation of the 2- and the 4-hydroxyl group. Under suitable conditions the glucitol–borate complex produces *ca.* 30% of L-xylose.

It has been shown [1] that the oxidation of D-mannitol, galactitol, and D-glucitol with a limited quantity of sodium periodate involves preferential attack on αT-glycol groups (Barker and Bourne's nomenclature [2]). Similar oxidation of erythritol showed that a $CH(OH)\cdot CH_2\cdot OH$ group was more readily cleaved than a $CH(OH)\cdot CH(OH)$ group, but the reverse was found for hexitols.[3] Of acyclic compounds the *threo*- (αT) was oxidised more rapidly than the *erythro*-isomer (αC) and for *threo*-compounds the rate of oxidation decreases with increasing length of the substituents on the glycol group.[4] Thus it became interesting to study quantitatively the relative ease of cleavage of the various C–C bonds of D-glucitol with periodate, particularly as D-glucitol contains two αT- and α-glycol groups, and one αC-glycol group. It was further thought that limited oxidation of D-glucitol in water and in phosphate (pH 10) and borate buffer (pH 10) would clarify the structure of the D-glucitol–borate complex. Buffer of pH 10 was chosen as the concentration of the complexes between polyhydroxy-compounds and borate is greatest in alkali.[5] Characterisation and determination of the products of oxidation were aided by the use of ^{14}C-tracer techniques.

D-Glucitol was separately oxidised in water and in 0·5M-phosphate (pH 10) and 0·5M-borate buffer (pH 10) with 0·25 mol. of sodium periodate. The molar ratio of borate or phosphate to D-glucitol was 3. The products expected from the cleavage of the various C–C bonds were disclosed by chromatographic, ionophoretic, and colorimetric methods. The results shown in Table 1 indicate that 97—100% of the carbon was accounted for.

When oxidised in water, 75% of the D-glucitol remained unchanged and less than 0·1% of the carbon appeared as formic acid (Table 1). As the amount of periodate taken was only 5% of that needed for the complete oxidation of D-glucitol it is likely that the other products arose almost exclusively by direct oxidation of D-glucitol rather than by further oxidation of primary products. It is thus possible to assess the susceptibilities of the various glycol groups of D-glucitol to periodate by calculating the relative amounts of periodate used for the production of the corresponding products.

Table 2 shows that the susceptibilities of the various glycol groups of D-glucitol to periodate fall in the following order: αT > αC > α. Of the two αT- and two α-glycol groups, the 3,4- and the 5,6-group, respectively, are cleaved more readily.

Various mechanisms for the oxidation of glycols by periodate and structures of the intermediate complex, compound or ion, have been suggested. The generally accepted theory [6] is that a cyclic ester intermediate, which may be neutral or mono- or di-negatively charged, is formed from the glycol and H_5IO_6 or its dissociation products.* Buist, Bunton,

* [Added 30.11.60.] Since this paper was submitted, Keen and Symons (*Proc. Chem. Soc.*, 1960, 383) have found that ions such as $H_4IO_6^-$ or, possibly, $H_2IO_5^-$ are the major component of saturated aqueous solutions of sodium periodate.

and Miles [7] suggested the formation of an intermediate with a puckered five-atom ring in which the iodine atom is octahedral. They discussed the effect of the stereochemistry and electronic factors of this cyclic intermediate on the equilibrium constant for the formation of the intermediate and the rate constant for the decomposition to products. None of the proposed structures has however been proved. Nor do our results assist towards this or the reaction mechanism; but they are best understood by assuming a planar zig-zag conformation with large substituents in staggered positions,[8] the glycol group being attacked by H_5IO_6 or its dissociation products to form an intermediate with a puckered five-atom ring in which the iodine atom is octahedral.[7]

The O–O distance [9] in crystalline ammonium trihydrogen paraperiodate, $(NH_4)_2H_3IO_6$, in which the iodine is octahedral, is 2·73 Å. This is close to the calculated distance (2·83 Å) for α- and αT-glycol groups in which the large substituents on adjacent carbon atoms are fully staggered.[10] Thus, α- and αT-glycol groups can form with H_5IO_6 cyclic ester intermediates (I and II, respectively) with almost strainless five-atom rings and

TABLE 1. *Oxidation of* D-*glucitol with* 0·25 *mol. of sodium periodate in water and in borate and phosphate buffers* (pH 10).

	In water		In borate		In phosphate	
	Yield		Yield		Yield	
Product	(mol.)	C (%)	(mol.)	C (%)	(mol.)	C (%)
(D-Glucitol)	0·750	75·00	0·912	91·20	0·898	89·80
D-Arabinose	0·005	0·42	0·008	0·66	0·005	0·42
L-Xylose	0·009	0·75	0·021	1·75	0·011	0·92
D-Erythrose	0·047	3·13	0·003	2·00	0·008	0·53
L-Threose	0·015	1·00	0·001	0·07		
DL-Glyceraldehyde	0·269	13·45	0·015	0·75	0·037	1·86
Glycolaldehyde	0·085	2·83	0·072	2·40	0·082	2·73
Formaldehyde	0·015	0·25	0·016	0·27	0·012	0·20
Formic acid	0·005	0·08	0·042	0·70	0·078	1·30
Total		96·91		99·80		97·76

TABLE 2. *Percentage of periodate* (0·25 *mol.*) *consumed by the various* C–C *bonds of* D-*glucitol in water.*

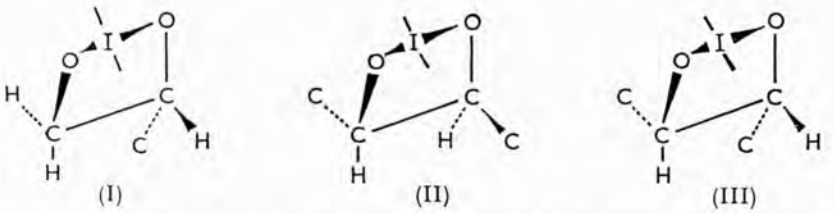
C–C Bond	Type of glycol	Based on yield of:	IO_4^- used (%)	C–C Bond	Type of glycol	Based on yield of:	IO_4^- used (%)
1,2	α	D-Arabinose	2·0	4,5	αC	L-Threose	6·0
5,6	α	L-Xylose	3·6	2,3; 4,5	αT, αC	Glycollaldehyde	34·0
1,2; 5,6	α, α	Formaldehyde	6·0	3,4	αT	Glyceraldehyde	53·8
2,3	αT	D-Erythrose	18·8	Oxidation of products		Formic acid	2·0

without significant distortion of the planar zig-zag conformation of the carbon chain. αC-Glycol groups, the calculated O–O distance [10] of which is 3·68 Å, can form a cyclic intermediate (III) only after considerable distortion of the carbon chain from the planar zig-zag conformation. As the non-bonded interaction in the three cyclic intermediates is (I) < (II) < (III) the order of susceptibility to cleavage by periodate should be α- > αT- > αC-glycol groups.[11] However, α-glycol groups of D-glucitol are least readily attacked by periodate. Whereas the oxygen atoms of αT-glycol groups seem to be already in a position required for the formation of the cyclic ester intermediate, owing to the almost unhindered rotation of the hydroxymethyl groups about the C–C bonds, only a fraction, f, of the molecules will have the oxygen atoms of α-glycol groups in this position and an entropy term, $\boldsymbol{R}T \ln 1/f$, has to be added to the free-energy change involved in the reaction. This could make α-glycol groups less susceptible to oxidation by periodate than αT-glycol groups of D-glucitol. For αC-glycol groups, a compression energy term has to be added to the free-energy change of the reaction. It is possible that the difference between these two terms makes the α-glycol groups of D-glucitol least readily attacked by periodate.

The difference in ease of cleavage of the two αT-glycol groups can be due to the stereochemistry of the ester intermediates. In (IV) and (V) the hypothetical intermediates

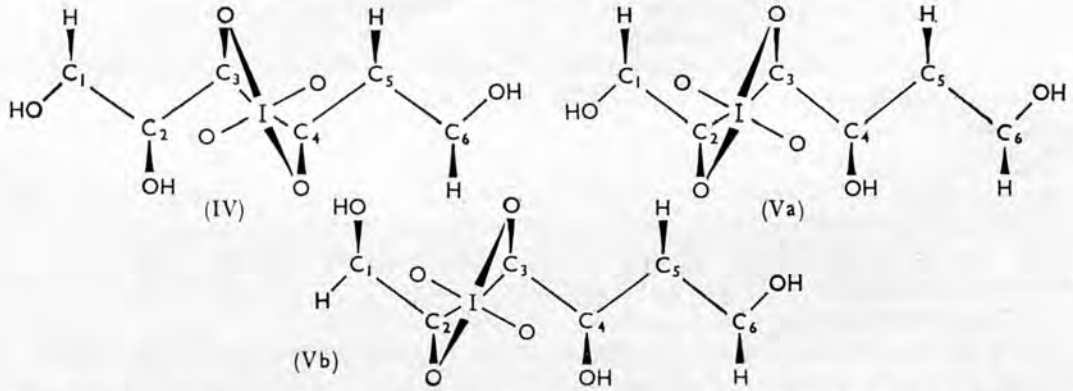
involving $C_{(3)}$–$C_{(4)}$ and $C_{(2)}$–$C_{(3)}$, respectively, are depicted with a puckered five-atom ring in which the iodine atom is octahedral. In (IV) and (Va) the 1- and 6-hydroxyl groups extend the planar zig-zag conformation of the carbon chain: in both cases a hydroxyl group and a hydrogen atom, $HO_{(2)}$, and $H_{(5)}$, $HO_{(4)}$ and $H_{(1)}$, respectively, lie close to two of the oxygen atoms attached to the iodine atom. The situation remains the same when the hydroxymethyl groups of (IV) rotate freely. However, a fraction of the molecules of (V) will have the 1-hydroxyl group in a position as shown in (Vb), in which two hydroxyl groups, $HO_{(1)}$ and $HO_{(4)}$, lie close to two of the oxygen atoms attached to the iodine atom. The resulting greater steric compression could make the αT-glycol group involving $C_{(2)}$ and $C_{(3)}$ less susceptible to oxidation by periodate than that involving $C_{(3)}$ and $C_{(4)}$.

Since it is established that αT-glycol groups are more readily oxidised by periodate than αC-glycol groups it is reasonable to assume that, in a statistical sense, the 5-hydroxyl group is more readily available for the formation of an intermediate involving $C_{(5)}$ and



(I) (II) (III)

Four of the oxygen atoms attached to the iodine atom are omitted. Two of these lie towards the observer in positions equivalent to the oxygen atoms attached also to the carbon atoms.



(IV) (Va) (Vb)

Atoms lying below the plane of the carbon atoms and two of the oxygen atoms attached to the iodine atom are omitted. The latter lie towards the observer in positions equivalent to O_3, O_4 and O_2, O_3, respectively.

$C_{(6)}$ than the 2-hydroxyl group for the formation of an intermediate involving $C_{(1)}$ and $C_{(2)}$. Thus, of the two α-glycol groups, the 5,6-bond is more readily cleaved than the 1,2-bond.

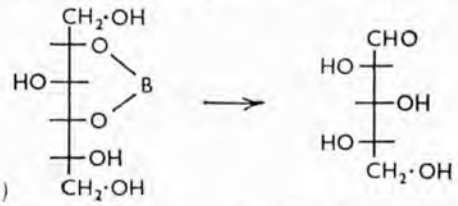
It was now possible to examine the effect of borate ions on the reaction. From the sequence of M_G values of substituted aldoses in borate solution at pH 10, Foster [5] concluded that the *aldehydo*-form of D-glucose is the principal one involved in complex formation and the pair of the 2- and the 4-hydroxyl group are sterically most favourable for complex formation. This, and the relative stabilities of cyclic acetals of D-glucitol,[10] suggested that a similar complex is favoured for D-glucitol. Partial periodate oxidation of such a complex (VI) should yield a larger quantity of L-xylose (VII) than a reaction in the absence of borate.

There was a possibility of anomalous reactions at pH 10.[7] Certain discrepancies have been found in the periodate oxidation in phosphate buffer at pH 7·5.[12] It was thought that similar discrepancies might arise with a borate buffer and that comparison between

the oxidation of D-glucitol in 0·5M-borate (pH 10) and 0·5M-phosphate buffer (pH 10) would be valid.

Oxidation of D-glucitol with 0·25 mol. of sodium periodate in the presence of borate (pH 10) or phosphate (pH 10) yielded very different results from those obtained with the unbuffered solution (Table 1). Only *ca.* 10% of the D-glucitol was oxidised in the alkaline solutions whereas the theoretical amount of 25% was oxidised in the unbuffered solution. Extensive secondary reactions (cf. formic acid production) under the alkaline conditions made detailed comparison with the unbuffered solution impossible.

The ratios of the yields of L-xylose to D-arabinose (primary reactions) in the unbuffered, borate-, and phosphate-buffered solutions are 1·8, 2·6, and 2·2, respectively. The ratios of the yield of L-xylose in borate- and phosphate-buffered solutions relative to that in the unbuffered solution are 2·3 and 1·2, respectively. This indicated that the yield of L-xylose could be significantly increased by the presence of borate ions. Accordingly, D-glucitol dissolved in 4M-borate buffer (pH 10·7) (borate to D-glucitol ratio 5 : 1) was oxidised with 2·5 mol. of sodium periodate. The component which had been characterised by carrier



(VI) (VII)

dilution analysis as L-xylose was the major product. This was separated by paper chromatography and determined quantitatively. It was found that the yield of L-xylose could be raised in this way to *ca.* 30%.

The results indicate that D-glucitol forms a complex with borate ions similar to that of D-glucose,[5] *i.e.*, participation of $HO_{(2)}$ and $HO_{(4)}$. However, a complex involving $HO_{(1)}$ and $HO_{(4)}$ is not excluded as the resulting 7-atom ring could have the 2- and the 3-hydroxyl group in *trans*-relation, when they would react with periodate more slowly than a $CH(OH)\cdot CH_2\cdot OH$ group.[13] On the other hand, it has been shown [14] that borate ions have no tendency to form complexes involving 7-atom or larger rings. The results clearly establish that the principal site of attack of periodate on the D-glucitol–borate complex is the 5,6-bond. The pair of hydroxyl groups involved in the formation of the complex are probably those on $C_{(2)}$ and $C_{(4)}$.

It is of interest that complex-formation with boric acid has been used similarly to effect selective substitution in D-glucose,[15,16] D-mannitol,[15,17] D-glucose diethyl mercaptal,[15] and methyl α- and β-glucopyranoside,[18] and to block the oxidation of carbohydrates in tissues by lead tetra-acetate.[19]

Experimental

Materials.—D-[^{14}C]Glucitol, uniformly labelled, was obtained from the Radiochemical Centre, Amersham. Glycollaldehyde was prepared as described by Powers *et al.*[20]

Chromatography.—The solvents used in chromatography were (*a*) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 2); (*b*) ethyl acetate–acetic acid–water (9 : 2 : 2); (*c*) methyl ethyl ketone saturated with water; [1] (*d*) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase).

Determination of Radioactivity.—Radioactivities were determined after conversion of the compound into carbon dioxide, and thence into barium carbonate.[21] The amount used was sufficient to give a thickness of greater than 20 mg. per cm.^{2}. The β-emission of a radioactive specimen was measured by means of a Geiger–Müller end-window counter and for times sufficient to give a standard counting error of less than ±2%.

Periodate Oxidations.—To mixtures of 0·166M-aqueous D-glucitol (1 mol.) with 0·5M-borate buffer (pH 10·6; 3 mol.) or 0·5M-phosphate buffer (pH 10·1; 3 mol.) (final pH 10·0) was added standard sodium periodate solution (0·25 mol.). After 10 min., Amberlite IR-120(H^+) was stirred into the buffered solutions to adjust the pH to 5.

Identification of Products.—D-Glucitol, D-arabinose, L-xylose, and DL-glyceraldehyde were identified by paper chromatography in solvents (*a*) and (*b*) and by treatment of the chromatograms with acetone–silver nitrate–alcoholic sodium hydroxide [22] or *p*-anisidine hydrochloride in butanol.[23] D-Erythrose and L-threose were separated from other products by paper chromatography in solvent (*c*),[1] resolved, and identified by paper ionophoresis in molybdate solution.[24] Glycollaldehyde was shown to be present by treatment with diphenylamine in acetic acid and measurement of absorption at 680 mμ.[25] Formaldehyde was shown to be present by the chromotropic acid method.[26] Formic acid was assumed to constitute the total titratable acid present.

Characterisation and Determination of Products.—(i) *D-Glucitol, D-arabinose, and L-xylose.* D-[^{14}C]Glucitol was separately oxidised with sodium periodate (0·25 mol.) in water, 0·5M-borate buffer (pH 10), and 0·5M-phosphate buffer (pH 10) as described above. A carrier compound (D-glucitol, D-arabinose, or L-xylose) was dissolved in each solution and allowed to equilibrate overnight. Boric acid was removed by repeated distillation with methanol, and phosphoric acid by precipitation with aqueous barium hydroxide. The products were separated by chromatography on Whatman paper no. 3 in solvent (*a*). D-[^{14}C]Glucitol was converted into the hexa-acetate which was recrystallised from ethanol until consecutive samples possessed constant m. p. and specific radioactivity. D-Arabinose and L-xylose were converted into their phenylosazones which were recrystallised twice from water and twice from benzene, after which consecutive samples possessed constant m. p. and specific radioactivity. The details of the analysis are shown in Table 3.

TABLE 3. *Carrier dilution analysis of products from oxidation of* D-[^{14}C]*glucitol with* 0·25 *mol. of sodium periodate.*

W_G (mg.)	S_o (μc per g.-atom of carbon)	Product	Medium	W_c (mg.)	M. p. of deriv.	S_i (μc per g.-atom of carbon)	Yield (mol.)
72	3164	(D-Glucitol)	Water	250	99°	561·90	0·750
72	3164	D-Arabinose	Water	254	163	3·43	0·005
72	3164	L-Xylose	Water	341·4	164	5·03	0·009
72	3164	D-Erythrose *	Water	242·3	185	29·40	0·047
72	3164	DL-Glyceraldehyde †	Water	254	72	117·52	0·269
72	3164	(D-Glucitol)	Borate	247	99	664·40	0·912
72	3164	D-Arabinose	Borate	233	163	6·14	0·008
72	3164	L-Xylose	Borate	226	164	17·54	0·021
72	3164	D-Erythrose *	Borate	256	185	1·77	0·003
72	3164	DL-Glyceraldehyde †	Borate	242·3	72	7·06	0·015
400	821	(D-Glucitol)	Phosphate	724·7	98	272·00	0·898
72	3164	D-Arabinose	Phosphate	275·1	163	3·24	0·005
72	3164	L-Xylose	Phosphate	195·5	164	10·25	0·011
72	3164	D-Erythrose *	Phosphate	255·3	185	4·71	0·008
72	3164	DL-Glyceraldehyde †	Phosphate	242·3	72	17·66	0·037

W_G = Weight of D-[^{14}C]glucitol oxidised. S_o = spec. radioactivity of D-[^{14}C]glucitol. W_c = weight of carrier added. S_i = spec. radioactivity of parent compound of isolated derivative.
* Carrier compound was erythritol. † Carrier compound was glycerol.

(ii) *D-Erythrose and DL-glyceraldehyde.* D-[^{14}C]Glucitol samples were oxidised with sodium periodate (0·25 mol.) as described above. The solutions were treated with potassium borohydride at room temperature for 12 hr. and then with Amberlite IR-120(H^+). Erythritol and glycerol were added as carrier compounds. After equilibration for 1 hr. boric acid was removed by repeated distillation with methanol. [^{14}C]Erythritol and [^{14}C]glycerol were separated by chromatography on Whatman paper no. 3 in solvent (*d*) and converted into the tetra- and tri-benzoate, respectively, which were recrystallised from aqueous pyridine and aqueous ethanol, respectively, until consecutive samples possessed constant m. p. and specific radioactivity. The details of the analysis are shown in Table 3.

(iii) *L-Threose.* D-[^{14}C]Glucitol (20 mg.) was separately oxidised with sodium periodate (0·25 mol.) in water and 0·5M-borate buffer (pH 10) as described above. The borate-buffered solution was treated with Amberlite IR-120(H^+), and the boric acid removed as above. L-[^{14}C]Threose and D-[^{14}C]erythrose were separated by paper chromatography in solvent (*c*). The eluted tetroses were subjected to ionophoresis in molybdate solution (pH 5).[24] *p*-Anisidine

hydrochloride in butanol and ultraviolet light [23] revealed the positions of the tetroses on the ionophoretograms. From a comparison of the β-emission of the two spots the ratio of the yields of L-threose and D-erythrose was found. The yield of L-threose was calculated from the results of the determination of D-erythrose. The results are shown in Table 4.

(iv) *Glycollaldehyde.* The results of Dische and Borenfreund [25] show that glycollaldehyde, after treatment with diphenylamine in acetic acid, can be determined by measurement of the optical density at 680 mμ and that the presence of glyceraldehyde will cause an error not greater than *ca.* 3%. Measurement of the optical density of standard solutions of glycollaldehyde (0·1%) containing also D-erythrose, formate, phosphate, borate, and iodate showed that only the latter interfered seriously with the determination of glycollaldehyde. Iodate was thus removed from the unbuffered and phosphate-buffered reaction mixtures by treatment with Amberlite IRA-400(OAc). To avoid possible loss of glycollaldehyde by adsorption on the resin as the glycollaldehyde–borate complex, the borate-buffered reaction mixture was treated with much glucitol, with which borate reacts preferentially, before treatment with the resin.

D-Glucitol (0·91 g.) was oxidised as described above. After removal of the iodate, glycollaldehyde was determined colorimetrically.[25] The measurement of the optical densities at 680 mμ (Ilford filter no. 608) of the solutions obtained from the unbuffered and borate- and phosphate-buffered mixtures corresponded to yields of 0·085, 0·072, and 0·082 mole of glycollaldehyde per mole of D-glucitol, respectively.

TABLE 4. *Determination of* L-*threose from oxidation of* D-[^{14}C]*glucitol with* 0·25 *mol. of periodate.*

Product	Medium	Radioactivity (counts per min.)	Ratio, L-threose : D-erythrose	Yield of L-threose * (mol.)
L-Threose	Water	216	0·31	0·015
D-Erythrose	Water	693		
L-Threose	Borate (pH 10)	50	0·23	0·001
D-Erythrose	Borate (pH 10)	222		

* Based on yield of D-erythrose.

(v) *Formaldehyde.* D-Glucitol (0·91 g.) was oxidised as described above. After removal by steam-distillation from the reaction mixtures, formaldehyde was determined by the chromotropic acid method.[26] Measurement of the optical densities (Ilford filter no. 606) of the solutions obtained from the unbuffered and borate- and phosphate-buffered mixtures corresponded to yields of 0·015, 0·016, and 0·012 mole respectively of formaldehyde per mole of D-glucitol.

(vi) *Formic acid.* D-Glucitol (0·91 g.) was separately oxidised as described above. Formic acid was determined by titration with 0·01N-sodium hydroxide, a pH-meter being used for end-point detection. A blank titre was found after oxidation of ethylene glycol with 0·125 mol. of sodium periodate. From the buffered solutions the formic acid was first separated by acidification and distillation of the reaction mixture. True titres: 2·5 ml. (water), 21·0 ml. (borate buffer), 39·0 ml. (phosphate buffer). These correspond to yields of 0·005, 0·042, and 0·078 mole, respectively, of formic acid per mole of D-glucitol.

Determination of L-*Xylose from Oxidation of* D-*Glucitol in* 4M-*Borate Buffer* (5 *Mol.*) *with* 2·5 *Mol. of Sodium Periodate.*—To a solution of D-glucitol (1·82 g.) in 4M-borate buffer (pH 10·7) (12·5 ml.) was added a solution of sodium periodate (5·45 g.) in water (25 ml.). After 10 min. sodium ions and boric acid were removed by treatment with Amberlite IR-120(H^+) and repeated distillation with methanol, respectively. The residue was extracted with methanol. Paper chromatography in solvent (*a*) showed that the major component had an R_F value identical with that which had been characterised by carrier dilution analysis as L-xylose. Elution and quantitative determination of this component by the anthrone method [27] showed a 30·6% yield.

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39.

PAPER CHROMATOGRAPHY OF CARBOHYDRATES AND RELATED COMPOUNDS IN THE PRESENCE OF BENZENEBORONIC ACID

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(Received October 18th, 1962)

The well-known reaction of polyhydroxy-compounds with borate ions to form anionic complexes has been used extensively for the separation of carbohydrates and related compounds by paper electrophoresis in borate solution[1] and chromatography on columns of anion exchange resins[2] and charcoal[3]. The presence of boric acid has also been shown to affect the paper chromatographic behaviour of carbohydrates[4], the increase or decrease in R_F value being dependent on the pH of the solvent[5]. We now report the paper chromatographic behaviour of carbohydrates and related compounds in the presence of benzeneboronic acid.

EXPERIMENTAL

Solvents

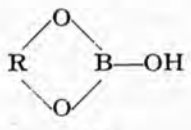
The solvents used for descending chromatography on Whatman No. 1 paper were (a) ethyl acetate–acetic acid–water (9:2:2 v/v) and (b) 0.55 % solution of benzeneboronic acid in ethyl acetate–acetic acid–water (9:2:2 v/v). The solvent front moves about 30 cm in 4–5 h.

Spray reagent

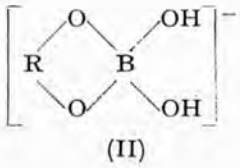
The compounds were detected on paper chromatograms with potassium periodatocuprate and rosaniline[6].

RESULTS AND DISCUSSION

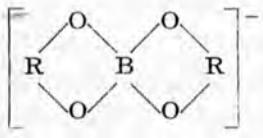
The products of the reactions between boric acid or borate ions and polyhydroxy-compounds have structures of the types I–IV.



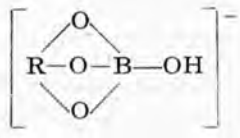
(I)



(II)



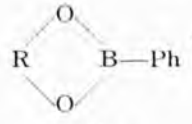
(III)



(IV)

Since boric acid, $B(OH)_3$, does not act as a proton donor but a Lewis acid[7], accepting the electron pair of the base, *e.g.* OH^-, to form the anion $B(OH)_4^-$, the compounds formed at acidic pH values are neutral esters (I) whereas those formed under alkaline conditions are anionic complexes (II–IV). Compounds with structure I should have higher R_F values in solvents with a stationary aqueous phase than those with structures II–IV. This is indeed confirmed by the chromatographic behaviour of D-glucitol in solvents containing (i) boric acid and acetic acid (R_G 2.2; movement with respect to glucose) and (ii) boric acid and pyridine (R_G 0.3)[5].

Benzeneboronic acid, $Ph \cdot B(OH)_2$, is known to react with several polyhydroxy-compounds to give esters with structure V[8–10]. The detailed structures of some of these have been elucidated[11,12].



(V)

The replacement of the hydroxyl group of I by a phenyl group should increase the affinity of the ester for the organic solvent and thus result in an increase in R_F value. The results (Table I) show that this is indeed the case; in solvent (b), in which the boric acid has been replaced by benzeneboronic acid, D-glucitol moves with an R_G value of 5.6.

TABLE I

R_F VALUES OF CARBOHYDRATES AND RELATED COMPOUNDS IN SOLVENTS (a) AND (b)

Compound	*R_F value*	
	Solvent (a)	*Solvent (b)*
Glycerol	0.32	0.35
Erythritol	0.23	0.31
D-Arabitol	0.14	0.50
1-deoxy-	0.45	0.71
5-deoxy-	0.46	0.85
Ribitol	0.14	0.48
2-deoxy-D-	0.32	0.46
Xylitol	0.14	0.45
Allitol	0.17	0.49
D-Altritol	0.16	0.51
1-deoxy-	0.36	0.85
1,6-dideoxy-	0.57	0.97
Galactitol	0.07	0.47
1-deoxy-D-	0.31	0.68
1,6-dideoxy-	0.58	0.85
D-Glucitol	0.08	0.45
2-deoxy-	0.22	0.60
3-O-methyl-	0.19	0.44
4-O-methyl-	0.30	0.40
D-Mannitol	0.08	0.43
1,6-dideoxy-	0.58	0.96
2-O-methyl-	0.22	0.70
1,2-di-O-methyl-	0.46	0.82

(continued on p. 255)

TABLE I (*continued*)

Compound	*R_F value*	
	Solvent (a)	*Solvent (b)*
DL-Glycerose	0.38	0.40
D-Erythrose	0.31	0.84
L-Threose	0.31	0.53
D-Arabinose	0.12	0.11
D-Lyxose	0.18	0.18
D-Ribose	0.25	0.50
2-deoxy-	0.40	0.41
D-Xylose	0.15	0.15
D-Altrose		
1,6-anhydro-β-pyranose	0.20	0.19
D-Galactose	0.06	0.08
6-deoxy-	0.19	0.18
1,6-anhydro-β-pyranose	0.33	0.38
D-Glucose	0.08	0.08
3-O-methyl-	0.21	0.23
5-deoxy-	0.28	0.27
methyl α-pyranoside	0.20	0.21
1,6-anhydro-β-pyranose	0.33	0.31
D-Gulose	0.13	0.27
1,6-anhydro-β-pyranose	0.31	0.30
L-Idose	0.09	0.16
D-Mannose	0.08	0.09
6-deoxy-	0.22	0.25
3,4-di-O-methyl-	0.55	0.59
methyl α-pyranoside	0.42	0.42
1,6-anhydro-β-pyranose	0.33	0.39
D-Fructose	0.11	0.12
L-Sorbose	0.10	0.16
allo-Inositol	0.04	0.11
dextro-Inositol		
3-O-methyl-	0.09	0.08
epi-Inositol	0.01	0.04
levo-Inositol	0.03	0.02
2-O-methyl-	0.07	0.07
muco-Inositol	0.05	0.05
1-deoxy-	0.08	0.08
myo-Inositol	0.02	0.02
1-deoxy-	0.07	0.06
scyllo-Inositol	0	0

Table I shows the R_F values of some carbohydrates and related compounds in the solvent containing the benzeneboronic acid [solvent (b)]. In all cases comparison was made with a solvent from which benzeneboronic acid was omitted [solvent (a)]. It can be seen that a number of useful separations are obtained, *e.g.* most aldoses and ketoses are well separated from their reduction products within 4 to 5 hours.

The isolated benzeneboronates of many polyhydroxy-compounds are easily hydrolysed, even during chromatography, with a solvent containing water, *e.g.* solvent (a). In this solvent the benzeneboronic acid, which can be detected under U.V. light, moves almost with the solvent front and hence is easily separated from the polyhydroxy-compounds. Thus, solvent (b) offers an advantage for separations on a preparative scale. Normally, boric acid is removed from an eluate by repeated distillation with methanol. However, the benzeneboronic acid can be separated from the polyhydroxy-

compounds by re-chromatography of the eluate in solvent (a), avoiding any destruction of the polyhydroxy-compounds which might occur when boric acid is removed by repeated distillation with methanol.

It seems reasonable to assume that only compounds which have at least two hydroxyl groups in an appropriate spatial arrangement to react with benzeneboronic acid will have significantly higher R_F values in solvent (b) than in solvent (a). However, comparison of the R_F values in the two solvents cannot be regarded as a satisfactory method to detect such an arrangement in a compound, since *e.g.*, the R_F values of glycerol and D-glucose are not appreciably altered by the presence of benzeneboronic acid, although crystalline benzeneboronates of these have been obtained[11,12]. It is likely that, under the conditions of the chromatography, the equilibrium does not favour the formation of certain benzeneboronates, which will of course have differing relative stabilities according to ring size, substituents, etc. On the other hand, Table I shows that the aldoses and cyclitols, the R_F values of which are markedly affected by the presence of benzeneboronic acid, have in their more stable conformation a 1 (*ax*), 3 (*ax*)-diol grouping. *muco*-Inositol and 1,6-anhydro-β-D-glucopyranose also possess such a diol grouping, but as mentioned earlier the conditions of the chromatography might not favour the formation of their benzeneboronates. 2-Deoxy-D-ribose, the R_F value of which is the same in both solvents, possesses such a diol grouping only in the C1 conformation (REEVES' nomenclature)[13] of its α-anomer. It is not possible to decide which anomer and conformation of D-ribose reacts with benzeneboronic acid.

During the course of this work GAREGG AND LINDBERG[14] reported the paper electrophoretic behaviour of carbohydrates in solutions of sulphonated benzeneboronic acid. Presumably, under the conditions used, the esters formed migrate due to the ionisation of the sulphonic acid group.

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SUMMARY

The R_F values of a number of polyhydroxy-compounds are markedly increased by the addition of benzeneboronic acid to the solvent. The increase is due to the formation of esters between benzeneboronic acid and the polyhydroxy-compounds. For certain carbohydrates and cyclitols the increase has been related to their structures. Acyclic polyhydroxy-compounds have, in general, much higher R_F values in the solvent containing benzeneboronic acid than the aldoses or ketoses from which they derive. This provides a rapid method for the separation of pairs of such compounds.

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695. *Phenylboronates of Acyclic Polyhydroxy-compounds*

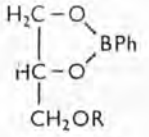
By E. J. Bourne, E. M. Lees, and H. Weigel

The preparation of phenylboronates of several acyclic polyhydroxy-compounds (and of D-glucose) is described. The derivatives of glycerol and galactitol have been shown to be the 1,2- and 1,3-4,6-diphenylboronate, respectively. Conformational aspects are discussed.

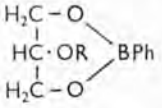
Phenylboronic acid reacts [1–5] with certain cyclic and acyclic diols and polyols to give cyclic phenylboronates. With the exception of phenylboronates of some glycosides,[4] detailed structures have not been assigned to the derivatives of polyols. Previously [6] we reported the effect of the presence of phenylboronic acid on the chromatographic behaviour of polyols. We now report the preparation of phenylboronates of some acyclic polyols (and D-glucose) and the structural analysis of the derivatives of galactitol and glycerol.

The properties of the phenylboronates are shown in Table 2. In the determination of the boron content of these compounds use was made of the absorption peak at 219 mμ of solutions of phenylboronic acid in aqueous methanol. Polymeric structures can be discarded on the basis of molecular-weight determinations made on the phenylboronates of glycerol, D-mannitol, and D-glucose.

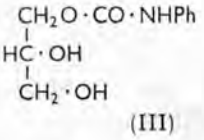
Glycerol Phenylboronate.—The two possible structures of a glycerol phenylboronate are (Ia) and (IIa). As in the case of galactitol bisphenylboronate (see below) it was not possible to effect satisfactory toluene-*p*-sulphonylation or methylation of the unsubstituted hydroxyl group. However, treatment with phenyl isocyanate gave an *O*-phenylcarbamoylglycerol phenylboronate (Ib or IIb). Its hydrolysis and the separation of the products (III or IV and phenylboronic acid) was effected by paper chromatography,[6] but the *O*-phenylcarbamoylglycerol, an oil, could not be distilled without decomposition.



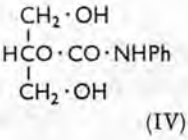
(Ia: R = H)
(Ib: R = CO·NHPh)



(IIa: R = H)
(IIb: R = CO·NHPh)



(III)



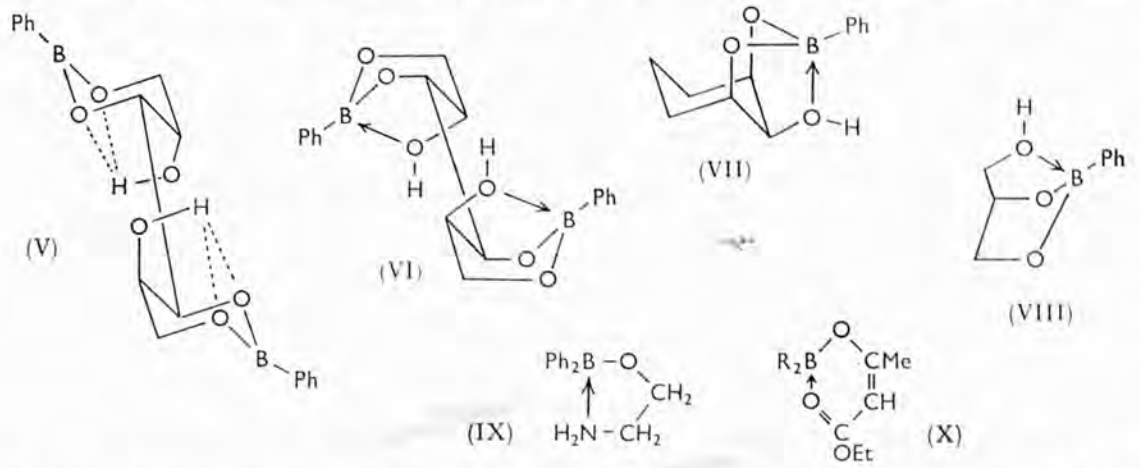
(IV)

In order to establish the structure of the *O*-phenylcarbamoylglycerol (III or IV) the mixture of *O*-phenylcarbamoylglycerol and phenylboronic acid, obtained by addition of water to a solution of the *O*-phenylcarbamoylglycerol phenylboronate (Ib or IIb) in dioxan, was treated directly with sodium periodate. The effect of the presence of phenylboronic acid was ascertained by periodate oxidation of glycerol phenylboronate, and phenylboronic anhydride $(PhBO)_3$. Under the same conditions *O*-phenylcarbamoylglycerol phenylboronate, glycerol phenylboronate, and phenylboronic anhydride consumed, respectively, 1·81, 2·82, and 2·40 mol. of periodate. Thus, the *O*-phenylcarbamoylglycerol consumed 1·0 mol. of periodate and in addition it produced 1·1 mol. of formaldehyde. These results are compatible with structure (III) and show that the glycerol phenylboronate was in fact the 1,2-phenylboronate (Ia). They also confirm the structure postulated by Bowie and Musgrave [7] on the grounds of relative rates of hydrolysis of cyclic phenylboronates with 5- and 6-membered heterocyclic rings.

Galactitol Bisphenylboronate.—By heating solutions containing varying molar proportions of galactitol and phenylboronic acid in anhydrous or aqueous acetone, Sugihara and Bowman obtained in each case a galactitol trisphenylboronate.[3] However, our method (method 1) always gave a bisphenylboronate, even when phenylboronic anhydride and galactitol were used in the molar ratio of 1 : 1.

As in the case of glycerol 1,2-phenylboronate, the galactitol bisphenylboronate could not be satisfactorily toluene-*p*-sulphonylated or methylated.

Benzoylation of galactitol bisphenylboronate, followed by treatment with an acetolysing mixture,[8] gave the known 1,3,4,6-tetra-*O*-acetyl-2,5-di-*O*-benzoylgalactitol. Treatment of galactitol bisphenylboronate with phenyl isocyanate gave a bis-*O*-phenylcarbamoyl-



galactitol bisphenylboronate which, on hydrolysis in aqueous dioxan, yielded a bis-*O*-phenylcarbamoylgalactitol. This compound proved to be not very suitable for studies with sodium periodate or lead tetra-acetate, because of its low solubility in water and its ready hydrolysis in glacial acetic acid.

For the above reason, and in view of the low yields of 1,3,4,6-tetra-*O*-acetyl-2,5-di-*O*-benzoylgalactitol and bis-*O*-phenylcarbamoylgalactitol bisphenylboronate, an alternative approach to elucidate the structure of the galactitol bisphenylboronate was the preparation of a bis-*O*-phenylcarbamoylgalactitol by an unambiguous route. Thus, 1,3-4,6-di-*O*-benzylidenegalactitol was converted into 1,3-4,6-di-*O*-benzylidene-2,5-bis-*O*-phenylcarbamoylgalactitol which, on hydrolysis, gave 2,5-bis-*O*-phenylcarbamoylgalactitol. This was identical with the bis-*O*-phenylcarbamoylgalactitol obtained from galactitol bisphenylboronate. The latter was thus galactitol 1,3-4,6-bisphenylboronate.

General Observations.—The above results show that reactions of acyclic polyols with phenylboronic anhydride yield both 5- and 6-membered ring systems. The galactitol 1,3-4,6-bisphenylboronate might have the conformation [(V); for clarity the $C_{(3)}$–$C_{(4)}$ bond is enlarged and C-bond H atoms are omitted] with intramolecular hydrogen bonding between the unsubstituted hydroxyl groups and the ring oxygen atoms. In this case, absorption at *ca.* 3580 cm.$^{-1}$ would be expected.[9,10] However, it exhibited only one sharp absorption band at 3636 cm.$^{-1}$, characteristic of a free hydroxyl group.[9] The complete absence of intramolecular hydrogen bonding suggests that the oxygen atoms on $C_{(2)}$ and $C_{(5)}$ co-ordinate with the corresponding boron atoms (VI). (The co-ordinating electron pair of the oxygen is not directly involved in the O–H bonding. Thus, the influence of this type of co-ordination on the O–H stretching frequency should be small.) This is supported by the fact that phenylboronic acid markedly affects the chromatographic mobility of only those six-membered cyclic polyhydroxy-compounds which possess a *cis-cis*-1,2,3-triol group.[6,11] Structure (VII) has been suggested as that being formed during the chromatographic process. It is evident that the hetero-ring systems in (VI) and (VII) are identical.

The glycerol 1,2-phenylboronate exhibited absorption bands at 3630 and 3597 cm.$^{-1}$, the ratio of intensities being *ca.* 1 : 2. The appreciable amount of intramolecular hydrogen bonding (3597 cm.$^{-1}$) probably arises in the same way as that in cyclohexane-1,2-diols, since the oxygen atom of the hydroxymethyl group and that on $C_{(2)}$ can be brought into the same spatial disposition as those in cyclohexane-1,2-diols. The smaller amount of

free hydroxyl absorption (3630 cm.$^{-1}$) may be due to (*a*) the ring strain inherent in forming a hydrogen bond from the extra-annular hydroxymethyl group and/or (*b*) co-ordination of the oxygen atom of the hydroxymethyl group with the boron atom, resulting in non-planarity of the phenylboronate ring (VIII).

Boron is strongly electrophilic by virtue of its tendency to fill the vacant orbital and complete the octet. Consequently, the properties of many boron compounds have been explained by postulating internal co-ordination of an electron-donating atom with boron, *e.g.*, 2-aminoethyl diphenylborinate (IX) [12] and 2-ethoxycarbonyl-1-methylvinyl di-*n*-butyl- and diphenyl-borinate (X, R = *n*-butyl or phenyl).[13] Such co-ordination would render the unsubstituted hydroxyl groups in galactitol 1,3-4,6-bisphenylboronate and glycerol 1,2-phenylboronate less reactive towards the reagents used (*i.e.*, methyl iodide, benzoyl chloride, toluene-*p*-sulphonyl chloride) than in the case of hydrogen bond formation. The failure or low yields of the reactions are thus compatible with structures (VI) and (VIII).

Experimental

Boron Analysis.—The plot of absorption at 219 mμ against concentration of solutions of phenylboronic acid (0·01—0·1mM) in 50% aqueous methanol gave a straight line. The graph was used to determine the quantity of boron (in the form of PhB<) in solutions of phenylboronates in 50% aqueous methanol.

Molecular Weights.—These were determined according to the method of Finch and Gardner [14] using benzene as solvent.

Preparation of Phenylboronates of Polyols.—The quantities used, the yields obtained, and properties of the phenylboronates are shown in Tables 1 and 2.

Table 1

Preparation of phenylboronates

		Phenylboronic anhydride		Polyol		
Parent polyol	Method	Weight (g.)	Solvent (ml.)	Weight (g.)	Solvent (ml.)	Yield (g.)
Glycerol	1	2·3	10 [a]	22	10 [b]	2·5
Erythritol	1	1·7	15 [a]	1·0	15 [b]	2·0
D-Arabinitol	1	0·69	5 [a]	0·50	5 [b]	0·90
Galactitol	1	5·0	25 [a]	4·4	150 [b]	7·0
D-Glucitol	1	8·6	25 [a]	5·0	25 [b]	13·0
2,4-*O*-benzylidene-	2	0·12		0·15	25 [c]	0·24
1,3-2,4-di-*O*-ethylidene-	2	0·09		0·20	25 [c]	0·27
D-Mannitol	1	8·6	25 [a]	5·0	25 [b]	13·0
1,6-di-*O*-benzoyl-	2	0·06		0·10	25 [c]	0·13
D-Glucose	1	5·8	20 [a]	5·0	20 [a]	7·0

[a] Methanol. [b] Water. [c] Acetone.

Table 2

Properties of phenylboronates

			M		C (%)		H (%)		B (%)	
Parent polyol	M. p.	Formula	Found	Reqd.	Found	Reqd.	Found	Reqd.	Found	Reqd.
Glycerol	74·5—76·5° *	$C_9H_{11}BO_3$	178	178·0	60·0	60·7	6·1	6·2	6·0	6·1
Erythritol	88	$C_{16}H_{16}B_2O_4$							7·4	7·4
D-Arabinitol	114—116	$C_{17}H_{18}B_2O_5$							6·7	6·7
Galactitol	125—130	$C_{18}H_{20}B_2O_6$			59·9	61·1	6·0	5·7	6·3	6·1
D-Glucitol	189	$C_{24}H_{23}B_3O_6$			62·8	65·5	4·8	5·3	7·4	7·4
2,4-*O*-benzylidene-	199 *	$C_{25}H_{24}B_2O_6$			67·5	67·9	5·0	5·5		
1,3-2,4-di-*O*-ethylidene-	88 *	$C_{16}H_{21}BO_6$			59·6	60·0	6·1	6·6	3·3	3·4
D-Mannitol	137 *	$C_{24}H_{23}B_3O_6$	440	439·9	62·9	65·5	4·9	5·3	7·4	7·4
1,6-di-*O*-benzoyl-	150 *	$C_{32}H_{28}B_2O_8$			67·9	68·4	4·9	5·0		
D-Glucose	166	$C_{18}H_{18}B_2O_6$	325—365	352·0	61·2	61·4	5·3	5·2	6·1	6·2

* Recrystallised from dry hexane.

The m. p. of the known phenylboronates, *i.e.*, of glycerol,[7] D-glucitol,[3] and D-mannitol,[3] agree with those reported in the literature.

Method 1. Phenylboronic anhydride in methanol was added to the polyol in water. After *ca.* 1 hr. the solid product was filtered off, washed with hot water and cold methanol, and dried.

Method 2. Phenylboronic anhydride and the polyol in acetone were refluxed for *ca.* 1 hr. The solvent was distilled off and the residue was recrystallised from dry hexane.

Paper Chromatography.—The solvent was butan-1-ol–ethanol–water (40 : 11 : 19). Under the conditions used the phenylboronates hydrolysed [2,6] to give the polyols, detectable with silver nitrate in acetone–alcoholic sodium hydroxide,[15] and phenylboronic acid, detectable under ultraviolet light.

Infrared Spectra.—These were measured in 4 cm. layers in CCl_4 solution on the Unicam S.P. 100 spectrometer using a 3000 l.p.i. grating. Concentration of compounds was $<0{\cdot}005$M.

Attempted Toluene-p-*sulphonylation of Galactitol Bisphenylboronate.*—Toluene-*p*-sulphonyl chloride (1·6 g.) was added to a solution of galactitol bisphenylboronate (1 g.) in dry pyridine (25 ml.) and kept at room temperature for 48 hr. Paper chromatography revealed components with R_F 0·18 (major, identical with galactitol), 0·37 (trace), and 0·55 (trace).

Benzoylation of Galactitol Bisphenylboronate.—Benzoyl chloride (0·66 ml.) was added to an ice-cooled solution of galactitol bisphenylboronate (1 g.) in dry pyridine (15 ml.), and the mixture kept overnight at room temperature. Pyridine was removed by distillation under reduced pressure. The syrupy residue was treated overnight with a mixture of acetic anhydride, glacial acetic acid, and concentrated sulphuric acid (35 : 15 : 1, vol./vol./vol.; 15 ml.) and then poured on ice. The solid was recrystallised from absolute ethanol to give 1,3,4,6-tetra-*O*-acetyl-2,5-di-*O*-benzoylgalactitol [8] (150 mg.), m. p. 156° (Found: C, 59·6; H, 5·7%. Calc. for $C_{28}H_{30}O_{12}$: C, 60·2; H, 5·4%). The infrared spectrum revealed the presence of carbonyl groups and the absence of hydroxyl groups.

2,5-*Bis*-O-*phenylcarbamoyl-galactitol* 1,3-4,6-*Bisphenylboronate.*—Phenyl isocyanate (3·5 ml.) was added to a suspension of galactitol bisphenylboronate (5 g.) in dry benzene (50 ml.). Heating gave a solution which was refluxed for 18 hr. The solid material obtained on cooling was recrystallised from benzene to give 2,5-*bis*-O-*phenylcarbamoylgalactitol* 1,3-4,6-*bisphenylboronate* (1 g.), m. p. 223—224° (Found: C, 64·7; H, 5·3; N, 4·8%. $C_{32}H_{30}B_2N_2O_8$ requires C, 64·9; H, 5·1; N, 4·7%).

2,5-*Bis*-O-*phenylcarbamoylgalactitol from* 2,5-*Bis*-O-*phenylcarbamoylgalactitol* 1,3-4,6-*Bisphenylboronate.*—Water (25 ml.) was added to a solution of 2,5-bis-*O*-phenylcarbamoylgalactitol 1,3-4,6-bisphenylboronate (0·5 g.) in dioxan (5 ml.). The *bis*-O-*phenylcarbamate* produced (0·27 g.) had m. p. 257° (Found: C, 57·8; H, 5·6; N, 6·6%. $C_{20}H_{22}N_2O_8$ requires C, 57·4; H, 5·3; N, 6·7%).

1,3-4,6-*Di*-O-*benzylidene*-2,5-*bis*-O-*phenylcarbamoylgalactitol.*—Phenyl isocyanate (3 ml.) was added to a solution of 1,3-4,6-di-*O*-benzylidenegalactitol [8] (5 g.) in dimethylformamide (50 ml.). Heating to 60° for 30 min. produced crystalline 1,3-4,6-*di*-O-*benzylidene*-2,5-*bis*-O-*phenylcarbamoylgalactitol* (5·5 g.), m. p. 343° (Found: C, 68·2; H, 5·3; N, 4·8%. $C_{34}H_{32}N_2O_8$ requires C, 68·4; H, 5·4; N, 4·7%).

2,5-*Bis*-O-*phenylcarbamoylgalactitol from* 1,3-4,6-*Di*-O-*benzylidene*-2,5-*bis*-O-*phenylcarbamoylgalactitol.*—A suspension of 1,3-4,6-di-*O*-benzylidene-2,5-bis-*O*-phenylcarbamoylgalactitol (5 g.) in dioxan (25 ml.) and water (250 ml.) was refluxed in the presence of Amberlite IR-120(H^+). After filtration the resin was washed with dioxan (50 ml.). 2,5-Bis-*O*-phenylcarbamoylgalactitol (0·48 g.) was deposited by addition of water (250 ml.) to the filtrate. It had m. p. 257°. Admixture with the material obtained from 2,5-bis-*O*-phenylcarbamoylgalactitol 1,3-4,6-bisphenylboronate caused no depression in m. p.

Attempted Toluene-p-*sulphonylation of Glycerol Phenylboronate.*—The procedure was that used for galactitol bisphenylboronate. Chromatography of the reaction mixture revealed glycerol as the only polyol component.

1-O-*Phenylcarbamoylglycerol* 2,3-*Phenylboronate.*—Phenyl isocyanate (3 ml.) was added to a solution of glycerol phenylboronate (5 g.) in dry benzene (100 ml.), and the whole refluxed for 6 hr. The residue obtained after evaporation was recrystallised from benzene–light petroleum (b. p. 60—80°) to give 1-O-*phenylcarbamoylglycerol phenylboronate* (5 g.), m. p. 117° (Found: C, 64·5; H, 5·3; N, 4·9%. $C_{16}H_{16}BNO_4$ requires C, 64·7; H, 5·4; N, 4·7%).

Periodate Oxidations.—Phenylboronic anhydride $(PhBO)_3$ (24·7 mg.), glycerol 1,2-phenylboronate (44·7 mg.), and 1-*O*-phenylcarbamoylglycerol 2,3-phenylboronate (52·3 mg.) were separately dissolved in dioxan (25 ml.). Water (65 ml.) and 0·1N-sodium periodate (10 ml.)

were added to each solution. After 28 hr. the periodate consumptions (mol.) were as follows: phenylboronic anhydride, 2·40; glycerol 1,2-phenylboronate, 2·82; 1-*O*-phenylcarbamoylglycerol 2,3-phenylboronate, 1·81. 1-*O*-Phenylcarbamoylglycerol 2,3-phenylboronate produced 1·10 mol. formaldehyde, estimated by the chromotropic acid method [16] and identified as its dimedone derivative. Under the same conditions ethyl *N*-phenylcarbamate did not consume periodate.[17]

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Synthesis of Deoxy-compounds from Carbohydrate Toluene-*p*-sulphonylhydrazones

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Toluene-*p*-sulphonylhydrazones (tosylhydrazones) of aldoses are, generally, obtained crystalline in high yields. The recent reports by Gaglioti *et al.*[1] that treatment of a tosylhydrazone with lithium aluminium hydride or sodium borohydride affords a conversion of the original carbonyl group into a methylene group prompted us to apply this reaction to tosylhydrazones of carbohydrates.

A solution of D-glucose tosylhydrazone[2] (5g.) and potassium borohydride (3g.) in methanol (150 ml.) was heated under reflux for *ca.* 12 hours. After destruction of the excess of borohydride and removal of potassium borate the reaction mixture was fractionated on a cellulose column using butan-1-ol saturated with water as eluant. The fraction containing a component which had $R_{GLUCOSE}$ 2·1 (butan-1-ol – ethanol – water, 40:11:19) and $M_S(Mo)$ 1·0[3] was evaporated to a syrup. Trituration with ethanol afforded crystalline 1-deoxy-D-glucitol (I) (1·0g.), m.p. 128–129° (from ethyl acetate). Acetylation gave a pentaacetate, m.p. 103–104°.

L-Arabinose tosylhydrazone[4] afforded, in a similar manner, 1-deoxy-L-arabinitol (II), m.p. 131–133°. This gave, on acetylation a tetraacetate, m.p. 113–114°, and on benzoylation a tetrabenzoate, m.p. 134–135°.

We could not obtain a crystalline tosylhydrazone of D-fructose. However, paper chromatographic and electrophoretic analysis of a reaction mixture obtained by treatment of a suspected syrupy D-fructose tosylhydrazone with potassium borohydride, revealed a component which had the same migration rate as 2-deoxy-D-*arabino*-hexitol.

Thus, this facile method seems to be generally applicable in the carbohydrate field. The structures of the tosylhydrazones of carbohydrates and the synthesis of other deoxy-compounds, as well as the mechanism of the reaction, are under investigation.

Satisfactory elemental analyses were obtained for compounds (I) and (II).

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