Carbohydrate Research, 31 (1973) 301–309
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TRITIUM INCORPORATION IN SACCHARINIC ACID PRODUCTION

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(Received March 1st, 1973; accepted for publication in revised form, June 14th, 1973)

ABSTRACT

D-Glucose, turanose, maltose, melibiose, isomaltose, and inulin were each degraded in aqueous barium tritioxide under nitrogen. The saccharinic acids subsequently isolated contained carbon-bound tritium. Maltose, similarly degraded in the presence of air, gave, in addition, *O*-D-glucosylaldonic acids which were not tritium-labelled. The application of these observations to the structural analysis of oligosaccharides is discussed.

INTRODUCTION

In a previous paper[1], we showed that the saccharinic acids produced on degrading carbohydrates in aqueous barium deuteroxide contained deuterium that was incorporated in accordance with the accepted mechanism of saccharinic acid formation[2]. The type of saccharinic acid produced on alkaline degradation is known to be specific for the type of inter-unit linkage undergoing cleavage. Thus, anaerobic degradation of 3-*O*-substituted hexoses gives mainly 3-deoxyhexonic acids[3–5], 4-*O*-substituted hexoses yield primarily 3-deoxy-2-*C*-(hydroxymethyl)-pentonic acids[3,6,7], and 1-*O*-substituted hexuloses degrade characteristically to 2-*C*-methylpentonic acids[3,8,9]. 6-*O*-Substituted hexoses give 2-*C*-methylglyceric acid as a characteristic degradation product[10].

The introduction of sensitive and specific colorimetric methods[11] for detecting these saccharinic acids provides the basis for a sensitive method of examining oligosaccharide structures. Furthermore, the degradation of oligosaccharides in aqueous barium tritioxide, with resulting incorporation of tritium into the saccharinic acids produced, should greatly enhance the sensitivity with which these specific degradation products can be detected. The incorporation of radioactive label is now described.

EXPERIMENTAL

Degradations. — Maltose, turanose, D-glucose, melibiose, and isomaltose (2.0 mg), and inulin (8.0 mg) were each degraded in sealed vessels at 50° in 0.25M barium tritioxide for 2 h (inulin, 3 h) under an atmosphere of previously dried, oxygen-free nitrogen; the barium tritioxide was prepared from barium oxide and tritium

TABLE I

PRODUCT YIELDS ON ALKALINE DEGRADATION[a]

Product	*Turanose*	*Maltose*	*Melibiose*	*Isomaltose*	D-*Glucose*	*Maltose (aerobic)*
3-Deoxy-2-*C*-(hydroxymethyl)-D-*erythro*-pentonic acid (1)		0.23*				0.12*
3-Deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid (2)		0.41*				0.21*
3,4-Dihydroxybutyric acid (3)		0.02*				0.06*
3-Deoxy-D-*ribo*-hexonic acid (5)	0.28*	0.03*		0.03*	0.13*	
3-Deoxy-D-*arabino*-hexonic acid (6)	0.40*	0.04*		0.05*	0.25*	
2-Deoxy-D-*erythro*-pentonic acid (7)	0.09*	0.01*			0.04*	
4-*O*-α-D-Glucopyranosyl-D-mannonic acid (11)						0.03
2-*C*-Methylglyceric acid (12)			0.17*	0.11*	0.15*	
3-Deoxy-D-*xylo*-hexonic acid (13)			0.15*			
3-Deoxy-D-*lyxo*-hexonic acid (14)			0.09*			
3-Deoxy-6-*O*-α-D-glucopyranosyl-D-*ribo*-hexonic acid 3-Deoxy-6-*O*-α-D-glucopyranosyl-D-*arabino*-hexonic acid				} 0.09*		
3-Deoxy-6-*O*-α-D-galactopyranosyl-D-*ribo*-hexonic acid (15)			0.09*			
3-Deoxy-6-*O*-α-D-galactopyranosyl-D-*arabino*-hexonic acid (16)			0.08*			
3-*O*-α-D-Glucopyranosyl-D-arabinonic acid						0.06
2-*O*-α-D-Glucopyranosyl-D-erythronic acid						0.03

[a]Yields expressed as moles of product/mole of substrate. *Radioactive products.

oxide (~5.4 mCi/ml, 1.0 ml; Radiochemical Centre, Amersham). In addition, maltose (2.0 mg) was similarly degraded, but under an atmosphere of air. Excess barium ions were removed from the degradation products by treatment with Dowex-50W X8 (H^+) resin (20–50 mesh; 10 ml). The residual solution and washings were freeze-dried to remove all exchangeable tritium, the solid residue was re-dissolved in water (5 ml), and the freeze-drying was repeated. After one repetition of this process, a solution of the resulting solid was fractionated by elution from a column (140.0 × 0.6 cm; bed volume, 37 ml) of Dowex-AG1 X8 (AcO^-) resin (200–400 mesh), using 0.5M acetic acid (0.8 ml/min)[21]. The automated fluorimetric method[22] of determining formaldehyde released on periodate oxidation was used to continuously examine the column eluate. In addition, the maltose (aerobic), isomaltose, and melibiose degradation products were continuously monitored on elution, by using an automated modification of the L-cysteine–sulphuric acid method[23].

Hydrolysis of isomaltose degradation product. — Isomaltose (2.0 mg) was degraded anaerobically and the reaction product fractionated as above. Those fractions responding to the L-cysteine–sulphuric acid assay[23] were combined, concentrated to dryness by rotary evaporation, taken up in 2M hydrochloric acid (2.0 ml), and hydrolysed overnight at 100°. Residual hydrochloric acid was removed by freeze-drying, and the resulting solid was taken up in 0.02M barium hydroxide (2.0 ml) and fractionated by elution from a column (22.0 × 0.5 cm; bed volume, 5 ml) of Dowex-AG1 X8 (AcO^-) resin (200–400 mesh), using a linear gradient of 0 → 0.2M ammonium acetate (100 ml, 0.60 ml/min). The column eluate was examined as above.

Assay for tritium by liquid scintillation counting. — Samples (1.0 ml) were added to a Triton X-100/toluene emulsifier (10.0 ml, 2:1 *v/v*) containing 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) as phosphor (15 g/l), shaken to obtain emulsification, and then counted at 10°.

DISCUSSION

The major products of the degradation of maltose under nitrogen are given in Table I and were found to be 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (**1**) and 3-deoxy-2-*C*-hydroxymethyl-D-*threo*-pentonic acid (**2**). 3,4-Dihydroxybutyric acid (**3**) was obtained from the cleavage of 4-deoxy-D-*glycero*-hexo-2,3-diulose (**4**), which is the intermediate in the formation of **1** and **2**. The D-glucose molecule released in the formation of **4** was degraded to give 3-deoxy-D-*ribo*-hexonic acid (**5**) and 3-deoxy-D-*arabino*-hexonic acid (**6**) as major products, together with 2-deoxy-D-*erythro*-pentonic acid (**7**) formed by the cleavage of 3-deoxy-D-*erythro*-hexosulose (**8**). Each of these deoxyaldonic acids (**1**–**3**, **5**–**7**) was seen to contain tritium (Fig. 1); this observation is in agreement with earlier work on deuterium incorporation[1]. The formation of radioactively labelled products is represented in Fig. 2.

A further degradation product was detected by radioassay alone. Its elution position on anion-exchange chromatography and its failure to yield formaldehyde on periodate oxidation suggested that it was lactic acid (**9**). This was confirmed by a

control experiment in which maltose was degraded in aqueous barium hydroxide under conditions otherwise identical to those used above. The fractionated reaction products were assayed by the L-lactate dehydrogenase method[12], which showed that 0.09 mole of L-lactic acid/mole of maltose was formed, *i.e.*, 0.18 mole/mole of a racemic mixture. The formation of **9** on degradation of carbohydrates in alkali is well-known[3,13] and is thought to occur by cleavage of the carbohydrate chain to yield triose fragments capable of rearrangement to **9**. For this cleavage to occur in hexoses, HO-4 must be free and thus all of the lactic acid found in the present degradation of maltose must have arisen from D-glucose released in the formation of **4**. The presence of 3-deoxy-4-*O*-glucosylhexonic acids in the degradation products of 4-*O*-substituted oligosaccharides has been reported previously[14,15], but no such products were detected here.

Alkaline degradations of carbohydrates performed in the presence of air or oxygen are known to give considerable yields of aldonic acids[16,17]. The mechanism

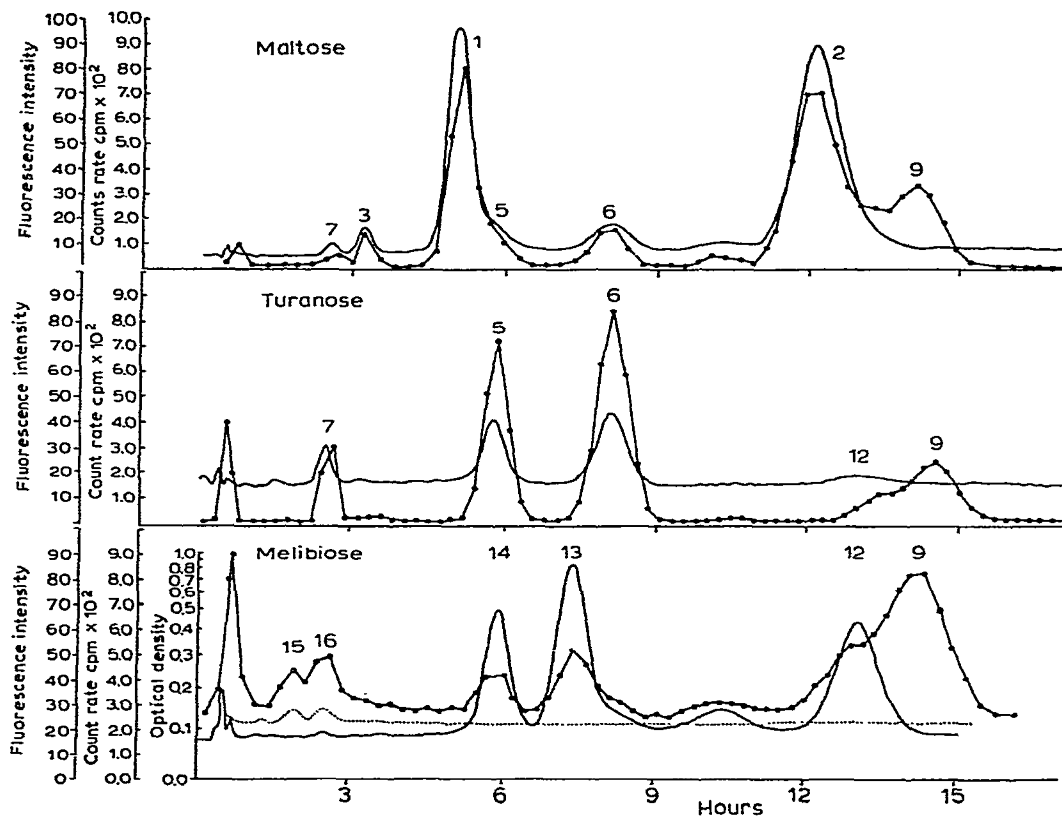

Fig. 1. Chromatographic fractionation of the degradation products of maltose, turanose, and melibiose: —•—•—•, radioassay; ———, detection of formaldehyde released on periodate oxidation; ...... L-cysteine–sulphuric acid assay.

of formation of these acids remains uncertain; if they are formed by cleavage of enediol species, it seems unlikely that they would incorporate tritium on formation in aqueous barium tritioxide. Aldonic acids can also arise by mechanisms that do not appear to involve chain cleavage; for example, 4-*O*-β-D-glucopyranosyl-D-mannonic acid was obtained from the oxidative alkaline degradation of cellobiose[17]. It was suggested that the intermediate in this epimerisation is 4-*O*-β-D-glucopyranosyl-D-*arabino*-hexosulose, and it is conceivable that any of the corresponding material, 4-*O*-α-D-glucopyranosyl-D-*arabino*-hexosulose (**10**), produced in the present degradation of maltose could be formed with incorporation of tritium which would subsequently appear in the product of rearrangement of **10**, namely, 4-*O*-α-D-glucopyranosyl-D-mannonic acid (**11**). Small amounts of **11** were found, together with 2-*O*-α-D-glucopyranosyl-D-erythronic acid and 3-*O*-α-D-glucopyranosyl-D-arabinonic

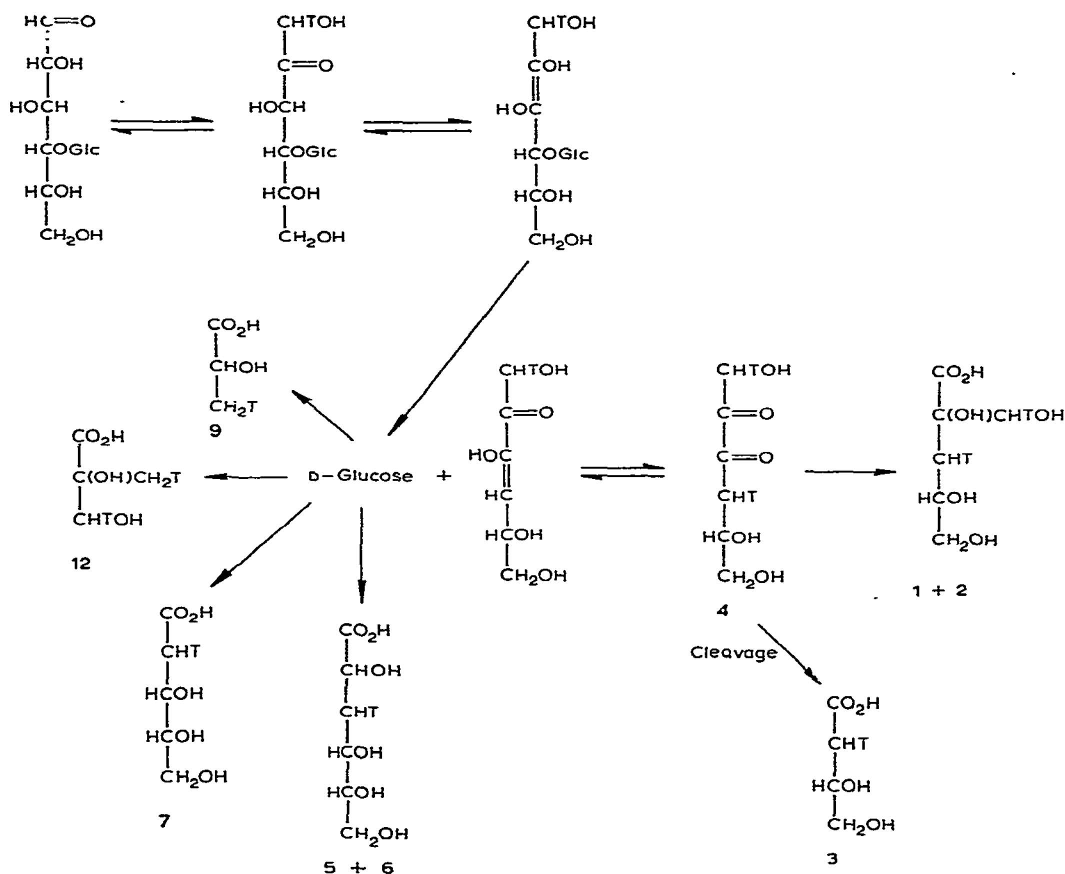

Fig. 2. Incorporation of tritium in the degradation of maltose. In Figs. 2–4, the positions at which tritium is depicted as taken up are shown by analogy with the specific positions of deuterium incorporation determined in earlier experiments[1]. The extent of tritium uptake at each individual site is represented qualitatively.

acid (Table I); each of these products has been identified previously in the aerobic degradation of maltose under similar conditions[18]. In the present experiment, no tritium could be detected in any of these three *O*-glucosylaldonic acids. Compounds **1–3**, **5**, and **9** were also detected in the aerobic degradation product. As in the corresponding anaerobic degradation, these deoxyaldonic acids were all radioactively labelled and were thus readily distinguishable from the aldonic acids also obtained.

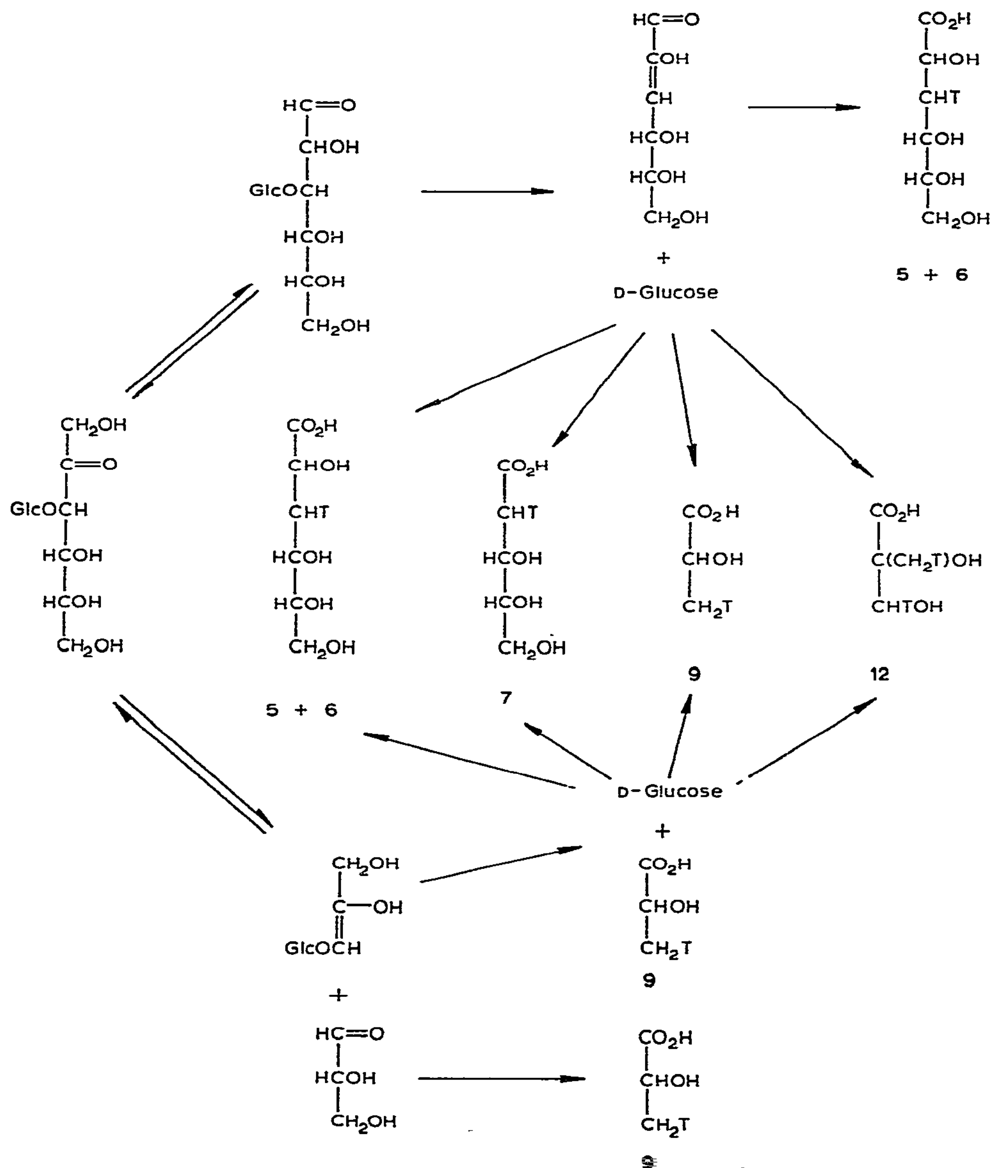

Fig. 3. Incorporation of tritium in the degradation of turanose.

The major products of the anaerobic degradation of turanose (Fig. 1) are listed in Table I and, as anticipated, they were **5**, **6**, and **7**. Compound **9** was also found, and three possible pathways exist (Fig. 3) for its formation (*cf.* the degradation of maltose, where **9** can only arise from released D-glucose). 2-*C*-Methylglyceric acid (**12**) was also detected. As a free HO-3 is almost certainly necessary for its formation[10], all the **12** detected amongst the degradation products of turanose must have arisen from the released D-glucose. Indeed, D-glucose degraded in aqueous barium tritioxide gave, as anticipated, **5**–**7**, **9**, and **12** (Table I). 2,4-Dihydroxybutyric acid was not detected amongst the reaction products, although this acid has been found previously on degradation of D-glucose under alternative conditions[13].

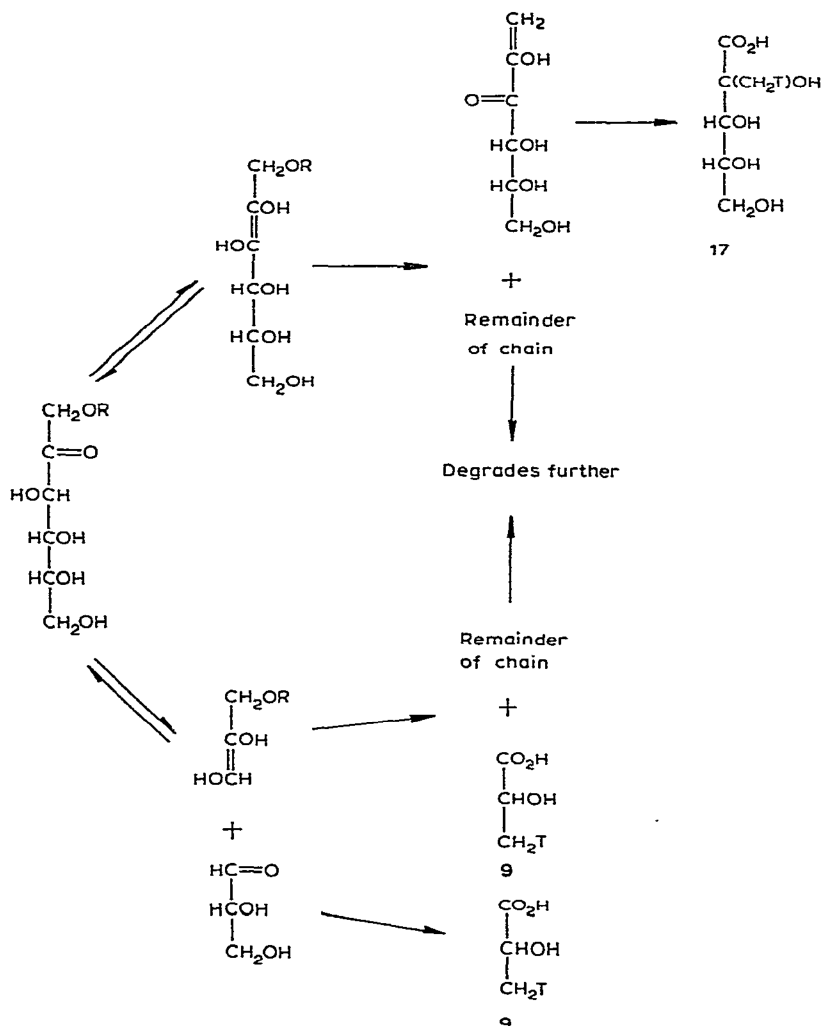

Fig. 4. Incorporation of tritium in the degradation of inulin.

Melibiose was degraded to give mainly radioactive 3-deoxy-D-*xylo*-hexonic acid (**13**) and 3-deoxy-D-*lyxo*-hexonic acid (**14**), together with **9** and **12** (Fig. 1); **13** and **14** were formed from the released D-galactose. 3-Deoxy-6-*O*-α-D-galactopyranosyl-D-*ribo*-hexonic acid (**15**) and 3-deoxy-6-*O*-α-D-galactopyranosyl-D-*arabino*-hexonic acid (**16**), previously characterised by acid hydrolysis to D-galactose, and **5** and **6**, respectively[19], were obtained by a "stopping" reaction in contrast to the "peeling" reaction which gave **12**. Similarly, isomaltose gave **5**, **6**, **9**, and **12**, together with the stopping-reaction products 3-deoxy-6-*O*-α-D-glucopyranosyl-D-*ribo*-hexonic acid and 3-deoxy-6-*O*-α-D-glucopyranosyl-D-*arabino*-hexonic acid (Table I). The last two acids were collected and, on acid hydrolysis and subsequent fractionation, gave inactive D-glucose and radioactive **5** and **6**.

When inulin was degraded (at a carbohydrate concentration slightly higher than that used in all the above experiments), only trace amounts of acids **5**, **6**, and **12** were obtained; these presumably arise as non-reducing, terminal units and are released and degraded. Much larger amounts of material corresponding in elution position to 2-*C*-methyl-D-ribonic acid (**17**) and **9** were the major products. The reaction scheme shown in Fig. 4, in which both **9** and **17** can be formed by sequential degradation of the polysaccharide chain, would account for these products. These observations are consistent with the formation of **9** and **17** as major products in the degradation of 1-*O*-methyl-D-fructose[9].

The carbohydrates examined here served as model compounds for oligosaccharides containing 1→3, 1→4, 1→6, and 2→1 inter-unit linkages, with each type of linkage giving characteristic saccharinic acids. The specific activity of each of these saccharinic acids obviously depends on the initial specific activity of the tritium oxide used. In all of the experiments described here, this activity was only ~5mCi/ml. No attempt was made to increase the specific activity of the reaction products by using tritium oxide of higher activity, although solutions of 5Ci/ml are available. Nevertheless, by merely modifying the fractionation procedures used here, it has been possible to successfully examine the inter-unit linkage in microgramme amounts of an unknown disaccharide[20]. Other degradation products, such as formic glyceric, erythronic, and threonic acids, were not determined in the present series of experiments.

ACKNOWLEDGMENTS

The authors thank Professors M. Stacey, C. B. E., F. R. S., and S. A. Barker for their interest in this work.

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