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STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XXIII[1]. TRANS-β-D-GLUCOSYLATION BY EXTRACTS OF *Tetrahymena pyriformis* AND *Ochromonas malhamensis*

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ABSTRACT

Extracts of *Tetrahymena pyriformis* contain an enzyme which can transfer β-D-glucosyl residues from cellobiose to C-6 of either D-glucose or the non-reducing residue in cellobiose, with the formation of either gentiobiose or 6^2-β-D-glucosylcellobiose. With an extract of *Ochromonas malhamensis*, transfer from cellobiose is mainly to C-4 and C-6 of the non-reducing residue of cellobiose, giving cellotriose or 6^2-β-D-glucosylcellobiose. The same extracts can also use gentiobiose as a D-glucosyl donor, giving higher gentiosaccharides as the major products.

INTRODUCTION

When β-D-glucosidase preparations from various biological sources are incubated with solutions containing more than 1% of cellobiose, the enzymic hydrolysis to D-glucose is usually accompanied by the formation of oligosaccharides (for a review, see Manners[2]). This arises from the fact that most carbohydrases are transglycosylases, and that hydrolysis represents a special example in which a glycosyl unit is transferred from an aglycone to water. With cellobiose, transfer may take place onto either D-glucose or cellobiose to give di- and tri-saccharides, respectively. The nature of the oligosaccharides formed is related to the acceptor specificity of the β-D-glucosidase. Since cell-free extracts of the ciliate *Tetrahymena pyriformis* and the flagellate *Ochromonas malhamensis* show marked β-D-glucosidase activity, it was of interest to examine the action of these preparations on concentrated solutions of cellobiose and gentiobiose under various conditions. A preliminary account of these results has been presented elsewhere[3].

MATERIALS AND METHODS

Materials. — We are indebted to Dr. J. F. Ryley for freeze-dried cells of *Tetrahymena pyriformis* and *Ochromonas malhamensis*. Details of the conditions of growth of *T. pyriformis* are described by Archibald and Manners[4]. Cultures of *O. malhamensis* were grown in daylight in a medium containing Oxoid Peptone (0.1%, w/v), Hepamino Liver Extract (0.1%), and D-glucose (0.1%) at pH 6.0. For bulk growth, 2-litre

conical flasks each containing 500 ml of medium were used. After growth for 7 days at 24°, the cells from 36 l of medium were collected by using a Sharples centrifuge, washed once in citrate buffer (pH 6.0), freeze-dried in 100 ml of buffer, and stored at −15°. Part of the freeze-dried preparation was purified by suspending 5 g in water (500 ml) at 20° with occasional stirring for 1 h, discarding insoluble material, and allowing the solution to autolyse at 37° for 3 days under toluene. After dialysis to remove impurities of low molecular weight, protein was precipitated by the addition of ammonium sulphate to 70% saturation. The precipitate was collected, dissolved in water (100 ml), dialysed against running tap water for 12 h, and centrifuged. The supernatant solution was made 20mM with respect to sodium citrate (pH 6.0) and freeze-dried to give 1.1 g of enzyme preparation.

General methods. — Details of the experimental methods for the separation and identification of oligosaccharides were given by Manners, Pennie, and Stark[5]. For paper chromatography, the following solvent systems were used (v/v): (*A*) ethyl acetate–pyridine–water (10:4:3); (*B*) butyl alcohol–pyridine–water (6:4:3); (*C*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4). The following spray reagents were used for diagnostic purposes: (*a*) reducing aldohexoses substituted at C-4 give a characteristic blue colour with diphenylamine–aniline[6]; (*b*) periodate–*p*-rosaniline forms a yellow colour with such sugars as 3-*O*-substituted aldohexoses which give rise to a malonaldehyde derivative on periodate oxidation[7]. Other sugars, *e.g.* 4-*O*-substituted aldohexoses, give a blue colour. From the colour obtained before and after borohydride reduction, it is therefore possible to differentiate between (1→2)-, (1→3)-, (1→4)-, and (1→6)-linked glucose disaccharides. Electrophoretic mobilities of oligosaccharides are expressed as M_G values (D-glucose = 1.00), and of the derived alcohols as M_S values (D-glucitol = 1.00), in borate and molybdate buffers, respectively[5].

A phenol–sulphuric acid reagent[8] was used to determine the amount of carbohydrate eluted from paper chromatograms. The degree of polymerisation (*DP*) of oligosaccharides was determined by the method of Peat and co-workers[9], except that the anthrone estimation was replaced by the above reagent. A D-glucose oxidase reagent[10] was used to follow D-glucosidase activity quantitatively.

Micro-scale methylation of disaccharides and gas–liquid partition chromatography of the derived methyl glycosides was carried out as described previously[5].

The nomenclature of oligosaccharides is that recommended by Whelan[11].

Incubation conditions. — All digests were incubated at 37° under toluene, to prevent microbial contamination. Qualitative digests contained solutions of substrate (1%) and freeze-dried protozoal enzyme preparation (1%) in a total volume of 0.5 or 1 ml.

RESULTS

Carbohydrase activity of protozoal extracts. — Aqueous extracts of the freeze-dried cells showed substantial carbohydrase activity. Both extracts readily hydrolysed

β-D-linked disaccharides (cellobiose, gentiobiose, laminaribiose, ββ-trehalose) but only slowly attacked methyl and phenyl β-D-glucosides, xylobiose, lactose, melibiose, and laminarin, and did not hydrolyse xylan.

In general, the level of activity towards β-D-glucosides was much less than that towards the corresponding α-D-glucosides. The rate of hydrolysis of various glucosides (0.1%) by the *T. pyriformis* extract (0.025%) at 36° was constant over 24 h; if the maltase activity is taken as 100 units, the relative activities were cellobiase 4.7, phenyl α-D-glucosidase 3.2, and phenyl β-D-glucosidase 1.5.

With the initial *O. malhamensis* extract, small quantities of D-glucose were produced by autolysis. This effect was overcome by partial purification using dialysis followed by precipitation with ammonium sulphate; no D-glucose was released on incubation of the purified preparation over a period of 14 days.

Action of T. pyriformis *extract on cellobiose.* — When a 10% solution of cellobiose was incubated with a 1% extract of *T. pyriformis*, several oligosaccharides were synthesised (Table I). The first of these could be detected after only 8 h.

TABLE I

SYNTHESIS OF OLIGOSACCHARIDES FROM CELLOBIOSE BY EXTRACTS OF *Tetrahymena pyriformis*[a]

Time of incubation (days)	R_G *value (solvent* A)[b]					
	0.76	*0.44*	*0.33*	*0.18*	*0.14*	*0.07*
0.3	−	−	+	−	−	−
1	−	+	++	+	−	−
2	−	++	+++	+	+	−
5	+	+++	+++	++	+	−
10	+	+++	+++	++	++	+

[a]Excluding the original cellobiose (R_G 0.58) and D-glucose. [b]The relative amounts of the products on paper chromatograms are indicated by: −, nil; +, light; ++, medium; +++, heavy.

The oligosaccharides were isolated by preparative paper chromatography from a digest consisting of cellobiose (5 g) and *T. pyriformis* extract (0.5 g) in water (50 ml) which had been incubated for 15 days. The properties and identities of the oligosaccharides are given in Table II. All the fractions were readily hydrolysed to D-glucose by almond β-D-glucosidase, showing the presence of β-D-glucosidic linkages, and the absence of α-D-glucosidic linkages.

The chromatographic and electrophoretic properties of Fractions *T1* and *2* identify these disaccharides as laminaribiose and sophorose, respectively. The identification of an additional disaccharide as unchanged cellobiose was confirmed by methylation analysis; a methanolysate contained the mixed glycosides of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-glucose.

Fraction *T3* was identified as gentiobiose by the properties in Table II, and by the presence of the glycosides of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-D-glucose in a methanolysate of the methylated sugar.

TABLE II

PROPERTIES OF OLIGOSACCHARIDES SYNTHESISED FROM CELLOBIOSE BY EXTRACTS OF *T. pyriformis* (*T.1–T.6*) AND *O. malhamensis* (*O.1–O.3*)[a]

Fraction No.	R_G *value* (*solvent* A)	*Yield* (*mg*)	M_G	M_S	*Colour*[b] *with diphenylamine reagent*	*Colour with periodate–p-rosaniline* (*a*)	(*b*)	*Identity*
T.1	0.76	10	0.60	0.01	g	y	y	Laminaribiose
T.2	0.62	5	0 26	0.78	g	y	b	Sophorose
T.3	0.44	100	0.56	0.80	g	b	b	Gentiobiose
T.4	0.33	45	0 25	0.20	b	—	—	6^2-β-D-Glucosyl-cellobiose
T.5	0.18	27	—	—	b–g	—	—	Gentiotriose
T.6	0.14	12	—	—	b	—	—	Unknown
O.1	0.40	2	—	—	g	—	—	Gentiobiose
O.2	0.33	17	0.25	0.20	b	—	—	6^2-β-D-Glucosyl-cellobiose
O.3	0.23	23	0 27	0.20	b	—	—	Cellotriose

[a]Excluding any residual cellobiose and D-glucose formed by hydrolysis. [b]b, Blue; g, grey, y, yellow.

The presence of a 4-*O*-substituted reducing end-group in Fraction *T4* was indicated by the various colour tests, and by the electrophoretic mobility which was the same as that for cellobiose. Partial, acid hydrolysis gave D-glucose, cellobiose, and gentiobiose; after borohydride reduction, a partial hydrolysate contained cellobiitol and gentiobiose. After methylation, a methanolysate contained the glycosides of 2,3,4,6-tetra-, 2,3,4-tri-, and 2,3,6-tri-*O*-methyl-D-glucose. Fraction *T4* is therefore 6^2-β-D-glucosylcellobiose (4-*O*-β-gentiobiosyl-D-glucose).

Fraction *T5* had similar chromatographic and electrophoretic mobilities and colour reactions to gentiotriose. However, after borohydride reduction and partial hydrolysis with acid, gentiobiose, gentiobiitol, and other disaccharides were produced, indicating that fraction *T5* was not homogeneous, and contained gentiotriose and another oligosaccharide. The fraction *T6* with R_G 0.14 gave a blue colour with diphenylamine–phosphoric acid, indicating the presence of a 4-*O*-substituted reducing end-group, but was not examined further.

Action of T. pyriformis *extract on gentiobiose.* — When a 10% solution of gentiobiose was incubated with the *T. pyriformis* extract, D-glucose and a sugar with the R_G value of gentiotriose were detected within 30 min, and the amount increased steadily. After 2 days, the major products were clearly gentiotriose and gentiotetraose, with smaller amounts of sugars having R_G 0.29 and 0.53, and traces of cellobiose, laminaribiose, and gentiopentaose. With gentiobiose, transfer was therefore mainly to C-6 of the non-reducing residue of gentiosaccharides, thereby forming the next higher oligosaccharide.

Action of O. malhamensis *preparation on cellobiose.* — Incubation of a 10% solution of cellobiose with the partially purified preparation from *O. malhamensis*

resulted in the synthesis of two new oligosaccharides within 1 day, and after 6 days, traces of a third oligosaccharide were detected (Table III). On further incubation, the concentration of the sugar with R_G 0.23 decreased slowly, whilst that of those with R_G 0.33 and 0.40 increased. However, additional oligosaccharides were not produced.

TABLE III

SYNTHESIS OF OLIGOSACCHARIDES FROM CELLOBIOSE BY AN ENZYME PREPARATION FROM *Ochromonas malhamensis*[a]

Time of incubation (days)	R_G *values (solvent* A)		
	0.40	*0 33*	*0.23*
1	–	+	+
2	–	+	++
4	–	++	++
6	+	+++	+++

[a]Excluding the residual cellobiose and also D-glucose formed by hydrolysis.

A digest containing cellobiose (2.5 g) and enzyme preparation (250 mg) in a total volume of 25 ml was incubated for 12 days. The pattern of synthesis was identical to that in Table III. Samples were withdrawn at intervals for quantitative paper chromatography. The results are given in Table IV. After 12 days, the enzyme was inactivated by heating, coagulated protein was removed, and the oligosaccharides were separated by preparative paper chromatography.

TABLE IV

CHANGES IN THE COMPOSITION OF A DIGEST CONTAINING CELLOBIOSE (10%) AND AN ENZYME PREPARATION FROM *Ochromonas malhamensis*

Time of incubation (days)	D-*Glucose* (%)	*Disaccharides* (%)	*Higher oligosaccharides* (%)
2	0.3	95.0	4 7
4	0.9	93.7	5.4
8	2.8	91.1	6.1
11	3 0	89.4	7.6

Fraction *O.1* had the same mobility as gentiobiose in solvents *A*, *B*, and *C*. It was hydrolysed to D-glucose by almond β-D-glucosidase, and gave a grey colour with diphenylamine–phosphoric acid reagent.

Fraction *O.2* was chromatographically pure, and had R_G 0.40 in solvent *B*. It was a trisaccharide (experimental *DP* 3.1) and was completely hydrolysed by almond β-D-glucosidase; on partial hydrolysis with acid, it gave D-glucose, gentiobiose, and cellobiose. After borohydride reduction, a partial hydrolysate contained D-glucose,

gentiobiose, and cellobiitol. The presence of a 4-*O*-substituted reducing group was also indicated by the blue colour with diphenylamine–phosphoric acid reagent. The oligosaccharide is therefore 6^2-*β*-D-glucosylcellobiose.

Fraction *O.3* had the chromatographic and electrophoretic properties of a 4-*O*-substituted derivative of D-glucose. It was a trisaccharide (experimental *DP* 3.3) and was completely hydrolysed by almond *β*-D-glucosidase; on partial hydrolysis, it gave only D-glucose and cellobiose, and had the same R_G value as authentic cellotriose in solvents *A*, *B*, and *C*.

The effect of additional D-glucose on the pattern of oligosaccharide production was examined in a digest containing cellobiose (8%) and D-glucose (4%). Traces of gentiobiose could be detected within 12 h, together with the other two oligosaccharides. On further incubation, the amount of gentiobiose steadily increased, and after 8 days, all three sugars were present in approximately the same amounts; thereafter, gentiobiose predominated. Laminaribiose and other oligosaccharides could not be detected.

Trans-β-D-glucosylation from cellobiose to pentoses. — Digests containing cellobiose (5%), various pentoses (5%), and the partially purified *O. malhamensis* preparation (1%) were prepared and examined by paper chromatography with solvent *A*. Small amounts of glucosyl-pentoses, which gave a pink colour with aniline oxalate, were detected after 7 days from D-xylose (R_G 0.64), D-arabinose (R_G 0.75), and D-lyxose (R_G 0.72 and 0.90). D-Ribose failed to act as an acceptor.

Action of O. malhamensis *preparation on gentiobiose.* — Incubation of a 10% solution of gentiobiose with the partially purified *O. malhamensis* preparation resulted in the formation of three oligosaccharides (R_G 0.30, 0.18, and 0.07; solvent *A*) within 12 h. On continued incubation, the concentration of gentiobiose and the sugar with R_G 0.30 decreased, whilst additional oligosaccharides with R_G 0.25 and 0.12 made transient appearances. Traces of laminaribiose (R_G 0.75) and cellobiose (R_G 0.57) appeared after 2 days, and slowly increased in amount. After 8 days, the relative proportions of sugars (determined by quantitative paper chromatography) were D-glucose, 35%; gentiobiose, 25%; other disaccharides, 7%; higher oligosaccharides, 34%.

The oligosaccharides were isolated from a digest of gentiobiose (1 g) which had been incubated with the enzyme preparation (0.1 g) in water (10 ml) for 10 days. The yields and probable identities are given in Table V. Although insufficient quantities of the oligosaccharides were available for full chemical characterisation, all the oligosaccharides except *G4* and *G6* had the same R_G values (in solvents *A*, *B*, and *C*) and M_G values as authentic samples of the appropriate reference sugar. *G4* was a mixture of two sugars; the major component had R_G 0.30 and M_G 0.30, suggesting the presence of a 4-*O*-substituted D-glucose residue. The minor component (R_G 0.26) had M_G 0.55, indicating a 3- or 6-*O*-substituted D-glucose residue. Neither sugar was an oligosaccharide of the cellosaccharide or laminarisaccharide series. The R_G values of fraction *G7* in solvents *A* and *B* corresponded to gentiotetraose.

Trans-β-D-glucosylation from gentiobiose to various pentoses. — Glucosyl-pentoses were readily detected when mixtures of gentiobiose (5%) and pentose (5%)

TABLE V

OLIGOSACCHARIDES SYNTHESISED FROM GENTIOBIOSE BY EXTRACTS OF *O. malhamensis*

Fraction No.	R_G *value (solvent* A)	*Yield (mg)*	*Tentative identity*
G.1	0.76	13	Laminaribiose
G.2	0.57	15	Cellobiose
G.3	0.42	204	Gentiobiose
G.4	0.30, 0.26	27	—
G.5	0.18	68	Gentiotriose
G.6	0.14	10	—
G 7	0.07	47	Gentiotetraose

were incubated with the *O. malhamensis* preparation. With each pentose, a mixture was produced; after 7 days, the digests were inactivated by heating, and the various sugars separated by preparative paper chromatography. The major products were eluted from paper chromatograms, and hydrolysed with acid, and the presence of approximately equal quantities of glucose and the original pentose was shown. The R_G values (solvent *A*) were as follows: D-xylose, 0.60 (major), 0.82 (minor product); D-lyxose, 0.71 (major), 0.88, 0.30 (minor products); D-arabinose, 0.65 (major), 0.28, 0.73 (minor products); D-ribose, 0.62 (major), 0.77, 0.86 (minor products).

DISCUSSION

The protozoa constitute a large class of unicellular organisms which show a wide range of morphological and biochemical features. *Tetrahymena pyriformis* is a free-living ciliate commonly found in fresh-water ponds, which can be readily grown in bacteria-free culture on a peptone–salt medium. The reserve carbohydrate is glycogen, and the cells contain the enzymes of the glycolytic pathway which anaerobically convert glycogen into succinic acid and other products[12]. *Ochromonas malhamensis* is also a fresh-water organism, but is a flagellate which stores fat and leucosin[13] [a β-(1→3)-D-glucan] as reserve materials. This organism can be grown on a medium containing acetate as carbon source. Cell-free extracts of both of the above organisms show a wide range of carbohydrase activities, which appear to differ in many respects from the admittedly limited information on other protozoa. For example, extracts of the sheep-rumen ciliate *Entodinium caudatum* show strong maltase and amylase activity, but only traces of other carbohydrases[14]. Extracts from the cattle-rumen ciliate *Epidinium ecaudatum* (Crawley) show strong xylanase, xylobiase, and α-D-galactosidase activity[15], in contrast to the lack of this activity in the present experiments.

Although the cell-free extracts of *T. pyriformis* and *O. malhamensis* contain mixtures of carbohydrases, it is possible to use these extracts directly to obtain information on the acceptor specificity of the enzymes, since the transfer of a glucosyl residue follows the formation of a specific glycosyl–enzyme complex by the appro-

TABLE VI

OLIGOSACCHARIDES[a] SYNTHESISED FROM CELLOBIOSE BY VARIOUS β-D-GLUCOSIDASE PREPARATIONS

Enzyme source	*Gentiobiose*	*Laminaribiose*	*Sophorose*	*Cellotriose*	*6²-β-D-Glucosyl-cellobiose*	*3²-β-D-Glucosyl-cellobiose*	*Gentiotriose*	*Ref.*
Alfalfa	++	+	−	+	++	+	−	16
Aspergillus niger	+	+	+	+	++	+	+	17
Barley	++	++	−	+	++	−	++	18
Cladophora rupestris	+	+	−	++	−	−	−	19
O. malhamensis	+	−	−	++	++	−	−	−
T. pyriformis	++	+	+	−	++	−	+	—

[a]++ Major, product; +, minor product.

priate enzyme and the carbohydrate donor[2,20]. α-D-Glucosidases, for example, do not interfere with the reaction between cellobiose and a β-D-glucosidase. Moreover, in view of the limited quantity of cells which were available, purification of the extracts was not practicable.

As a preliminary to these studies, the ability of the extracts to polymerise D-glucose was examined. As reported previously[4], the *T. pyriformis* preparation had no action on a 20% solution of D-glucose. By contrast, the *O. malhamensis* preparation slowly synthesised small quantities of a sugar having the R_G value of gentiobiose from D-glucose. This fact has been taken into account in assessing the trans-β-D-glucosylase activity of the extract (Tables III and IV). This enzyme preparation also produced D-glucose by the autolysis of leucosin; this effect was removed by partial purification of the preparation.

When incubated with 10% solutions of cellobiose, the two extracts produced different patterns of oligosaccharides. These are considered to be true products of enzymic transfer reactions, and not artefacts due to microbial contamination of the enzyme digests. The *T. pyriformis* extract gave a mixture of gentiobiose and 6^2-β-D-glucosylcellobiose with smaller amounts of laminaribiose and gentiotriose, and traces of sophorose and higher oligosaccharides. This contrasts with the *O. malhamensis* extract which gave cellotriose and 6^2-β-D-glucosylcellobiose, and a very small amount of gentiobiose. This indicates that the OH groups at C-4 or C-6 of the non-reducing residue of cellobiose are the most favourable acceptor sites, whereas with the *T. pyriformis* enzyme extract, C-6 of D-glucose and the non-reducing residue of cellobiose are preferred; the OH groups at C-3 or C-2 of D-glucose are less favourable.

The results obtained in the present study are compared in Table VI with those reported by various workers using plant or fungal enzyme preparations. The *O. malhamensis* enzyme also differs from other β-D-glucosidases in its failure to use pentoses as effective, alternative acceptors. For example, when enzyme preparations from *Aspergillus niger*[21] or *Cladophora rupestris*[19] are incubated with mixtures of cellobiose and xylose, 3-*O*-β-D-glucopyranosyl-D-xylose is readily synthesised, whilst barley β-D-glucosidase produces 4-*O*-β-D-glucopyranosyl-D-xylose from a mixture of salicin and xylose[22].

Since the enzyme extracts from *T. pyriformis* and *O. malhamensis* showed significant gentiobiase activity, their action on a 10% solution of gentiobiose was also examined. In both cases, a mixture of oligosaccharides —mainly gentiosaccharides— was produced, and the pattern of synthesis was different from that for cellobiose. Moreover, in the presence of pentoses, glucosyl-pentoses were formed more readily by the *O. malhamensis* extract from gentiobiose than from cellobiose. These results may indicate the presence of separate cellobiase and gentiobiase enzymes in the extracts.

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REFERENCES

1 Part XXII. D. J. MANNERS AND J. J. MARSHALL, *J. Inst. Brewing*, 75 (1969) 550.
2 D. J. MANNERS, *Bull. Soc. Chim. Biol.*, 12 (1960) 1789.
3 D. J. MANNERS, J. R. STARK, AND D. C. TAYLOR, *Abstr. Sixth FEBS Meeting, Madrid* (1969) 128.
4 A. R. ARCHIBALD AND D. J. MANNERS, *Biochem. J.*, 73 (1959) 292.
5 D. J. MANNERS, I. R. PENNIE, AND J. R. STARK, *Carbohyd. Res.*, 7 (1968) 291.
6 S. SCHWIMMER AND A. BEVENUE, *Science*, 123 (1956) 543.
7 F. E. HARDY AND J. G. BUCHANAN, *J. Chem. Soc.*, (1963) 5881.
8 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
9 S. PEAT, W. J. WHELAN, AND J. G. ROBERTS, *J. Chem. Soc.*, (1956) 2258.
10 J. W. WHITE AND M. H. SUBERS, *Anal. Biochem.*, 2 (1961) 380.
11 W. J. WHELAN, *Ann. Rev. Biochem.*, 29 (1960) 105.
12 J. F. RYLEY, *Biochem. J.*, 52 (1952) 483.
13 A. R. ARCHIBALD, W. L. CUNNINGHAM, D. J. MANNERS, J. R. STARK, AND J. F. RYLEY, *Biochem. J.*, 88 (1963) 444.
14 A. R. ABOU AKKADA AND B. H. HOWARD, *Biochem. J.*, 76 (1960) 445.
15 R. W. BAILEY, R. T. J. CLARKE, AND D. E. WRIGHT, *Biochem. J.*, 83 (1962) 517.
16 D. H. HUTSON, *Biochem. J.*, 92 (1964) 142.
17 S. A. BARKER, E. J. BOURNE, G. C. HEWITT, AND M. STACEY, *J. Chem. Soc.*, 73 (1955) 3734.
18 F. B. ANDERSON AND D. J. MANNERS, *Biochem. J.*, 71 (1959) 407.
19 W. A. M. DUNCAN, D. J. MANNERS, AND J. L. THOMPSON, *Biochem. J.*, 73 (1959) 295.
20 M. A. JERMYN, *Rev. Pure Appl. Chem.*, 11 (1961) 92.
21 S. A. BARKER, E. J. BOURNE, G. C. HEWITT, AND M. STACEY, *J. Chem. Soc.*, (1957) 3541.
22 D. J. MANNERS AND J. R. STARK, *Carbohyd. Res.*, 3 (1966) 102.