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Carbohydrases of a Rumen Strain of *Lactobacillus bifidus*

2. THE INTRACELLULAR α-1→6-GLUCOSIDASE (ISOMALTODEXTRINASE)*

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Bailey & Clarke (1959) gave an account of an extracellular dextranase secreted by several strains of *Lactobacillus bifidus*, two of which had been isolated from the rumen of a cow (Clarke, 1959). Hydrolysis of dextran by the dextranase was unusual in that it did not liberate glucose or isomaltose, which are the main products from the action of all other known dextranases on dextran (see Bailey & Clarke, 1959). Instead the enzyme hydrolysed dextran, by random cleavage of the α-1→6-glucosidic links, to a mixture of isomaltotriose, -tetraose, -pentaose and higher isomaltodextrins. The organism itself grew readily in a medium containing isomalto-tetraose and -pentaose as sole carbohydrates, suggesting the presence of an intracellular enzyme capable of hydrolysing these short-chain isomaltodextrins. Such an enzyme would be of interest, as none of the other dextran-fermenting organisms would need to produce it.

The present paper contains an account of the α-1→6-glucosidase activity present in extracts prepared from *Lb. bifidus* cells that had been grown on dextran.

EXPERIMENTAL

Lactobacillus bifidus, strain D13/293 (Clarke, 1959), was used after revival from a freeze-dried culture that had been prepared from cells grown in dextran-free, glucose–tomato juice medium (Clarke, 1959). The revived cells grew readily in the glucose–tomato juice medium but failed completely to grow in the dextran medium (Bailey & Clarke, 1959). The organism was, therefore, cultured in a modified dextran medium containing glucose (0·1 %). After several attempts, each time with an inoculum from a glucose–tomato juice culture and prolonged (4–6 days) incubation of the cultures, the growth of the organism suddenly changed from very slow to vigorous. It was then able to grow in the normal dextran medium with good production of its typical dextranase. The organism was maintained in this condition by frequent subculture in the normal dextran medium. All cultures were incubated, in McIntosh & Fildes jars containing $H_2 + CO_2$ (95:5, v/v), at 37–38°.

Cell extracts. Dextran medium (500 ml.) was inoculated with the organism capable of rapid growth and incubated for 48 hr., by which time the pH of the culture had fallen from 7·0 to 5·8 and the cell-free culture fluid contained an appreciable amount of dextranase activity. Cells were harvested by centrifuging for 30 min. (25 000*g*) and washed twice by shaking them in buffer (acetate or phosphate–citrate, pH 6·0) for 10 min. and recentrifuging. The washed cells were suspended in the same buffer (10 ml.), disintegrated at 2° in a Nossal (1953) shaker, diluted with more buffer (15–20 ml.), extracted for 5 min. at 0° in a Teflon tissue homogenizer and finally centrifuged for 30 min. (25 000*g*) at 0°. The final extract, from the cells from a culture of 500 ml., was diluted with buffer to 40 ml. Extracts were stored at 2° under toluene.

Three standard extracts were prepared; *A* in acetate buffer (0·05M) and *B* and *C* in phosphate–citrate buffer (McIlvaine, 1921). All three preparations hydrolysed the same range of sugars and hydrolysed isomaltodextrins at about the same rate and in the same manner. Reaction studies, at pH 6·0, indicated that, compared with phosphate or citrate, acetate had a slightly depressing effect on the α-1→6-glucosidase activity (see below). Except where stated otherwise, the results reported in this paper were obtained with the phosphate–citrate extracts. Extract *B*, which was used in all of the quantitative results described below, contained 0·73 mg. of N/ml.

Carbohydrates. All carbohydrates used were from the usual commercial sources except for the isomaltodextrins and the following compounds: panose (*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose), isolated from a *Leuconostoc mesenteroides* sucrose–maltose culture (Bailey, Barker, Bourne & Stacey, 1957); leucrose (5-*O*-α-D-glucopyranosyl-D-fructopyranose), isolated from a *Streptococcus bovis* sucrose culture (Bailey & Bourne, 1959); dextran, also from a *S. bovis* sucrose culture (Bailey, 1959); isomaltulose (6-*O*-α-D-glucopyranosyl-D-fructofuranose), nigerose (3-*O*-α-D-glucopyranosyl-D-glucopyranose) and sophorose, provided by Dr F. H. Stodola, Dr D. H. Hutson and Dr M. J. Clancy respectively. Isomaltodextrins were isolated from a partial acid hydrolysate of *S. bovis* dextran (Bailey & Clarke, 1959).

All sugars were shown, by paper chromatography, to be pure or to contain only traces (less than 0·1 %) of other sugar impurities. The anhydro-glucose content of each isomaltodextrin was measured by an anthrone method suitable for hexoses (Bailey, 1958). The amounts of isomaltodextrins used in the various quantitative experiments, given as mg. of pure sugar, were corrected for the trace of non-sugar impurity present and, in the case of isomaltose, for the moisture content.

Reducing sugars. These were measured in enzyme digests by the cuprimetric method of Shaffer & Hartmann (1921); all results were calculated as glucose. In this method a

* Part 1: Bailey & Clarke (1959).

Table 1. *Hydrolysis of sugars by extracts from dextran-fermenting cells of* Lactobacillus bifidus

Solutions containing cell extract (0·25 ml.), phosphate–citrate buffer (pH 6·0; 0·25 ml.) and carbohydrate (5 mg.) were incubated at 38° for 24 hr. and analysed by paper chromatography. The degree of hydrolysis was assessed by grading visually the intensity of sugar spots, corresponding to hydrolysis products, on the scale: − (no (hydrolysis) and + (faint spot, trace hydrolysis) to + + + + + + (very intense spots, original substrate entirely hydrolysed).

Added sugar	Degree of hydrolysis	Probable identity of hydrolysis products
Isomaltose	+ + + + + +	Glucose
Isomaltotriose	+ + + + + +	Glucose, isomaltose (weak)
Isomaltotetraose	+ + + + + +	Glucose, isomaltose and isomaltotriose (both weak)
Panose	+ + + +	Glucose, maltose
Isomaltulose	+ + + +	Glucose, fructose
Melibiose	+	Glucose, galactose
Nigerose	+ + + +	Glucose
Gentiobiose, cellobiose, sophorose	+ + + + +	Glucose
Arbutin, salicin, amygdalin	+ + + + + +	Glucose
Maltose, turanose, αα-trehalose, sucrose, lactose	+	Expected monosaccharides
Methyl α-glucoside, leucrose, raffinose	−	.

heating time of 15 min. had been shown to be satisfactory for isomaltodextrins (Bailey & Clarke, 1959).

Paper-chromatographic analyses. For routine analysis papers were developed with ethyl acetate–water–pyridine (2:2:1, by vol.; Jermyn & Isherwood, 1949) and sprayed with $AgNO_3$ (Trevelyan, Procter & Harrison, 1950). In most cases solutions were first desalted with Bio-Deminrolit (Permutit Co. Ltd.) which had been carbonated with CO_2 gas; with the above solvent system, however, phosphate–citrate buffer did not interfere with the location of mono-, di- or tri-saccharides. Other solvents and sprays used in the identification of hydrolysis products were: Solvents; *n*-butanol–ethanol–water (40:11:19, by vol.; Andrews, Ball & Jones, 1953), a modified version of the ethyl acetate–water–pyridine solvent (Malpress & Hytten, 1958); Sprays; aniline hydrogen phthalate (Partridge, 1949), *p*-anisidine (Hough, Jones & Wadman, 1950), diphenylamine–aniline (Bailey & Bourne, 1960) and benzylamine–ninhydrin (Bayly & Bourne, 1953).

RESULTS

Carbohydrase activity of cell extracts from Lactobacillus bifidus *cells grown on dextran*

Action on glucose. A solution containing cell extract (*B*; 0·5 ml.), phosphate–citrate buffer (pH 6·0; 1·3 ml.) and glucose (5 mg.) was incubated at 38° and at intervals (30 min.) portions (0·4 ml.) were analysed for glucose. The results showed that there was no loss of glucose during 1·5 hr. of incubation.

Hydrolysis of various carbohydrates. Standard solutions were prepared containing cell extract (*A* or *B*; 0·25 ml.), phosphate–citrate buffer (pH 6·0; 0·25 ml.; McIlvaine, 1921) and carbohydrate (5 mg.). Appropriate control solutions omitting the cell extract and added carbohydrate respectively were also prepared. All of the solutions were incubated, under toluene, at 38° for 24 hr. and analysed by paper chromatography both before and after incubation. The results obtained are listed in Table 1.

Only added compounds were detected in the solutions where no hydrolysis occurred and in the carbohydrate controls. Likewise the controls containing cell extract only showed no detectable sugar either before or after incubation. Of the α-linked sugars, the only ones hydrolysed to any extent, apart from nigerose, were those containing α-1→6-links. The extracts were, therefore, used without further purification for a more detailed study of the α-1→6-glucosidase.

Hydrolysis of isomaltodextrins. Standard cell-extract (*A* or *B*) solutions containing one each of a series of isomaltodextrins (5 mg.) were incubated at 38° and analysed by paper chromatography at intervals. Pure isomaltodextrins of degree of polymerization (D.P.) $n = 2$–7 respectively and a mixture of isomaltodextrins $n = 8$ and 9 were used; controls showed that they were not hydrolysed when incubated alone in the buffer. During the first hour of incubation all of the solutions contained, in addition to the added sugar, easily detected amounts of glucose and the isomaltodextrin of D.P. $= n-1$. In solutions containing isomaltotetraose or higher isomaltodextrins, homologues of D.P. $= n-2$ or lower could scarcely be detected during the first hour and only increased gradually as the incubation proceeded. There was some evidence for the formation, presumably by transferase action, of a higher homologue in solutions containing isomaltodextrins of D.P. $n = 2$, 3, 4 and 5. The rate of accumulation of glucose in the solutions suggested that the isomaltodextrins of D.P. $n = 2$, 3, 4 and 5 were hydrolysed at about

the same rate but that the higher homologues were hydrolysed at slower rates.

Hydrolysis of dextran. The cell extracts were tested for extracellular dextranase as described by Bailey & Clarke (1959). Only a trace of dextranase activity was detected and the amount was not considered sufficient to affect the quantitative results obtained with isomaltodextrins of D.P. $n = 5$ or higher. Chromatographic examination of the dextran–cell extract solutions during the first 1–3 hr. incubation showed no sign of glucose or any oligosaccharide.

Hydrolysis of isomaltotetraitol. A more definite indication of the mode of action of the α-1→6-glucosidase on isomaltodextrins was obtained by examining its action on the tetrasaccharide alcohol prepared by reducing isomaltotetraose. Isomaltotetraose (30 mg.) in water (30 ml.) was treated with sodium borohydride (100 mg.) at room temperature for 2 hr. After removal of sodium with IR-120 resin and boric acid by distillation with methanol the tetraitol was purified by preparative chromatography on thick paper (Whatman no. 3MM). The sugar was shown to be non-reducing by its slow reaction with the silver nitrate spray and failure to react with the *p*-anisidine and aniline hydrogen phthalate sprays.

A standard cell-extract solution (extract *B*) containing isomaltotetraitol (5 mg.) was incubated at 38° and analysed by paper chromatography at intervals. The chromatograms showed that glucose and non-reducing trisaccharide were present in the solution after only 30 min. incubation. The glucose content increased steadily as the incubation proceeded but reducing components corresponding to isomaltose or isomaltotriose were not detected at any stage. The identity of the glucose was confirmed with the various spray reagents. Inspection of the intensity of the glucose spots on the papers indicated that the enzyme hydrolysed isomaltotetraitol at about the same rate as isomaltotetraose.

Hydrolysis of sugars by extracts from Lactobacillus bifidus *cells grown on various carbohydrates.* After passage through several dextran cultures the organism was subcultured twice in media in which the dextran had been replaced by glucose (1 %, w/v), sucrose (2 %, w/v) or starch (1 %, w/v), and finally grown in each of these media (500 ml.). Cells were harvested, disintegrated and extracted into phosphate–citrate buffer in the usual way. Standard cell-extract solutions were prepared containing isomaltotetraose, arbutin, sucrose or nigerose, incubated under toluene at 38° for 24 hr. and analysed by paper chromatography. The results obtained are shown in Table 2.

Effect of various conditions on the α-1→6-glucosidase activity

In preliminary experiments solutions containing cell extract, phosphate–citrate buffer and isomaltose, isomaltotriose or isomaltotetraose were incubated at 38° and the reducing-sugar content was measured at intervals. The results showed that the rate of increase in reducing sugar was linear with time provided that not more than 40 % of the glucosidic links had been hydrolysed. In measuring the effect of various conditions on enzyme activity, with tri- or tetra-saccharide as substrate, digest composition was so arranged that not more than 30–40 % of the links were hydrolysed in 1 hr. at 38°. In comparing the rate of hydrolysis, by the enzyme, of isomaltodextrins of different D.P.'s, concentrations were chosen so that, assuming, from the chromatographic evidence, a multi-chain endwise cleavage of glucose units, not more than 30–40 % of the link at the non-reducing end of the added sugar was hydrolysed in 1 hr. at 38°. In all of the quantitative work the solutions used were preheated for 15 min. at 38° before being measured and mixed. Solutions were covered with toluene during incubation.

Effect of buffer ions on enzyme activity. Solutions containing cell extract (*A*; 0·8 ml.), buffer (0·1 M; pH 6·0; 0·9 ml.) and isomaltotriose (4·6 mg. in water; 0·1 ml.) were incubated at 38° and reducing sugars measured in portions (0·5 ml.) after incubation for 0, 30 and 60 min. When the added buffer was phosphate or citrate the rate of increase in reducing-sugar concentration was the same; from 1·54 to 2·52 mg. in 1 hr. With acetate buffer the increase was from 1·54 to 2·38 mg.

Effect of pH *on enzyme activity.* Solutions con-

Table 2. *Hydrolysis of sugars by extracts from* Lactobacillus bifidus *cells grown on various carbohydrates*

Solutions containing cell extract (0·25 ml.), phosphate–citrate buffer (pH 6·0; 0·25 ml.) and sugar (5 mg.) were incubated at 38° for 24 hr. and analysed by paper chromatography. Scale for degree of hydrolysis was as in Table 1.

Carbohydrate in culture medium	Degree of hydrolysis by cell extracts			
	Isomaltotriose	Arbutin	Sucrose	Nigerose
Dextran	+ + + + + +	+ + + + + +	+	+ + + +
Glucose	+ + + +	+ + + +	–	+ +
Sucrose	+ +	+ + + +	+ + + + +	+ + +
Starch	+ + +	+ + + + + +	–	+ + +

taining cell extract (*B*; 0·5 ml.), phosphate–citrate buffer (pH 4·0–6·0; 1·2 ml.) and isomaltotriose (4·6 mg. in water; 0·1 ml.) were incubated at 38° and analysed for reducing sugars after 0, 30 and 60 min. The results obtained, as a plot of increase in reducing-sugar concentration against pH, are given in Fig. 1. There was an apparent loss of reducing sugars in solutions buffered below pH 5·0; a heavy white precipitate, possibly insoluble protein, formed during the incubation of these solutions, and it may have affected the reducing-sugar measurements.

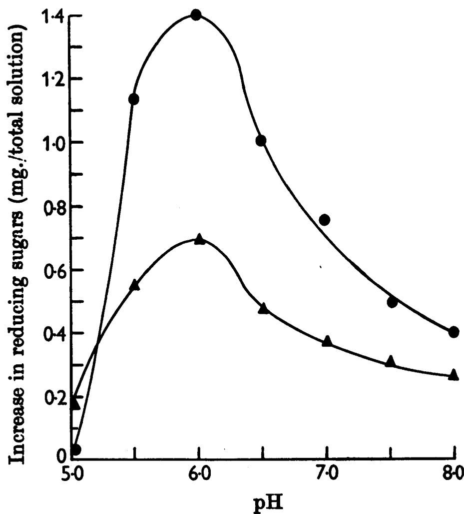

Fig. 1. Effect of pH on α-1→6-glucosidase activity. Solutions containing cell extract (*B*; 0·5 ml.), phosphate–citrate buffer (1·2 ml.) and isomaltotriose (4·56 mg. in water, 0·1 ml.) were incubated at 38°, and reducing sugars measured after 0, 30 and 60 min. Increase in reducing sugars, as glucose, after 30 min. (▲), and 60 min. (●).

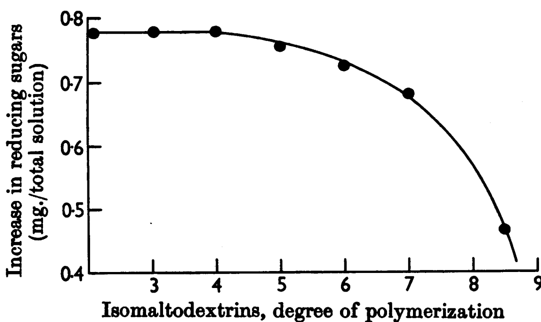

Fig. 2. Rate of hydrolysis of isomaltodextrins by α-1→6-glucosidase. Solutions containing cell extract (*B*; 0·5 ml.), phosphate–citrate buffer (pH 6·0; 1·4 ml.) and isomaltodextrin (0·023 m-mole, in water, 0·4 ml.) were incubated at 38° and reducing sugars measured after 0, 20, 40 and 60 min. Increase in reducing sugars, as glucose, after 40 min.

Effect of temperature on enzyme activity. Solutions similar to those used for measuring the effect of pH on activity were prepared except that, in view of a shortage of trisaccharide, tetrasaccharide was used as substrate and the pH of the buffer was 6·0. The solutions were incubated at various temperatures (30–50°), and reducing sugars measured after 0, 30 and 60 min. The results obtained, after both time intervals, showed that the enzyme possessed optimum activity within the temperature range 36–41° and that above 41° activity declined rapidly.

Effect of D.P. of the isomaltodextrin on enzyme activity. Solutions containing cell extract (*B*; 0·5 ml.), phosphate–citrate buffer (pH 6·0; 1·4 ml.) and isomaltodextrin (0·023 m-mole, in water, 0·4 ml.) were incubated at 38°, and the reducing-sugar content was measured after 0, 20, 40 and 60 min. Pure isomaltodextrins of D.P. 2–7 respectively and a fraction containing approximately equal amounts of isomaltodextrins of D.P. 8 and 9 were used. With each isomaltodextrin a plot of increase in reducing-sugar concentration against time was a straight line during 1 hr. In Fig. 2 the results obtained are shown as a plot of increase in reducing-sugar concentration, after 40 min. incubation, against the D.P. of the isomaltodextrin.

Transferase activity of the cell extract

Solutions containing cell extract (*B*, 0·1 ml.), phosphate–citrate buffer (pH 6·0; 0·2 ml.) and isomaltotriose (0·75, 1·5, 3·0 and 15·0 mg. respectively) were incubated at 38° for 1 hr. and analysed by paper chromatography. Spots corresponding to glucose and isomaltose were present on chromatograms of all four solutions. A single reducing component, moving at the expected rate for isomaltotetraose, was also present on the papers of the solutions containing 1·5 mg. (weak spot) and 3·0 and 15·0 mg. (both strong spots) of isomaltotriose, but was absent from the chromatogram of the solution containing only 0·75 mg. of sugar. When the solutions contained isomaltose instead of isomaltotriose, a component moving at the expected rate for isomaltotriose could be detected on papers if the original solution contained 3 mg. or more of isomaltose.

DISCUSSION

The occurrence in *Lb. bifidus* cells grown on dextran of an enzyme hydrolysing isomaltodextrins to glucose is as was expected from the known liberation of these oligosaccharides from dextran by the organism's extracellular dextranase. The difficulties encountered in reviving the organism as a dextran fermenter are consistent with

the selection of a mutant capable of using dextran. The decline in α-1→6-glucosidase activity in cells grown on sucrose (Table 1) is consistent with this suggestion. That the enzyme is an α-1→6-glucosidase rather than an α-1→6-glycosidase is indicated by the results listed in Table 1. Thus melibiose is hydrolysed at a very much lower rate than the isomaltodextrins, isomaltulose or the α-1→6-link of panose. Apart from an α-1→3-glycosidase or possibly glucosidase the cell extracts prepared appeared to contain only traces of other α-glycosidases; on the other hand, they were rich in β-glycosidase activity.

The slow hydrolysis of dextran by the extracts was most probably due to the presence of traces of the normal extracellular dextranase for the following reason. Hydrolysis of isomaltodextrins by the α-1→6-glucosidase is by the successive cleavage of single glucose units. Glucose, however, could not be detected in cell-extract–dextran solutions during the early stages of incubation and only appeared after the expected release of short-chain isomaltodextrins by normal dextranase action. Presumably some dextranase remains closely associated with the *Lb. bifidus* cells and cannot be removed by simple washing.

Hydrolysis by the cell extract of isomaltodextrins of D.P. 2–5 at about the same rapid rate and a progressive decline in the rate of hydrolysis of isomaltodextrins of increasingly higher D.P. are complementary to the reported action of the extracellular dextranase on dextran. Thus the dextranase hydrolysed unbranched *S. bovis* dextran to a mixture of isomaltodextrins of D.P. 3–6 and these compounds would, therefore, be the substrate for the cells. In addition isomaltoheptaose and isomalto-octaose are the smallest polymers readily attacked by the dextranase (Bailey & Clarke, 1959). From Fig. 2 it would seem that the α-1→6-glucosidase would hydrolyse isomaltodextrins of D.P. > 10 at a very low rate.

The presence of a possibly constitutive α-1→3-glycosidase in the cell extracts is not unexpected. Although the dextran used in the present work was virtually unbranched (Bailey, 1959), dextrans are commonly branched through α-1→3- and, to a less extent, α-1→4-links. The *Lb. bifidus* dextranase hydrolyses many of these branched dextrans but cannot hydrolyse the α-1→3-links themselves which remain in branched oligosaccharides of D.P. 4 and higher (Bailey, Hutson & Weigel, 1960). The α-1→3-glycosidase in the cells would, therefore, enable the organism to utilize these branched oligosaccharides if it was growing in a medium containing branched dextran.

Temperature and pH optima of the α-1→6-glucosidase are in agreement with the conditions of growth of the organism (Bailey & Clarke, 1959; Clarke, 1959). The α-transferase action of the cell extract is of the type normally encountered with glycosidases, although it occurs at a relatively low concentration (1% or less) of isomaltodextrin. It was not possible to establish whether transfer was to carbon atoms other than C-6 of the non-reducing glucose of the acceptor sugar.

So far as the mode of action of the α-1→6-glucosidase is concerned, chromatographic evidence from enzyme solutions containing isomaltodextrins suggests an endwise cleavage of single glucose units from the isomaltodextrin chain. With, for example, isomaltopentaose and isomaltohexaose random cleavage of the chains would have resulted in the rapid appearance of large amounts of isomaltose and isomaltotriose. This did not happen; there was instead a rapid production of isomaltotetraose plus glucose and isomaltopentaose plus glucose respectively. This rapid release of glucose and the isomaltodextrin one glucose unit smaller than the added sugar was obtained from isomaltodextrins of D.P. 2–9. These results also suggest that, under the experimental conditions used in the present work, the hydrolysis was multi-chain rather than single-chain; the latter type of hydrolysis should have led to the rapid appearance of all of the isomaltodextrins of D.P. less than that of the added substrate. Hydrolysis of panose to glucose plus maltose indicated that the cleavage of the glucose units was from the non-reducing end of the molecule. This was confirmed by incubating isomaltotetraitol with the enzyme; there was a rapid release of glucose plus a trisaccharide alcohol but no production of reducing di- or tri-saccharide. Finally the ready hydrolysis of isomaltulose also indicates that it is the configuration of the non-reducing end of the oligosaccharide that is important to the enzyme.

Extracts from many organisms are able to hydrolyse α-1→6-glycosidic links. Some of the enzymes are non-specific α-glycosidases or α-glucosidases; none of the ones that are true α-1→6-glucosidases have been tested against a wide range of isomaltodextrins. Thus the enzyme isolated from pig intestinal mucosa by Larner & McNickle (1955) and designated by them as oligo-α-1→6-glucosidase was tested against isomaltose and panose but not against isomaltodextrins of D.P. > 3. An apparently constitutive α-1→6-glucosidase extracted from dextran-synthesizing cells of *Leuconostoc mesenteroides* and *S. bovis* by one of us (R.W.B.) hydrolysed isomaltotriose more slowly than isomaltose. Since the dextranases of all of the known dextran-fermenting organisms, except *Lb. bifidus*, hydrolyse dextrans to mixtures of various amounts of glucose, isomaltose and isomaltotriose, none of these organisms would need to form an intracellular

enzyme capable of hydrolysing isomaltodextrins of D.P. > 3; *Lb. bifidus* cells would, however, require such an enzyme to convert the products of the extracellular dextranase activity into glucose.

In view of the range of isomaltodextrins hydrolysed by the cell extracts we feel that the enzyme can justifiably be termed an isomaltodextrinase rather than merely an α-1→6-glucosidase. We think that it is the first enzyme of its kind to be reported.

SUMMARY

1. Cell extracts prepared from a rumen strain of *Lactobacillus bifidus*, grown on dextran, contain an α-1→6-glucosidase that readily hydrolyses isomaltodextrins of degree of polymerization 2–9 to glucose.
2. Hydrolysis of the isomaltodextrins is by the cleavage of single glucose units from the nonreducing end of the molecule.
3. Isomaltodextrins of degree of polymerization 2–5 are hydrolysed at the same rate; as the degree of polymerization of the isomaltodextrins progressively increases above 5 the rate of hydrolysis decreases.
4. The enzyme possesses optimum activity within the pH range 5·5–6·5, and temperature range 36–41°; it may also act as a transferase if sufficient isomaltodextrin is present.
5. The extracts also hydrolyse nigerose and a variety of β-linked sugars but scarcely attack melibiose, maltose, turanose, leucrose, $\alpha\alpha$-trehalose or methyl α-glucoside.

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Tissue Components of the Domestic Fowl

5. PHOSPHOMONOESTERASES IN THE SEMINAL PLASMA OF THE COCK*

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During attempts to preserve fertile avian spermatozoa *in vitro*, it was found that cock seminal plasma possesses high acid-phosphomonesterase activity (acid phosphatase). There was also a little alkaline-phosphatase activity. The former finding was somewhat surprising since birds are devoid of those accessory reproductive organs that, in most mammals, are believed to contribute the bulk of the acid phosphatase(s) to the chemically different semen of the latter class (Mann, 1954). Lake (1960*b*, 1962) showed, concomitantly, that the acid phosphatase(s) of the cock was mainly derived from secretions of the vasa efferentia and vasa deferentia with a possible contribution from the

* Part 4: Bell (1960).