BERNAERTS, M. J. & DE LEY, J. (1960). *J. gen. Microbiol.* 22, 137–146

The Structure of 3-Ketoglycosides formed from Disaccharides by Certain Bacteria

BY M. J. BERNAERTS

Central Laboratory, Ministry of Economic Affairs, Brussels, Belgium

AND J. DE LEY

Biochemical Laboratory, Veterinary College, State University, Ghent, Belgium

SUMMARY: Bacteria, provisionally called *Alcaligenes faecalis*, oxidize lactose, maltose and the corresponding bionates aerobically to 3-ketoglycosides. Several properties of the pure compounds are described, as well as the arguments, leading to the proposed chemical formula.

In a preliminary paper (Bernaerts & De Ley, 1958) we announced the discovery of 3-ketoglycosides, formed by the action of some bacteria on lactose, maltose and the corresponding bionates. In the paper accompanying the present one (Bernaerts & De Ley, 1960) we described the procedure for the formation and isolation of these new compounds. The present report gives a description of the experiments which lead to the proposed structure of these substances.

METHODS

Preparation and purification of the oxidation products. The methods have been described in the foregoing paper (Bernaerts & De Ley, 1960). The compounds were prepared by aerobic fermentation with a strain of bacteria presumed to be *Alcaligenes faecalis*. The oxidation products of lactose (RL), lactobionate (RLB) and maltobionate (RMB) were obtained in good yield in the culture media and used chiefly in the chromatographically pure state. As the yield of the oxidation product from maltose (RM) was always very small, it was not purified.

Paper chromatography. The following solvents were currently used to separate mixtures of sugars: the top layer of ethyl acetate, pyridine, water (10 + 4·5 + 10; solvent C); *n*-butanol, pyridine, water (45 + 25 + 40; solvent D); methylethyl ketone, acetic acid, water saturated with boric acid (9 + 1 + 1; solvent E); top layer of *n*-butanol, ethanol, water (4 + 1 + 5; solvent F). Other solvents were occasionally used and will be mentioned in the text.

Aldohexoses were revealed with *p*-anisidine HCl in *n*-butanol, lactones with the spray reagent of Abdel-Akher & Smith (1951). Most of the classical sprays for reducing sugars were also used. However, the *o*-phenylenediamine HCl spray (Lanning & Cohen, 1951) for revealing reducing substances and keto-functions was preferred, since a variety of specific colours was obtained.

Mixtures of aldohexoses of the order of 100 mg. or more were separated with a Grycksbo filter-paper column described by Hagdahl & Danielson (1954).

It was used according to the instructions of the manufacturers (L.K.B.-Produkter, Stockholm). Large volumes of solvent E were necessary for the required separation. The isolation of the relatively small amounts of sugars from the large excess of solvent was effected by distilling off both methylethyl ketone and acetic acid *in vacuo*. Boric acid was eliminated by distilling off at 70° methyl borate, formed following the addition of a large excess of methanol. The sugars were extracted with water from the resinous residue after evaporation of the methanol and the sugar solution further purified with Merck I and II ion exchangers.

Chromatography on active clay. Sugar acids and polyalcohols were separated from sugars on columns of active clay. Several samples of clays were tested. The best results were obtained with a sample of Florex XXF (Floridine Co., Warren, Pennsylvania, U.S.A.), which was washed, dried, passed through a 200-mesh sieve and mixed with 17 % (w/w) Celite, as described by Binkley & Wolfrom (1948). However, separation of related carbohydrates was not as good as that obtained by Lew, Wolfrom & Goepp (1946). The absorbent column was packed and the chromatogram developed as described by these authors. To obtain a reasonable flow under a slight vacuum, the height of the column never exceeded 12 cm. The diameter of the column was chosen according to the quantity of material to be chromatographed. The ratio of one part of absorbed substance to 100 parts of absorbent was ordinarily used. Sugar acids and sugar alcohols were more strongly absorbed than the hexoses present in our mixtures. The column was developed with a mixture of ethyl alcohol and water, as described below, till all the reducing carbohydrates had passed through. Afterwards the absorbent was pushed out of the tube. The zones of absorbed substances were located by brushing with alkaline permanganate and the column was cut into cylinders. The absorbed substances were eluted. Four times the volume of water used in the preparation of a thin slurry was adequate to elute completely even the most strongly absorbed sugar acids. The different fractions were purified, either with ion exchangers or by extracting the dried residue with methanol, and concentrated by lyophilization.

Reduction of the carbonyl function. Several reduction methods have been used with varying success: by passing a stream of hydrogen through the aqueous solution of the compound at 70° in the presence of freshly prepared Raney nickel (Karabinos & Ballun, 1953), or with $NaBH_4$ in alkaline (Abdel-Akher, Hamilton & Smith, 1951), or acid medium (Wolfrom & Anno, 1952). The excess of $NaBH_4$ was destroyed afterwards by acidification and the boric acid distilled off as methyl borate at 70°.

Melting points. These were determined microscopically and are reported uncorrected.

RESULTS

General properties of the oxidation products. The oxidation products of lactose (RL), lactobionate (RLB) and maltobionate (RMB) showed one component only in paper chromatography and were considered pure. They were colourless to slightly yellow glassy materials. Several attempts to obtain crystalline

products were unsuccessful; only the crude oxidation product from maltose (RM) was used.

Their most striking chemical characteristic, which was the origin of their discovery, was their very strong reducing property with the usual alkaline copper sugar reagents (e.g. Fehling, Scales) which were reduced at room temperature in a few seconds.

Several reactions proved the presence of a carbonyl function: the condensation at room temperature with phenylhydrazine derivatives, the formation of an enediol in alkaline and acid conditions, the condensation with *o*-phenylenediamine. The Schiff reagent was not reduced.

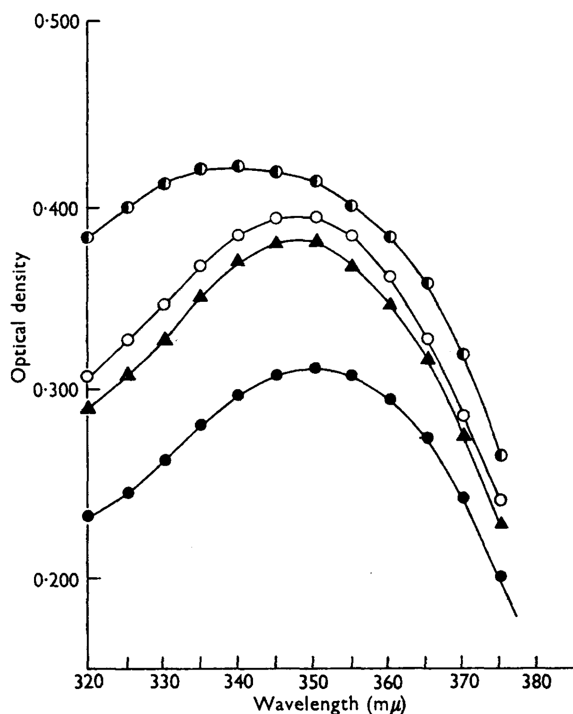


Fig. 1. Absorption spectra of condensation products of *o*-phenylenediamine HCl with RL (◐—◐), RLB (●—●), RMB (▲—▲) (all three: 33·3 μg./ml.), and methyl-β-3-keto-D-glucopyranoside (16·65 μg./ml.) (○—○).

The condensation product from the reaction with *o*-phenylenediamine HCl at 100° (Lanning & Cohen, 1951) showed an absorption maximum at 350 mμ for RMB and RLB, and at 335 mμ for RL and RM (Fig. 1). We found that methyl-β-3-keto-D-glucopyranoside, described by Lindberg & Theander (1954), also reacted in the same conditions and that the condensation product had a maximum at 350 mμ. Our compounds also reacted with semicarbazide (MacGee & Doudoroff, 1954), the final product having an absorption maximum at 230 mμ.

Although the oxidation products displayed nearly identical chemical

properties, they were different as shown by their R_F values (0·56 for RL; 0·48 for RLB) on paper chromatograms with methanol, pyridine, water (6+2+2). Each disaccharide and its corresponding bionate was thus not oxidized to the same product, as could be expected by analogy with oxidation of glucose and gluconate to 2- or 5-ketogluconate. Paper chromatography showed that only one strongly reducing compound was formed from each substrate. In several solvents the spots of RMB and RLB showed some tailing, probably due to the ionization of the carboxyl function. This phenomenon was currently observed in the chromatography of sugar acids. The presence of a carboxyl group in RLB and RMB was further indicated, because they remained partially absorbed on weak anion exchangers (Merck II). RL and RM passed through these columns. The four compounds were adsorbed on strong anion exchangers, such as Merck III or Amberlite IRA 400, and they were altered by this treatment. After elution, which was possible only with strong acids, they now reduced 2,6-dichlorophenolindophenol, iodine, etc., a property which they did not possess before ion-exchange treatment, and indicated strongly the transformation of the carbonyl function into an enediol.

The structure of the oxidation products from lactose and lactobionate (RL and RLB)

Hydrolysis with mineral acids; identification of glucose, or gluconate. When the hydrolysis was carried out in acid conditions, the solutions of both RL and RLB darkened rapidly and black precipitates were formed. Samples (*c.* 100 mg.) of chromatographically pure RL or RLB were hydrolysed for 1 hr. at 100° in 20 ml. 1·5 N-H_2SO_4. After cooling, the acid was neutralized with $BaCO_3$, the precipitates filtered off and washed. The filtrate and the washings were combined. The solutions no longer displayed the strongly reducing properties. In the filtrate from the hydrolysate of RL, reducing substances were now present which reduced Fehling and Scales solutions upon boiling. In contrast, the filtrate obtained from RLB was scarcely reducing.

The filtrate obtained from RL contained glucose, which was identified as follows. The solution was first treated with an excess of Merck I and II ion exchanger, followed by a small amount of charcoal and dried by lyophilization (yield 35 mg.). A sample was analysed by descending paper chromatography, on no. 2043*a* Schleicher & Schull paper, with solvent D. With *p*-anisidine HCl and other spray reagents only one spot was observed, which was identical to glucose in every respect. Galactose was absent. The lyophilizate was dissolved in 35 ml. water and a sample oxidized with notatin (Boehringer, Mannheim) in the Warburg apparatus according to Keilin & Hartree (1948). (This notatin preparation contained catalase.) The sample contained about 60 % of glucose.

In the solution derived from RLB, gluconate was detected as follows. By paper chromatography the presence of two gluconolactones could be detected. The solution was treated with an excess of MerckI cation exchanger, neutralized with NaOH to open the lactone rings and lyophilized. The powder was dissolved in the smallest amount of a mixture of 70 ml. absolute ethanol + 30 ml.

of water. The ethanolic solution was poured on a Florex column (length 8 cm.; diameter 1·6 cm.), impregnated with the same solvent. The chromatogram was developed with 25 ml. of the same ethanolic solution and the column pushed out of the tube; by brushing it with $KMnO_4$+KOH one main zone showed up. The column was cut into 1 cm. disks and every portion eluted at once four times with 5 ml. portions of water. The extracts were dried separately by lyophilization. Gluconate was detected as the lactone Fe-hydroxamate, according to Hestrin (1949). The reaction was positive only in those fractions which corresponded to the main absorption band. The presence of gluconolactones was confirmed, after lactonization, by paper chromatography with solvent F. Two spots were obtained, whose R_F values (0·42 and 0·23) were identical with those given by authentic γ- and δ-gluconolactones. No reducing sugars were present. The gluconate fractions were pooled, weighed (20 mg. from 92 mg. RLB) and dissolved in 20 ml. of water. A sample was oxidized by a specific particulate gluconate oxidase from *Acetobacter suboxydans* (De Ley & Stouthamer, 1959) in the Warburg apparatus at 30°. The rate of O_2 uptake was compared with that obtained by using a standard solution of authentic gluconate of the same concentration. The sample contained about 40 % of pure gluconate.

Detection of glucose after hydrolysis of RL with β-galactosidase. Pure RL (100 mg.) was dissolved in 1 ml. 0·02 M phosphate buffer (pH 7) to which 30 mg. of a β-galactosidase preparation and a small drop of toluene were added. The mixture was incubated at 30° and every 24 hr. a sample was analysed by paper chromatography. Control experiments without substrate or with potassium lactobionate were also run. The solution with RL turned brown; after 72 hr. it was very dark. The chromatograms in several solvents showed different reducing substances, one of which was identified as glucose. This spot was particularly distinct after 72 hr of enzymic hydrolysis. A small amount was isolated by paper chromatography. Using solvents C or D, it was separated from galactose. The R_F values were 0·38 and 0·36 for glucose and 0·34 and 0·31 for galactose. Galactose was also detected in the control experiments with potassium lactobionate. No carbohydrate was present in the control without substrate.

Identification of glucose, galactose, gluconate and sorbitol after reduction of RL and RLB, followed by acid hydrolysis. The previous experiments showed that either enzymic or acid hydrolysis occasioned the decomposition of the galactose moiety of RL and RLB. Glucose or gluconate had been recovered unchanged, and hence it was obvious that the carbonyl function was located in the galactose part of the molecule. In order to locate this group, we decided to reduce it and to determine the nature of the sugars formed. If, for example, C_2 bears the C=O group, reduction will yield galactose and talose; if it is located at C_3, galactose and gulose are to be expected. The reduction of RL with $NaBH_4$ in alkaline conditions at room temperature transforms the glucose moiety into sorbitol, as described for the reduction of lactose to lactitol (Abdel-Akher *et al.* 1951). The gluconate part of RLB will remain unchanged.

When solutions of pure RL or RLB (*c.* 100 mg. in 2 ml. water) were reduced

in this way (15 mg. $NaBH_4$ in 1 ml. water) they lost their reducing properties completely after 1–2 hr. After destroying the $NaBH_4$ and eliminating the boric acid, the syrup was hydrolysed with sulphuric acid as described above. The usual copper sugar reagents were now reduced only upon boiling.

The solution obtained from RL was neutralized with $BaCO_3$, purified with Merck I and II mixed ion exchangers and lyophilized. The residue was re-dissolved in a small amount of a mixture of 90 ml. absolute ethanol + 10 ml. water and poured on a pre-wetted Florex column (height 8 cm.; diameter 1·6 cm.). The chromatogram was developed with the same aqueous ethanol concentration until all the reducing substances had passed through. The column was then cut into 1 cm. disks and the adsorbed substances recovered as described above. A non-reducing compound was obtained in good yield (32 mg.); it was identified as sorbitol. After acetylation, crystals of the hexa-acetate were obtained (m.p. 98°, not recrystallized; reported in the literature 99·5°). The mixed melting point with authentic sorbitol hexa-acetate was unchanged.

The solution obtained from RLB was treated in a slightly different manner. It was neutralized with $BaCO_3$, purified with an excess of cation exchangers, neutralized with NaOH and lyophilized (yield: 32 mg.). The powder was dissolved in a small amount of a mixture of 70 ml. absolute ethanol + 30 ml. water and reducing substances were eliminated by chromatography on a Florex column. From this column gluconate was obtained after elution; it was identified as above. The yield was about 80 %.

The reducing substances which passed through the columns were further analysed by paper chromatography after purification with an excess of Merck I and II ion exchangers. After development with solvents C or D, two spots of similar intensity and a third much weaker spot were obtained from both solutions (derived from RL of RLB) after reduction and hydrolysis. The first spot behaved like galactose (R_F values: 0·34 and 0·31), the second spot like gulose or mannose (which had the same R_F values in these solvents: 0·40 and 0·41), and the weak one like talose (R_F values: 0·46 and 0·53). Solvent E on descending chromatograms for 48 hr. separated gulose from mannose, the latter moving 70 % as fast as the former. In this way it was shown that the second spot behaved like gulose.

When the reduction was carried out with borohydride in acid medium, the presence of glucose, galactose and gulose was easily shown, but talose was absent. When H_2 and Raney nickel were used, galactose and gulose were found, talose being again absent.

To obtain larger amounts of the sugars for the final identification, the following procedure was adopted. The time course of lactobionate assimilation in a culture (200 ml. containing 4 % of this carbohydrate) was followed until only a faint trace of the substrate remained. After centrifugation, precipitation of the proteins and elimination of the salts present, the clear supernatant liquid was reduced at once at 70° with H_2 in the presence of *c.* 40 g. Raney nickel. The reduction was continued until the reducing property of the solution had disappeared. Hydrolysis was carried out as usual. The remaining gluconate

was adsorbed on a large column (height 9 cm.; diameter 3·7 cm.) of active clay, the reducing fraction of the eluate purified with ion exchangers and concentrated (yield 1·1 g.). The remaining syrup was extracted with 20 ml. methanol and a Whatman MM disk impregnated with the latter solution. After drying the disk was chromatographed on a Grycksbo paper column with solvent E. Gulose was eluted after 15 l. solvent had run through; 172 mg. of pure gulose in 5 l. solvent were collected. Galactose appeared later and about 17 l. were still necessary to elute it nearly completely. A first fraction of 103 mg. galactose contained some gulose as impurity. Afterwards 108 mg. pure galactose were obtained. 100 mg. of the syrup presumed to be gulose were converted to the phenylhydrazone (Fischer & Stahel, 1891), which was recrystallized from absolute ethanol and dried. The yellow crystals melted at 139° (reported 143°). Authentic D-gulose was prepared by reduction of gulono-γ-lactone with $NaBH_4$ in acid medium, the solution neutralized and gulonic acid removed on a column of active clay. The phenylhydrazone, prepared as described above. melted at 138°; the mixed melting point being unchanged.

The presence of galactose was confirmed as follows: 108 mg were converted into the 1-methyl-1-phenyl hydrazone (Hirst, Jones & Woods, 1947) and the white crystals recrystallized from ethanol; m.p. 183–184° (reported 186°).

The structure of the oxidation products from maltose and maltobionate (RM and RMB)

Hydrolysis with mineral acids; identification of gluconate from RMB. When RMB was hydrolysed with acid, the solution darkened as described for RL and RLB. Nevertheless, gluconate was detected by paper chromatography and also as the Fe-hydroxamate of the lactone.

Identification of allose, glucose and gluconate after reduction of RM and RMB followed by acid hydrolysis. As RMB was very sensitive to the action of alkali, the reduction with $NaBH_4$ in alkaline medium was not used but H_2 and Raney nickel instead. Pure RMB (100 mg.) was completely reduced in less than 1 hr. and hydrolysed. The strong reducing property had completely disappeared and the usual sugar reagents were only reduced upon boiling. The solution was treated in the same way as described for RLB. Gluconate remained absorbed on the clay column and was identified in the eluate by paper chromatography and as the Fe-hydroxamate. It was shown on descending chromatograms run for 48 hr. with solvent E that the reducing sugars which had passed through the clay column consisted of two aldohexoses. As expected, the first behaved like glucose and the second like allose. With solvent E the migration speed of glucose is 77 % of that of allose. When a solution of crude RM was treated in the same way, the same two sugars were detected.

Larger amounts of glucose and allose were prepared from RMB by a procedure nearly identical with that described for RLB. The crude sugar mixture (1·2 g.) freed from gluconate, was put on the top of the column and the development performed with solvent E. Allose appeared after 19 l. solvent had passed through; 9 l. were necessary to elute 297 mg. chromatographically

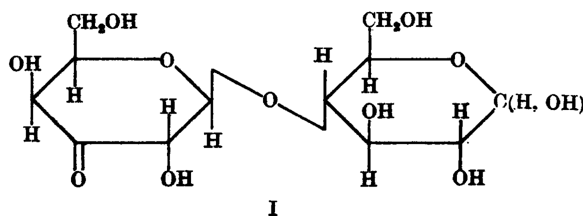
pure sugar. The following 8 l. contained 200 mg. glucose with traces of allose. After a total of 36 l. solvent had been used, glucose was still being eluted. The presumed allose was dissolved in a small amount of water to a thick syrup and thrice its volume of methanol added. After a few days 106 mg. of white needles had separated; m.p. 128° (reported 129°); mixed melting point with pure D-allose unchanged. Some (50 mg.) of these crystals were converted to the phenylhydrazone; after recrystallization from a mixture of equal volumes of absolute ethanol and ether, the crystals melted at 169° (reported 173°).

A sample of the pooled and lyophilized glucose fractions was dissolved in water (0·1 % solution) and samples oxidized with glucose oxidase. About 80% of the syrup was pure glucose.

In other experiments, mixtures of both aldohexoses were used. Glucose was fermented by a strain of *Saccharomyces cerevisiae* but allose was not attacked. To remove the salts required for the fermentation, the solution was purified with ion exchangers and further on a clay column. Allose crystallized after a few days from the concentrated solution.

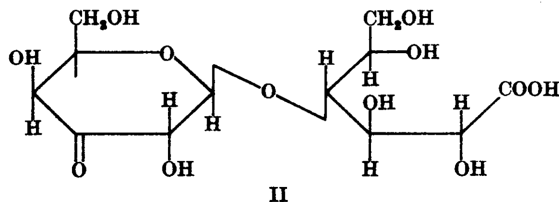
DISCUSSION

The oxidation products formed from lactose, maltose and the corresponding bionates by the action of bacterial strains presumed to be *Alcaligenes faecalis* during growth in aerobic conditions are carbohydrate derivatives which have not before been described. We suggest that the oxidation product RL, derived from lactose, is 4-0-β-3-keto-D-galactosido-D-glucose:



I

and that RLB, derived from lactobionate, is 4-0-β-3-keto-D-galactosido-D-gluconate:



II

The compound RMB, derived from maltobionate, is 4-0-α-3-keto-D-glucosido-D-gluconate. Although the yield of the oxidation product of maltose (RM) was too small to allow a thorough chemical study, the evidence suggests that it has a similar structure, namely, 4-0-α-3-keto-D-glucosido-D-glucose.

These conclusions are based on the following arguments:

(1) Acid hydrolysis liberates glucose from RL and gluconate from RLB and RMB. The remaining part of the molecules must be very unstable, since it yields dark condensation products, even with such a mild treatment as with β-galactosidase. 3-Ketoglucose or 3-ketogalactose could not be isolated as such. Several unidentified compounds were detected after chemical or enzymic hydrolysis; this agrees with the expected instability of a 3-keto-sugar.

(2) These compounds exhibit strongly reducing properties with the usual sugar reagents at room temperature, a behaviour similar to that of ascorbic acid, osones and methyl-β-3-keto-D-glucopyranoside. The presence of a keto group could be shown by the reaction with phenylhydrazine derivatives, *o*-phenylenediamine, semicarbazide and by the formation of an enediol function. This keto-group cannot be located in the aglycon moiety of the compounds.

(3) Reduction with H_2 and Raney nickel at 70° or with $NaBH_4$ results presumably in the formation of two non-reducing disaccharide derivatives from each compound: galactosido- and gulosido-sorbitol from RL, galactosido- and gulosido-gluconate from RLB, glucosido- and allosido-gluconate from RMB and glucosido- and allosido-sorbitol from RM. It is hoped to report on the physical and chemical properties of these compounds later. Hydrolysis of these compounds yields sorbitol, gluconate and the expected reducing sugars. This shows that the original oxidation products are compounds in C_{12}. The formation of nearly identical amounts of galactose and gulose, and of glucose and allose, shows that in each case the C=O function is located at C_3 of the galactosyl or glucosyl-part of the respective molecules. The formation of a trace of talose after alkaline reduction and hydrolysis of both RL and RLB shows that a slight amount of an enediol between C_2 and C_3 had been formed.

It seems highly improbable that changes other than oxidation at C_3 could have occurred; otherwise other sugars would have been obtained after reduction and hydrolysis. The oxidation of the C_6 of the glycosyl moiety to an aldehyde could not be detected after reduction and hydrolysis, but it should have been detectable by the reduction of the Schiff reagent, as described by Lindberg & Theander (1954).

The formation of these new compounds is interesting for several reasons. They point to a new and entirely unsuspected pathway of disaccharide catabolism in some bacteria; it is the first example of an enzymic oxidation which occurs in the glycosyl moiety of disaccharides and derivatives, whereas it would normally be expected in the aglycon part; they constitute the first examples of 3-ketosugar derivatives being isolated as intermediates in a metabolic pathway.

One of us (J. De L.) is an Associé du Fonds National de la Recherche Scientifique (Belgium).

Thanks are due to Professor Dr J. Wiame, the University of Brussels, for the gift of a sample of β-galactosidase and to Dr H. S. Isbell, National Bureau of Standards, Washington, D.C., U.S.A., for providing samples of D-talose and D-allose. One of us (M. J. B.) acknowledges with pleasure the technical assistance of Mr J. Furnelle.

REFERENCES

ABDEL-AKHER, M., HAMILTON, J. K. & SMITH, F. (1951). The reduction of sugars with sodium borohydride. *J. Amer. chem. Soc.* **73**, 4691.

ABDEL-AKHER, M. & SMITH, F. (1951). The detection of carbohydrate esters and lactones after separation by paper chromatography. *J. Amer. chem. Soc.* **73**, 5859.

BERNAERTS, M. J. & DE LEY, J. (1958). 3-Ketoglycosides, new intermediates in the bacterial catabolism of disaccharides. *Biochim. biophys. Acta*, **30**, 661.

BERNAERTS, M. J. & DE LEY, J. (1960). Microbiological formation and preparation of 3-ketoglycosides from disaccharides. *J. gen. Microbiol.* **22**, 129.

BINKLEY, W. W. & WOLFROM, M. L. (1948). *Chromatography of Sugars and Related Substances.* New York: Sugar Research Foundation, Inc.

DE LEY, J. & STOUTHAMER, A. H. (1959). The mechanism and localisation of hexonate metabolism in *Acetobacter suboxydans* and *Acetobacter melanogenum*. *Biochim. biophys. Acta*, **34**, 171.

FISCHER, E. & STAHEL, R. (1891). Zur Kenntniss der Xylose. *Ber. dtsch. chem. Ges.* **24**, 528.

HAGDAHL, L. & DANIELSON, C. E. (1954). A new column for preparative chromatography. *Nature, Lond.* **174**, 1062.

HESTRIN, S. (1949). The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. *J. biol. Chem.* **180**, 249.

HIRST, E. L., JONES, J. K. & WOODS, E. A. (1947). The quantitative determination of galactose, mannose, arabinose and rhamnose. *J. chem. Soc.* p. 1048.

KARABINOS, J. V. & BALLUN, A. T. (1953). Direct reduction of aldoses and ketoses by Raney nickel. *J. Amer. chem. Soc.* **75**, 4501.

KEILIN, D. & HARTREE, E. F. (1948). The use of glucose oxidase (notatin) for the determination of glucose in biological material and for the study of glucose-producing systems by manometric methods. *Biochem. J.* **42**, 230.

LANNING, M. C. & COHEN, S. S. (1951). The detection and estimation of 2-ketohexonic acids. *J. biol. Chem.* **189**, 109.

LEW, B. W., WOLFROM, M. L. & GOEPP, R. M. (1946). Chromatography of sugars and related compounds. *J. Amer. chem. Soc.* **68**, 1449.

LINDBERG, B. & THEANDER, O. (1954). The oxidation of glycosides. IV. Oxidation of methyl β-D-glucoside with dichromate. *Acta chem. scand.* **8**, 1870.

MACGEE, J. & DOUDOROFF, M. (1954). A new phosphorylated intermediate in glucose oxidation. *J. biol. Chem.* **210**, 617.

WOLFROM, M. L. & KIMIKO, A. (1952). Sodium borohydride as a reducing agent in the sugar series. *J. Amer. chem. Soc.* **74**, 5583.

(*Received* 6 *July* 1959)