BIOSYNTHETIC RELATION BETWEEN THE SOLUBLE AND INSOLUBLE DEXTRANS PRODUCED BY *LEUCONOSTOC MESENTEROIDES* NRRL B-1299

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1. Introduction

The structures and properties of bacterial dextrans vary with the organism [1, 2] and certain bacteria elaborate simultaneously two or more dextrans that differ in structure and water solubility [3]. As little is known of the manner by which more than one dextran type is produced by a single organism it was of interest to examine the simultaneous synthesis of two dextrans by *Leuconostoc mesenteroides* NRRL B-1299 [3]. The data presented here show that both dextrans are synthesised by structure-bound dextransucrase and that a large proportion, at least, of the insoluble dextran acts as a precursor of the water-soluble dextran.

2. Materials and methods

Leuconostoc mesenteroides B-1299 was cultured aerobically at 23°, with sucrose (2%) as carbon source as by Tsuchiya et al. [4]. The cells were collected after incubation for 25 hr by centrifugation at 7000 *g* for 15 min. A gelatinous insoluble dextran and 95% of the total dextransucrase activity were sedimented with the cells. The sediment was washed several times with sodium acetate (0.3 M) buffer pH 5.0, resuspended in the same buffer and sonicated for 2 min at 0° using a Biosonic sonicator. No dextransucrase activity was detected in the supernatant after centrifugation. The residue therefore, was sonicated in the same way two more times and the final residue was frozen and thawed. However, no dextransucrase was solubilised by this treatment and 85% of the original activity was recovered in association with the insoluble residue. Microscopic examination revealed that the residue contained no intact cells. Treatment with detergents, lysozyme, trypsin or concentrated salt solutions failed to solubilize the enzymic activity. A suspension of the enzyme in sodium acetate buffer pH 5.0 was used in all experiments.

Dextransucrase activity was assayed by incubating suitable amounts of the suspension at 25° in digests containing sucrose (100 mg/ml) and sodium acetate (0.1 M) buffer, pH 5.0. Samples removed at intervals were added directly to alkaline copper reagent and the reducing sugar released was estimated as by Nelson [5]. One unit is defined as that amount of enzyme that releases one μmole of fructose per minute. Total dextran synthesis was determined from the apparent release of fructose in the dextransucrase digests.

U-^{14}C-Sucrose was purchased from New England Nuclear. The incorporation of U-^{14}C-glucose into the total dextran was measured by placing aliquots of ^{14}C-sucrose–dextransucrase digests on squares of filter paper, and washing the paper with 66% ethanol and then acetone to remove all low molecular weight radioactive molecules, as by Kindt and Conrad [6]. The papers were dried and counted in scintillation solution containing 5 g diphenyloxazole and 0.5 g *p*-bis [2-(5-phenyloxazolyl)]-benzene per liter of toluene, using a Packard Tri-carb scintillation spectrometer. Incorporation into soluble dextran was measured in the same way on aliquots taken from the supernatant of digests centrifuged at 3000 *g* for 5 min. The difference between the ^{14}C-glucose incorporated into the total dextran and into the soluble dextran was taken as the ^{14}C-glucose incorporated into the insoluble dextran component. Con-

Table 1
Dextransucrase reaction in the presence of ^{14}C-labelled soluble dextran.

Reaction time (min)	Total dextran synthesis (mg/ml)	Total ^{14}C-dextran (10^5 cpm/ml)	Soluble ^{14}C-dextran (10^5 cpm/ml)
5	1.0	2.3	2.2
32	4.1	2.1	2.1
92	8.1	2.3	2.2
210	15.6	2.2	2.1

Digest (1 ml) contained dextransucrase suspension (0.5 units), sodium acetate buffer (0.06 M) pH 5.0, sucrose (40 mg) and ^{14}C-labelled soluble dextran (2 mg, 2.1 X 10^5 cpm). The digest was incubated at 25° and at the times indicated portions (0.2 ml) were diluted with water to 0.5 ml. Samples (0.05 ml) of the dilute solution were used to estimate total dextran synthesis (from the release of total reducing power) and the radioactivity of the soluble and total dextran components were determined as described in Materials and methods. The ^{14}C-labelled soluble dextran was prepared by the action of dextransucrase on ^{14}C-sucrose under the conditions described in fig. 1.

canavalin A was prepared from jack bean [7] and the precipitation reactions with dextrans were conducted as by Goldstein et al. [8]. The concentrations of dextran and maltose solutions were determined by acid hydrolysis [9] followed by estimation of glucose released with glucose oxidase [10] or Nelson's reagent [5].

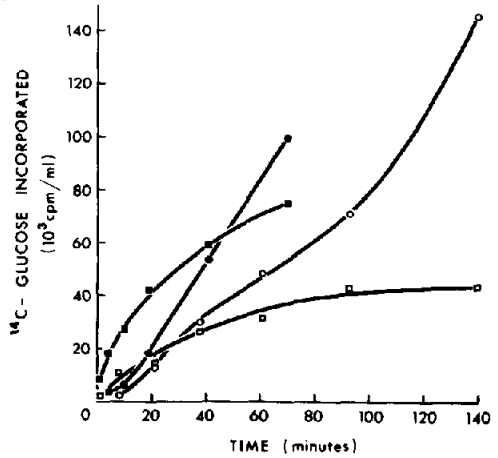

Fig. 1. Incorporation of ^{14}C-glucose into soluble and insoluble dextran. Dextransucrase suspension (1.0 unit) was incubated at 25° in digests (2 ml) containing ^{14}C-sucrose (40 mg, 4.2 X 10^5 cpm) and sodium acetate (0.06 M) buffer, pH 5.0. Samples (0.2 ml) were removed at intervals and made to 1.0 ml with water. Samples of this solution before and after centrifugation at 3000 *g* for 5 min were used, as described in Materials and methods, to determine the incorporation of ^{14}C-glucose into the soluble and insoluble dextran components of the digest. Soluble dextran (●—●), insoluble dextran (■—■). The corresponding open symbols represent a similar digest containing one half the amount of enzyme suspension.

3. Results and discussion

During 60 min incubations of the dextransucrase suspension with sucrose (20 mg/ml) the rate of release of total reducing sugars was constant and was directly proportional to the amount of enzyme present. Glucose oxidase determinations revealed, however, that glucose represented about 10% of the reducing sugars released. In order to examine the polysaccharides produced, the enzyme suspension was incubated in a digest (10 ml) containing 100 mg of sucrose/ml. After 5 hr at 30°, 230 mg equivalent of fructose (total reducing power) and 25 mg of glucose were released in the digest. The digest was centrifuged at 3000 *g* for 10 min and the residue washed repeatedly with water. The washings were combined with the digest supernatant and dialysed at 4° against five changes of distilled water over a period of 72 hr. Portions of dialysed solution and a suspension of the washed residue were hydrolysed at 100° for 6 hr in 1.5 N-H_2SO_4 and the total reducing sugar and glucose contents of the hydrolysates were determined. Glucose was found to be the only reducing sugar present. A similar result was obtained when a partial hydrolysis was conducted under much milder conditions (0.5 N-H_2SO_4 at 100° for 1 hr. This indicates that only a glucose polymer (dextran) is present in the soluble and insoluble fractions and the failure to detect any fructose polymer (levan) makes it unlikely that the observed glucose release in the sucrose digest is mediated by a levansucrase.

Glucose was not released over 6 hr when the

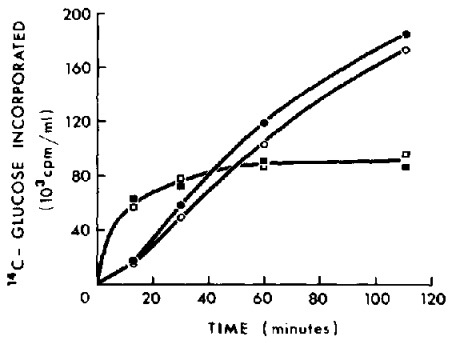

Fig. 2. Incorporation of ^{14}C-glucose into soluble and insoluble dextran in the presence of preformed soluble dextran. Dextransucrase suspension (1.3 units) was incubated in the presence of added soluble dextran (3.7 mg) under the conditions described in fig. 1 and the ^{14}C-glucose incorporation into soluble dextran (●—●) and insoluble dextran (■—■) was determined in the same manner. The corresponding open symbols represent a second digest in which soluble dextran was omitted.

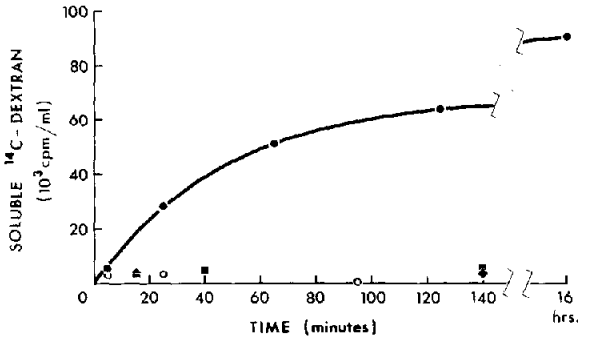

Fig. 3. Release of soluble dextran from ^{14}C-dextran-dextransucrase complex in the presence of sucrose. Insoluble ^{14}C-dextran-dextransucrase was prepared by incubating enzyme suspension with ^{14}C-sucrose. The complex was recovered by centrifugation, washed five times with sodium acetate (0.15 M) buffer, pH 5.0 and then resuspended in buffer. Portions of the radioactive suspension (0.4 ml, 2 X10^5 cpm) containg 1 unit dextransucrase were incubated with sucrose (40 mg) as described in fig. 1. Similar digests in which sucrose was omitted or was replaced by maltose (20 mg) or soluble dextran (2 mg) were prepared. Samples (0.4 ml) were removed from each digest at intervals, diluted to 1.0 ml with water and the amount of soluble ^{14}C-dextran released was determined as described in fig. 1. Sucrose (●—●); maltose (■—■); soluble dextran (◆—◆); no sucrose (○—○)

dextransucrase was incubated alone or with previously synthesised soluble dextran, thus establishing that the enzyme preparation does not contain a significant dextranase activity. The glucose release therefore is probably caused by the direct action on sucrose of an invertase-like enzymic impurity. This explanation is consistent with the lower yield of total dextran isolated (160 mg) than was expected by calculation from the release of total reducing sugars (207 mg).

The amount of dextran associated with the insoluble dextransucrase residue increased from 11 to 32 mg during the 6 hr incubation with sucrose, indicating that insoluble and soluble dextrans are synthesised simultaneously by the enzyme preparation. A possible explanation for the apparent synthesis of insoluble dextran was that the increasing molecular weight of the soluble dextran synthesised rendered it insoluble as the reaction proceeded. However, when enzyme and sucrose were incubated with ^{14}C-labelled soluble dextran (previously synthesised by the enzyme from ^{14}C-sucrose) no significant incorporation of ^{14}C-label into the insoluble component was detected, even after prolonged synthesis (table 1).

The incorporation of ^{14}C-glucose into the soluble and insoluble components of the dextran therefore was followed in digests containing ^{14}C-sucrose and two concentrations of enzyme (fig. 1). In both cases there was a significant lag period before the soluble dextran synthesised became radioactively labelled, after which there was a continuous and rapid incorporation of label into the soluble fraction. In contrast the insoluble component was rapidly labelled in the initial stages of the reaction but as the reaction proceeded the incorporation of ^{14}C-glucose slowed and apparently approached a maximum. When a similar experiment was carried out in the presence of unlabelled soluble dextran a small increase of incorporation of ^{14}C-label into the soluble fraction was seen but there was little or no change in the extent of the lag period (fig. 2). This makes it unlikely that the initial slow synthesis of soluble dextran results from a requirement of this reaction for a soluable dextran acceptor or primer.

The alternative possibility that the insoluble dextran is the precursor of the soluble polysaccharide is supported by the rapid release of radioactive label that occured when a ^{14}C-labelled dextran–dextransucrase insoluble complex (previously prepared by incubation of the enzyme suspension with ^{14}C-sucrose) was incubated with non-radioactive sucrose

Table 2
Release of soluble dextran from ^{14}C-dextran–dextransucrase complex following heat-inactivation.

Time of inactivation (min)	Temperature (°C)	Inactivation (%)	^{14}C-Dextran released (%)
1	50°	14	1.2
2.5	50°	47.5	3.1
5	50°	76.5	3.7
3	100°	100	10
30	100°	100	31

^{14}C-Dextran–dextransucrase was prepared and suspended in sodium acetate (0.15 M) buffer pH 5.0 as described in fig. 3. Portions (0.5 ml) containing 0.06 units of dextransucrase and 2 X10^3 cpm were treated as indicated. The suspensions were assayed for enzyme activity and then centrifuged at 3 X10^3 *g* for 5 min. The ^{14}C-dextran remaining in the supernatant was counted as described in Materials and methods.

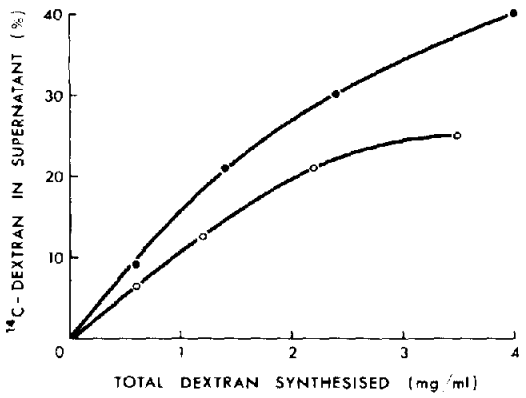

Fig. 4. Release of soluble dextran from partially heat-inactivated ^{14}C-dextran–dextransucrase complex in the presence of sucrose. ^{14}C-Dextran–dextransucrase suspension was heated at 50° for 2.5 min. Samples (0.3 ml) of the untreated suspension (0.3 dextransucrase units, 10^5 cpm) and the heat treated suspension (0.17 dextransucrose units, 10^5 cpm) were incubated with sucrose (20 mg) in 1 ml digests under the remaining conditions described in fig. 1. Samples were removed at intervals for estimation of the total dextran synthesis (from increase in reducing power), and the release of soluble ^{14}C-dextran into the supernatant was determined as described in fig. 1. Untreated suspension (●——●); heat-treated suspension (57.5% active) (○——○).

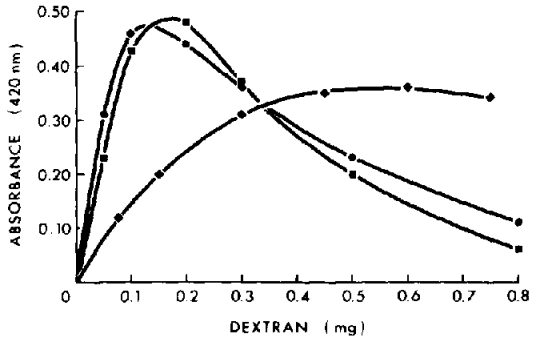

Fig. 5. Interaction of dextrans with concanavalin A. Dextransucrase suspension was incubated with sucrose under the conditions described in fig. 1. After 4 hr the digest was centrifuged at 5000 *g* for 10 min. (a) The residue was washed five times with water and then heated at 100° for 1 hr in 30% KOH. The solution was cooled and the dextran was precipitated with 66% ethanol. The precipitate was dissolved in water, centriguged to remove insolubles and the soluble dextran isolated by ethanol precipitation. (b) The digest supernatant was dialysed overnight against several changes of water and the soluble dextran isolated by ethanol precipitation. Varying amounts of the KOH-extracted dextrain (■——■), soluble dextran (●——●) and *Leuconostoc mesenteroides* B-1355-S dextran (◆——◆) were mixed with 1 mg concanavalin A in a total volume of 3 ml. The absorbance of the turbidity produced after 10 min was read on a Spectronic 20 spectrophotometer at 420 nm.

(fig. 3). No release of radioactivity into the supernatant was seen in digests in which sucrose was omitted or replaced by maltose or soluble dextran. This suggests that neither a hydrolytic nor a transferase reaction is directly responsible for the solubilisation of the radioactive dextran and that active dextran synthesis from sucrose is essential for the release to occur.

Denaturation of the ^{14}C-dextran–enzyme complex at 50° and pH 5.0 does not result in solubilisation of the ^{14}C-labelled dextran although an appreciable amount of radioactivity appears in the supernatant following treatment at 100° for 30 min (table 2). Partial heat denaturation of the enzyme complex results,

however, in the release of a decreased proportion of ^{14}C-dextran when it is incubated in the presence of sucrose. This is shown in fig. 4, where it can be seen that, even when the total dextran synthesised is identical in each reaction, the amount of ^{14}C-dextran released into the supernatant by the partially denatured enzyme is proportionally less than the corresponding amount released by the untreated enzyme. An explanation may be found in the possibility that only that dextran associated with active enzyme is capable of being released in a soluble form.

Extraction of the insoluble dextran–dextransucrase complex with hot 30% KOH resulted in the recovery of more than 60% of the dextran component in a water-soluble form. The extracted dextran interacts with a phytohemagglutenin, canavalin A, in a manner almost identical to that of the dextran synthesised as the soluble component (fig. 5). Both precipitin curves contrast with that of NRRL B-1355-S dextran, which is included in fig. 5 for comparison. The concanavalin A interaction is highly specific towards dextran structure [11] so that the near identity of the interactions of the soluble and previously insoluble dextran may indicate a close structural similarity.

These findings suggest that a large proportion of the gelatinous "insoluble dextran" observed in the *Leuconostoc mesenteroides* NRRL B-1299 culture represents a complex between the cell-bound dextransucrase (or a closely related cell wall component) and "soluble dextran" which, on completion of synthesis, is released into the supernatant. This complex takes on greater significance in view of the proposal that a covalently linked enzyme–dextran complex is involved in an "insertion type" mechanism of dextran synthesis (12). However, knowledge of the real nature of the complex and the mechanism of synthesis and release of the dextran, must await further investigations.

A similar association between dextran and the bacterial cell wall also may be of significance to the role of dextrans in dental caries. Guggenheim and Schroeder [13] suggest that the synthesis of water-insoluble dextran by the cariogenic oral *Streptococci* is an absolute condition for the formation and maintaince of microbial plaque on the smooth surface of teeth. A strong association between the cell surface and a water-soluble dextran however, may serve equally well to impart the gelatinous and adhesive properties required for the formation of this type of dental plaque.

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