DEGRADATION OF DEXTRANS BY ENZYMES OF INTESTINAL BACTERIA[1]

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In a previous report we (Hehre and Sery, 1952) showed that large numbers of dextran-splitting anaerobic bacteria, belonging to the *Bacteroides* genus, occur in the human colon and account for the dextran-degrading capacity of feces first noted by Engstrand and Åberg (1950). Examination of a series of isolates of these intestinal anaerobes revealed that, although growing cultures utilize dextran with the production of acid, sterile filtrates from dextran-broth cultures depolymerize the polysaccharide with the liberation of some reducing sugar (Hehre and Sery, 1952). We have now examined more closely the soluble dextran-splitting enzyme system produced by a selected strain of intestinal bacteroides. This system differs from most previously described dextranases. It hydrolyzes dextrans, apparently in endwise fashion under certain circumstances, to dextrose as the sole or major product.

Dextran-splitting enzymes elaborated by several types of fungi (Nordström and Hultin, 1948; Hultin and Nordström, 1949; Tsuchiya *et al.*, 1952*a*; Whiteside-Carlson and Carlson, 1952; Kobayashi, 1954), and by the bacterium, *Cellvibrio fulva* (Ingelman, 1948), have been found to cause rapid lowering of viscosity and molecular weight of the substrate before releasing much reducing sugar. The ultimate products of this type of degradation, as shown by Jeanes and her associates (1952, 1953) for dextranase from *Penicillium funiculosum* strain 1768, are oligosaccharides (isomaltose, isomaltotriose, etc.) rather than dextrose. Tsuchiya *et al.* (1952*a*) have reported dextran hydrolysis to dextrose by an amylase preparation from *Aspergillus niger* strain 330, and have otherwise contrasted the activities of the *A. niger* and *P. funiculosum* systems toward several substrates, including three dextrans of different chemical composition. In the present work, the bacteroides system has been examined in some detail for its action on a number of structurally different dextrans and related saccharides. The importance of enzymatic studies in the development of knowledge of the starch-glycogen polysaccharides suggests that such studies may, similarly, provide new information on the closely related dextran polysaccharides.

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MATERIALS

Bacteroides enzyme preparation. From 24 strains of enteric dextran-splitting bacteroides, one (FA-1A) was selected for study because it yielded somewhat more potent enzyme preparations than the others. Stock cultures were maintained on "chocolate" blood agar, i. e., on a meat infusion, yeast extract agar to which 5 per cent sterile citrated human blood was added, and the whole heated at about 80 C until brown.

Dextran-splitting enzymes were prepared from FA-1A cultures in broth comprising: dextran,[3] 2 per cent; tryptose, 1 per cent; yeast extract, 0.2 per cent; NaCl, 0.5 per cent; KH_2PO_4, 0.25 per cent; and Na_2HPO_4, 0.68 per cent. Suitable anaerobic conditions for growth were obtained as follows: to 800 ml of medium, autoclaved in a 1-L flask and cooled to about 70 C, sufficient separately sterilized medium was added (while hot) to partly fill the neck of the flask.[4] The medium was then cooled to 37 C and inoculated,

[3] The dextran in the medium, essential to induction of the enzyme, was the high molecular weight polymer (512-E) formed from sucrose by cell-free dextransucrase of *Leuconostoc mesenteroides* NRRL B-512 (Tsuchiya *et al.*, 1952*b*); it possessed 95 per cent "terminal plus 1,6-linked" units, as calculated from behavior on periodate oxidation.

[4] This procedure can be simplified by the use of a special culture vessel made by sealing the mouth of a 1-L Florence flask into an opening in the base of a second similar flask. Medium is added to fill the lower reservoir, with a slight excess (about 50 ml) extending into the upper reservoir; the system may then be autoclaved without danger of overflow.

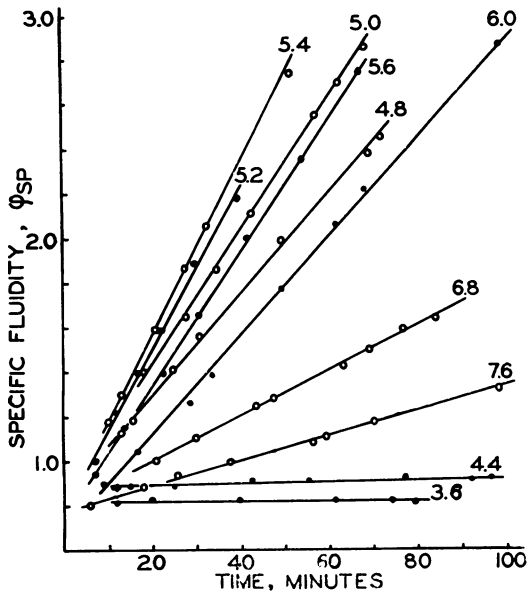

Figure 1. Increase in specific fluidity (reciprocal of specific viscosity) of enzyme-dextran mixtures incubated at different pH.

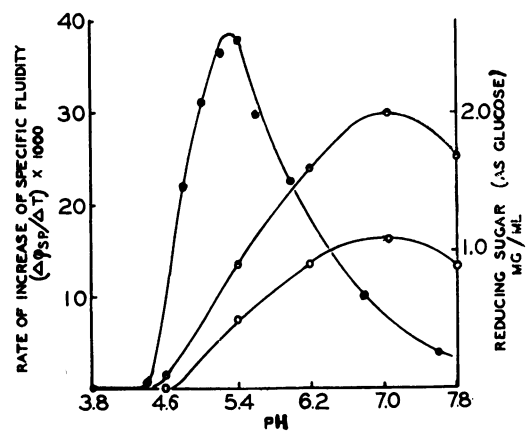

Figure 2. pH-activity curves for the Bacteroides enzyme system, with activity represented as rate of increase in specific fluidity ●, or as reducing sugar liberated (○, ◓) during 5 or 8 hours incubation at 30 C.

using fresh cultures grown 2 or 3 days in an anaerobic jar; the bacteria from one or two "chocolate" blood agar plates were suspended in a small amount of the 2 per cent dextran broth and introduced into the main flask without mixing; less viscous suspension fluids permitted the inoculum to rise to the surface of the culture medium.

After incubation at 37 C for 18 to 24 hr, each L of culture was treated with 375 g of ammonium sulfate, held at 4 C for 1 hr, then centrifuged in the cold. The sediment was taken up in 50 ml of distilled water, the bacterial cells separated by centrifugation, and the clear fluid stored at minus 70 C. Generally, 1 ml of this enzyme solution, when incubated with an equal volume of 1 per cent dextran 512-E for 2 hours at 30 C and pH 7.4, caused the release of 1.5 to 2.0 mg of reducing sugar (as dextrose).

EXPERIMENTAL RESULTS

Divergent effect of pH on enzyme activity. The action of the bacteroides enzyme system on dextran was early found to be complex in the sense that rapid reduction of viscosity is the most prominent effect produced in systems held at low pH, whereas reducing sugar liberation is the chief feature in systems incubated at higher pH.

The influence of pH on the capacity of the bacteroides system to lower dextran viscosity was examined with a series of mixtures comprising equal volumes of enzyme (1:4 dilution) and of 1 per cent dextran 512-E in different buffer solutions (acetate for the range pH 3.8 to 5.4, phosphate for the range pH 5.6 to 7.8). Each mixture was introduced into a No. 100 Ostwald-Cannon-Fenske viscometer held at 30 C, and measurements of the outflow times made during a 2-hr period. Figure 1 shows the family of straight lines obtained by plotting, for each mixture, the specific fluidity, φ sp ($= 1/\eta$ sp), against reaction time (see Hultin and Nordström, 1949; Ingelman, 1948). Since the individual lines tend to a common origin, their slopes were used as indices of relative enzyme activity (Levinson and Reese, 1950) in constructing a pH-activity curve (figure 2). It is evident that viscosity-depressing activity is maximal in the region of pH 5.4, drops off sharply at lower pH values, and reaches zero in the region of pH 4.4; the drop above pH 5.4 is more gradual, but the enzyme shows only slight activity at pH 7.6.

The effect of pH on reducing sugar liberation is quite different. Mixtures of enzyme and dextran 512-E, prepared as above but with enzyme diluted 1:2, were examined after 5 and 8 hours at 30 C. Dextrose was the only sugar detected on chromatograms prepared on Whatman no. 1 filter paper, using multiple ascents in 6:4:3 *n*-butanol: pyridine:water (Jeanes *et al.*, 1951) and a spray of ammoniacal silver nitrate (Hough, 1950). From

the quantities of dextrose (Hagedorn and Jensen, 1923) formed in the mixtures at different pH values, pH-activity curves of a second type were constructed. As figure 2 illustrates, the release of reducing sugars was maximal in the region of pH 7, where the viscosity-depressing effect of the enzyme was poor; and poor in the range of pH 5.0 to 5.5, where the viscosity-depressing effect was maximal.

The divergent influence of pH on the two activities was confirmed by tests on a number of different native and clinical dextrans (table 1). Paired mixtures containing equal volumes of enzyme (1:2 dilution) and of 1 per cent dextran in 0.1 M phosphate buffer, pH 5.4 or 7.4, were examined for changes in viscosity during the first hr, and for reducing sugar liberation after 6 hr at 30 C. The rate of increase in fluidity, in those instances where it could be measured, was from 2 to 4 times higher at pH 5.4 than at pH 7.4, whereas the amount of reducing sugar released was greater at pH 7.4 than at pH 5.4 for all but one of the 10 dextrans tested. This biphasic pH-activity relationship is hardly explainable except by the presence of two distinct dextran-splitting enzymes in the bacteroides preparation.

Extent of degradation of structurally different dextrans. The release of dextrose from different dextrans, under conditions (pH 7.4) providing relatively slight initial loss of viscosity, strongly suggests a preferential enzymatic attack on the outer parts of the dextran molecules. If so, the extent of hydrolysis of a particular dextran could be greatly influenced by such structural features as the number and length of its outer chains, extent and type of branching, etc. The availability of a number of purified dextrans differing from each other in behavior toward periodate oxidation, and in other respects, enabled this point to be examined. In the following experiment, the course and extent of hydrolysis of eight structurally different dextrans was followed by measurements of the dextrose liberated and of the residual polysaccharide.

The test mixtures comprised: enzyme, 4 ml; 2 per cent dextran, 2 ml; and 0.2 M phosphate buffer (pH 7.4) containing 400 units of penicillin per ml, 2 ml. (Pretesting showed that penicillin does not affect enzyme activity.) Control mixtures of each dextran without enzyme, and of enzyme plus buffer, were incubated with the test mixtures at 30 C. Analyses for reducing sugar release were made at 6, 24, and 48 hr; determinations of polysaccharide precipitable by 40 per cent (by volume) methanol, and of that precipitable in the range of 40 and 65 per cent methanol, were made at 48 hrs. The methanol-treated mixtures were kept at 4 C for 2 hr, then centrifuged in the cold; the sediments were drained, dried, and hydrolyzed in sealed tubes by N HCl at 120 C for 20 min. From the dextrose contents (Hagedorn and Jensen, 1923) of the neutralized hydrolyzates, the percentage of each of the polysaccharide residues was calculated, taking as 100 per cent the polysaccharide content of the corresponding dextran-buffer control. The results of the tests at 48 hr are summarized in table 2.

It is evident that the different dextrans were degraded to different degrees and, more important, that a general correlation exists between the degree of degradation on prolonged incubation

TABLE 1

Influence of pH on the enzymatic capacity to increase the fluidity of and to release dextrose from different dextrans

Dextran*		Rate of Increase of Specific Fluidity†		Rate of Release of Reducing Sugar‡	
		pH 5.4	pH 7.4	pH 5.4	pH 7.4
Native	1	28	7	6	16
	2	23	5	6	11
	3	6	3	4	14
	4	6	2	3	2
	5			8	13
	6			1	5
	7			1	2
Clinical	8			9	19
	9			9	17
	10			9	11

* Dextrans 1, 4, 6, 7, kindly supplied by Dr. Allene Jeanes, were from *Leuconostoc mesenteroides* NRRL B-512, -1298, -742 and -1355; dextrans 2, 3, 5 were from *L. mesenteroides* strain B (Sugg and Hehre, 1942), *Acetobacter capsulatum* NCTC 4943 (Hehre, 1951), and *Streptococcus* sp. strain 50 (Hehre, 1952): dextrans 8, 9, 10 were products of Commercial Solvents Corporation (Terre Haute, Indiana), A. B. Pharmacia (Uppsala, Sweden), and Dextran Corporation (Yonkers, New York).

† $\frac{\Delta\varphi sp}{\Delta\tau} \times 10^3$; determined only for mixtures containing dextrans of high initial viscosity.

‡ Mg reducing sugar liberated in 6 hr per 10 ml enzyme-dextran mixture; dextrose was the only sugar detected on chromatograms of the mixtures.

TABLE 2

Degradation of structurally different dextrans by the bacteroides enzyme system at pH 7.4

Dextran*		Dextrose Released†	Residual Polysaccharide	
Source	Terminal + 1,6-linked dextrose units		Precipitated by 40–65 per cent methanol	Precipitated by 40 per cent methanol
	per cent	*per cent*	*per cent*	*per cent*
Leuconostoc mesenteroides, NRRL B-512-E	95	100	0	0
Acetobacter capsulatum, NCTC 4843 (Hehre, 1951)	90	104	0	0
L. mesenteroides, B (Sugg and Hehre, 1942)	91	100	2	0
L. mesenteroides, NRRL B-1382	81	77	2	21
L. mesenteroides, NRRL B-1196	80	56	0	40
L. mesenteroides, NRRL B-742	73	47	4	49
L. mesenteroides, C (NRRL B-1298)‡	61	42	40	13
L. mesenteroides, NRRL B-1355	58	12	0	90

* Dextran 512-E was the enzymatically synthesized sample, kindly furnished by Dr. Henry M. Tsuchiya, used to induce the bacteroides enzyme. Preparations 1382, 1196, 742, C (or 1298) and 1355 were kindly supplied by Dr. Allene Jeanes, who also provided the periodate oxidation data from which the percentages of terminal plus 1,6-linked glucose units were calculated.

† Calculated from the amount of reducing sugar liberated through enzymic action, compared with the amount of released on acid hydrolysis of the corresponding dextran control. Chromatograms of all mixtures showed a heavy spot migrating as dextrose (*ca* 30 cm); in enzyme mixtures containing dextrans B and C, a trace spot (R_G = 0.2) of oligosaccharide other than isomaltose was also observed.

‡ Dextrans of strain C (Neill *et al.*, 1941; Hehre, 1943) and NRRL-B-1382 are characterized by strong reactivity with type 12 pneumococcus antiserum.

with enzyme, and the percentage of "terminal plus 1,6-linked" glucose units in the molecule as determined from periodate oxidation. Both the 512-E dextran and the dextran from *A. capsulatum* strain 4943, with 96 and 90 per cent "terminal plus 1,6-linked" units, were completely split to dextrose. Likewise, dextran B, comprising 91 per cent of such units, was nearly completely hydrolyzed—traces only of oligo- and polysaccharide resisting enzymic action. The remaining dextrans, containing lower proportions of "terminal plus 1,6-linked glucose" units (i. e., 81, 80, 73, 61 and 58 per cent), were hydrolyzed to respectively lesser degrees (77, 56, 47, 42, and 12 per cent). The polysaccharide residues of these incompletely hydrolyzed dextrans were, with one exception (dextran C), materials mostly precipitated from solution by 40 per cent methanol, rather than by 40 to 65 per cent methanol. Persistence of a high molecular weight "resistant core" might be expected from a preferential enzymatic attack on the outer portions of highly branched dextran molecules, with little or no scission of internal linkages.

In the exceptional case of dextran C, the bulk of the polysaccharide residue was precipitated in the range of 40 to 65 per cent methanol, rather than by 40 per cent methanol. This particular dextran would appear to have an internal structure quite different from that of the other dextrans tested. The unusual type of serological activity of dextran C (Neill *et al.*, 1941; Hehre, 1943) does not seem correlated with its anomalous behavior towards enzymatic action; dextran 1382, with serological properties of the same type as C, yielded a residual polysaccharide that was predominantly precipitable by 40 per cent methanol.

The data in table 2 refer to enzyme-dextran mixtures incubated 48 hr. That cessation of enzyme action had not invariably occurred in that period, however, is indicated by figure 3, which shows the course of glucose liberation in the same enzyme-dextran mixtures. Three of the preparations (512-E, 4943, B) were hydrolyzed rapidly and essentially completely in 48 hr. However, four others (1382, 1196, 742, C) were hydrolyzed more slowly and steadily, and did not reach a stationary level of degradation in 48 hr. It is uncertain whether these dextrans would eventually be hydrolyzed completely or leave truly refractory, "limit polysaccharides."

With dextran 1355 one can be more certain that a limit polysaccharide was produced, since dextrose liberation ceased entirely after 6 hr (al-

though the enzyme was found active after 48 hr). This dextran differs from all the others tested in possessing an unusually high proportion (35 per cent) of dextrose units, presumably 1,3,6-linked branch points, resistant to oxidation by periodate (Jeanes *et al.*, 1954).

The complete hydrolysis of dextran from *Acetobacter capsulatum* strain NCTC 4943 is worthy of special comment since this dextran, unlike all the others, was synthesized from dextrins rather than from sucrose.

Enzyme action on amylopectins and glycogens. Corn and potato amylopectins, and oyster and rabbit liver glycogens were tested as substrates, following the procedure of the preceding experiment. Table 3 shows that prolonged (48-hr) incubation with the enzyme at pH 7.4 caused notably greater hydrolysis of the amylopectins (22 and 26 per cent) than of the glycogens (4 and 6 per cent). In all cases, the only sugar detected chromatographically was dextrose, while nearly all of the residual polysaccharide was precipitable by 40 per cent methanol and, hence, evidently of high molecular weight. Supplementary measurements, made with the Klett-Summerson photometer and green filter no. 54, showed retention of iodine-coloring capacity after enzymatic treatment (as compared with the capacity before treatment) corresponding to 46 per cent for potato amylopectin, 56 per cent for corn amylopectin, 81 per cent for oyster glycogen, and 86 per cent for rabbit liver glycogen.

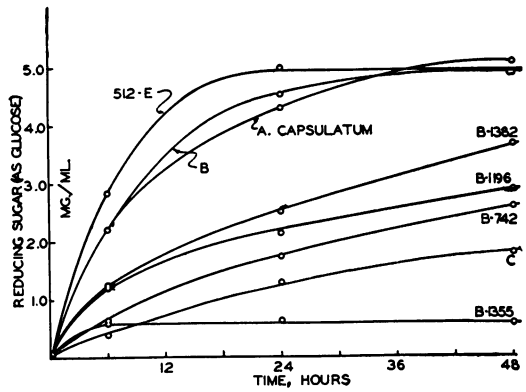

Figure 3. Release of reducing sugar (as dextrose) from structurally different dextrans (table 2) during incubation for 48 hr with the enzyme.

TABLE 3

Degradation of amylopectins and glycogens by the bacteroides enzyme system

Polysaccharide	Dextrose Released*	Residual Polysaccharide	
		Precipitated by 40–65 per cent methanol	Precipitated by 40 per cent methanol
	per cent	*per cent*	*per cent*
Corn amylopectin†	22	2	79
Potato amylopectin	26	2	74
Oyster glycogen	6	2	90
Rabbit liver glycogen	4	2	92

* Calculated from the amount of reducing sugar liberated through enzymatic action, compared with the amount released on acid hydrolysis of the corresponding polysaccharide control. Paper chromatograms in every instance showed a single heavy spot migrating as dextrose (*ca* 30 cm).

† Pentasol-soluble fraction, kindly supplied by Dr. T. J. Schoch.

Enzyme action on oligosaccharides. Tests for susceptibility to enzyme action, under the same conditions, also were made of several oligosaccharides possessing an unsubstituted (terminal) D-glucopyranosyl component. Paper chromatograms and reducing sugar measurements, at various intervals of enzyme-substrate incubation,

TABLE 4

Action of bacteroides enzyme on dextrose-terminated saccharides

Substrate*	Extent of Hydrolysis†	Sugars on Chromatograms, 48-hr Mixtures	
		Substrate	Glucose
Isomaltose	Complete in 3 hr	0	+
Maltose, panose	Complete in 48 hr.	0	+
Turanose, sucrose cellobiose, trehalose, glucose-1-phosphate	None detected in 48 hr	+	0‡

* Isomaltose, crystalline panose, and crystalline turanose were kindly supplied by Dr. Allene Jeanes, Dr. S. C. Pan and Dr. Nelson K. Richtmyer.

† Determined from the amount of reducing sugar released through enzymic action, compared with the amount released on acid hydrolysis.

‡ No increase over the trace of glucose present in the enzyme solution; no fructose detected in enzyme-turanose or -sucrose mixtures.

showed that isomaltose, maltose, and panose were completely hydrolyzed by the enzyme (table 4). Isomaltose, possessing the structural linkage that predominates in dextrans, was the most rapidly hydrolyzed. In the case of panose, chromatograms showed the presence of maltose and dextrose (but not isomaltose) during the first 6 hr of incubation; at 48 hr only dextrose was present. Thus, the α-1,6-glucosidic bond of panose, like that of isomaltose itself, is more rapidly split by the enzyme system than is the α-1,4-bond of panose and maltose. The lack of any significant hydrolysis of turanose, trehalose, sucrose or glucose-1-phosphate indicates that the bacteroides enzyme is not an α-glucosidase of broad specificity.

DISCUSSION

The bacteroides system is distinguished from nearly all previously described dextranases on the basis that dextrose is the sole or dominant sugar product. The enzyme system of *Aspergillus niger* strain 330 (Tsuchiya *et al.*, 1952*a*) alone resembles the bacteroides system in this respect, being similar also in its great affinity for isomaltose as a substrate. The two differ, however, in several important respects. The maximal release of glucose is at pH 7.0 to 7.5 with the bacterial enzyme, but at pH 4.4 with the mold preparation; also, starch (at least its major component, amylopectin) is hydrolyzed less rapidly and completely than most dextrans by the bacteroides system, but much more rapidly than the dextrans by the *A. niger* amylase (Tsuchiya, *personal communication*); finally, transglucosylase activity for maltose and cellobiose, not detected with the bacterial preparation, is a prominent feature of the *A. niger* 330 system (Tsuchiya *et al.*, 1950). In work yet unpublished, W. L. Bloom and his associates (*personal communication*), have obtained from the duodenal and jejunal mucosa of several species, including man, another dextranase that yields dextrose as the major hydrolytic product. It will be of great interest to compare the properties of this intestinal mucosal enzyme with the present system from one of the predominant types of intestinal bacteria. The bacteroides enzyme, otherwise, appears clearly different from the "debranching" enzymes and α-1,6-glucosidases of various sources, which cleave α-1,6-glucosidic linkages in starch-type polysaccharides or in oligosaccharides but not in dextrans.

Although knowledge of the structures of polysaccharides of the starch-glycogen class has been greatly enriched by studies on the enzymic degradation of these substances, only one previous report has dealt with the relationship between enzymic breakdown and the structure of dextrans. Tsuchiya, Jeanes, Bricker and Wilham (1952*a*) compared the rates of sugar liberation, by enzymes from *P. funiculosum* strain 1768 and *A. niger* strain 330, for three different dextrans. Both enzymes attacked B-512 dextran (95 per cent 1,6-linkages) more rapidly than B-742 dextran (75 per cent 1,6-linkages). The enzyme from *P. funiculosum* degraded the third dextran, B-523 (75 per cent 1,6-linkages), at the same rate as B-512 dextran,[5] while the enzyme from *A. niger* hydrolyzed it at a rate intermediate between those of B-512 and -742.

With the *Bacteroides* enzyme, good correlation was found between the degree of hydrolysis of different dextrans and their content of "terminal plus 1,6-linked" glucose units determined by periodate oxidation. Furthermore, those dextrans with lower proportions of units linked in this manner, after prolonged incubation with the enzyme, yielded residues precipitable by 40 per cent methanol and, presumably, of high molecular weight. These features strongly indicate that the bacteroides enzyme, at pH 7.4, preferentially attacks the outer portions of dextran molecules. They also furnish independent support for the concept, derived from chemical studies, that certain dextrans are highly branched molecules. Dextran 1355, for example, appears highly branched not only because of its high content (35 per cent) of dextrose units refractory to oxidation by periodate and low content (58 per cent) of "terminal plus 1,6-linked" units (Jeanes *et al.*, 1954), but also because it yielded a "limit" dextran, resistant to the enzyme, after only 12 per cent hydrolysis.

The complete or nearly complete breakdown to dextrose of several dextrans composed mainly but not exclusively of 1,6-linked units, as well as the cleavage of dextrose from the long outer chains of corn and potato amylopectins, indicates that the action of the bacteroides system is not confined to hydrolysis of α-1,6-glucosidic

[5] Dr. Jeanes (*personal communication*) states that *P. funiculosum* 1768 dextranase hydrolyzes dextrans having 96 to about 70 per cent 1,6-linkages in proportion to this percentage.

linkages. Tests with isomaltose, panose, and maltose have provided clear evidence that the specificity extends to the α-1,4-glucosidic linkage, although cleavage of the α-1,6-linkage is decidedly more rapid.

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SUMMARY

A soluble enzyme system, capable of hydrolyzing dextrans to dextrose as the sole or major product, has been obtained from an intestinal bacterium of the *Bacteroides* genus. This system evidently contains two different dextranases, since it can either "liquify" or "saccharify" dextrans. (At pH 5.0 to 5.5, the principal effect is rapid lowering of viscosity; at pH 7.0 to 7.5, the major effect is rapid release of glucose.)

At pH 7.4 the bacteroides system was found able to hydrolyze both α-1,6-and α-1,4-glucosidic linkages in oligosaccharides, and to cause the complete or nearly complete hydrolysis of dextrans containing 90 to 95 per cent "terminal plus 1,6-linked" glucose units. Dextrans with smaller proportions of such units (as well as amylopectins and glycogens) were partially hydrolyzed to dextrose under the same conditions. Since these incompletely hydrolyzed substances, with one exception, yielded polysaccharide residues readily precipitated by alcohol, it would appear that the enzymic attack was generally limited to the outer portions of the molecules. The exceptional dextran (from *Leuconostoc mesenteroides* strain C) presumably has an internal structure quite different from those of the other dextrans examined and, hence, is worthy of further chemical study.

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