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A Quantitative Study of the Production of Dextran from Sucrose by Rumen Strains of *Streptococcus bovis*

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SUMMARY: Freshly isolated and old stock strains of *Streptococcus bovis* originating from the rumen will produce dextran at 37° in liquid sucrose-containing media. For good yields the presence of CO_2 in some form is necessary. The CO_2 may be provided as HCO_3^- at the start or during the life of the culture or by incubation in a CO_2 atmosphere. The dextran has $[\alpha]_D^{20}$ +187° to +190° and is similar chemically to the leuconostoc dextran save that branching of the $\alpha 1 \rightarrow 6$ linked anhydro-glucose chain is rarer. With some strains practically no dextran is formed in H_2 as gas-phase or in a closed system without HCO_3^- from which air is excluded. Other strains seem to have a limited power of producing dextran under these conditions, possibly because their action is not entirely homofermentative. Tween 80 will partially replace CO_2 even with the first kind of strain. Highest yields of dextran, up to 80% of the anhydro-glucose provided, are obtained when the life of the culture is prolonged by repeated neutralization and when additional sucrose is supplied. This is best achieved by the continuous neutralization obtained when solid $CaCO_3$ is present in the culture. Dextran production is always accompanied by accumulation of fructose in the culture liquid together with a reducing fructose-containing disaccharide. Dextran can sometimes be produced in a simple liquid sucrose + proteose peptone medium with no phosphate buffering. Other things being equal, the presence of CO_2 or HCO_3^- does not greatly increase the yield of bacterial protein in sucrose media.

Streptococcus bovis or a similar amylolytic group D streptococcus is always present in the rumen (see MacPherson, 1953; Hungate, 1957). Strains of this organism have been reported to produce at least two types of polysaccharide: a capsular polysaccharide and a water-soluble polyglucosan. The capsular polysaccharide contains galactose, rhamnose and uronic acid units (Hobson & MacPherson, 1954) and is produced in media which contain glucose or presumably any other fermentable carbohydrate. There is no requirement of CO_2 for its formation and in older cultures it may also be found free in solution although admittedly in very low concentrations. As the capsular compound does not contain glucose there should be no difficulty in determining which of the two types of polysaccharide is responsible for the sliminess of any given S. *bovis* culture.

Hehre & Neill (1946) showed, chiefly by serological tests, that many strains of 'viridans' streptococci isolated from the blood of patients with subacute bacterial endocarditis could convert sucrose into a polysaccharide indistinguishable from leuconostoc dextran. *Streptococococcus bovis* is in many respects also a 'viridans' streptococcus (Topley & Wilson, 1946). Niven, Smiley & Sherman (1941) originally stated that several strains of *S. bovis*, of unrecorded origin, produced from sucrose a soluble polysaccharide which was described as 'dextran' because it yielded glucose on hydrolysis. More recently, Dain,

Neal & Seeley (1956) reported that the generality of *S. bovis* strains, including those isolated from cattle rumen, formed large glucosan-containing mucoid colonies (as distinct from the usual non-mucoid pinhead 'typically streptococcal' colonies) on nutrient agar only when provided with sucrose and a carbon dioxide atmosphere. The polysaccharide was shown to be a polyglucosan but the major linkage and hence the polysaccharide was not identified. In corresponding liquid cultures they stated that 'the cells tended to remain in suspension even after several days and viscosity of the medium was increased although in no case did complete solidification occur.' Hence these authors did not show that the organism is capable of producing the polyglucosan in liquid media nor that the polyglucosan produced on agar is dextran. In contrast to the above work Dr J. C. Appleby, formerly of the Rowett Research Institute, informed us that in 1956 she isolated from two sheep rumens a strain of *S. bovis* which produced much polyglucosan on sucrose nutrient agar in a hydrogen atmosphere. There was apparently no stimulation of polyglucosan production by CO_2. It seems likely therefore that such strains are not uncommon.

Most of the work on dextrans has hitherto been done on material produced by *Leuconostoc* spp. Following these investigations dextrans may be defined as water-soluble polyglucosans in which the major linkage is the $\alpha 1 \rightarrow 6$ glucosidic link (see Barker *et al.* 1955). Varying proportions (0–20 %) of $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 4$ glucosidic linkages have been reported to be present in the dextrans produced by different *Leuconostoc* spp. (Jeanes & Wilham, 1952; Barker *et al.* 1954). These secondary linkages are believed to occur as branch points along the main chain of $\alpha 1 \rightarrow 6$ linked glucose units. The presence of the $\alpha 1 \rightarrow 6$ glucosidic link, as the major linkage, must be demonstrated to show that any polyglucosan is in fact dextran.

Leuconostoc spp., which, unlike *Streptococcus bovis*, are heterofermentative (i.e. CO_2 producers) require neither added CO_2 in the gas phase nor HCO_3^- to give good dextran yields from sucrose-containing liquid media. It seemed therefore desirable to establish whether CO_2, in some form, was necessary for dextran production by *S. bovis*, particularly in liquid media. It also appeared necessary to show clearly to what extent the polysaccharide resembled true leuconostoc dextran. Such results are of practical interest since in the rumen *S. bovis* lives in a bicarbonate buffer in the presence of a gas-phase containing CO_2. When the animal consumes a sucrose-containing ration such as spring grass or clover the organism may produce a dextran slime. Hungate, Fletcher, Dougherty & Barrentine (1955) in fact believed that slime production in the rumen 'may be associated in an important way with bloat in ruminants'.

In this paper we show that dextran, produced from sucrose only, is responsible for the greatly increased viscosity of suitable liquid cultures of rumen strains (old and freshly isolated) of *Streptococcus bovis*. There is an absolute requirement for CO_2 to give appreciable dextran production by most, but not all, strains of this organism no matter how highly the medium may be buffered by phosphates, etc. We also record preliminary observations on the structure of the *S. bovis* dextran and on the nature of the soluble sugars found

in high dextran-producing cultures. An account of the properties of *S. bovis* dextransucrase is reserved for a subsequent publication. For the purpose of the present paper it has been considered sufficient to show that the polymer is a polyglucosan and that the major linkage present is the $\alpha 1 \rightarrow 6$ glucosidic linkage. This has been done by the chromatographic identification of isomaltose in partial hydrolysates and glucose in total hydrolysates of the dextran. Further confirmation has been obtained by periodate oxidation and by measurements of $[\alpha]_D$ values.

METHODS

Strains of rumen Streptococcus bovis *and of* Leuconostoc mesenteroides

Old stock cultures. Seven strains of *Streptococcus bovis* were obtained, freeze dried, from the Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland. They had been isolated from the rumen of sheep and calves in work described by Macpherson (1953); Mann, Masson & Oxford (1954); Mann & Oxford (1955). Their reference numbers are 18/C2, 18/M2, 19/C1, 2B, 16B, 2SA, and 1010Y.

Freshly isolated strains of Streptococcus bovis. These were isolated from muslin-strained rumen liquor obtained from three fistulated cows feeding on rye grass or red clover at this laboratory. Two isolation procedures were used.

(1) A loopful of a 1/2500 dilution of rumen liquor in sterile 0·5% (w/v) yeast extract (Difco) was inoculated into liquid 2% (w/v) sucrose Bacto-thioglycollate medium (10 ml., Difco; containing 0·075% (w/v) agar but no glucose) and incubated for 1 day in air at 37°. A loopful of the shaken culture was streaked on several plates of 3% (w/v) sucrose + 3% (w/v) proteose peptone no. 3 (Difco) agar and the plates incubated at 37° for 1 day in CO_2 (see below). The first plate invariably showed confluent mucoid growth but single well-separated mucoid colonies were always found on the other plates. No other organisms were ever encountered under these conditions.

(2) Crude rumen liquor was centrifuged for 30 min. at 40,000 *g*, and 0°. The supernatant fluid was discarded. One small loopful (0·001 ml.) of the yellow slimy layer on the surface of the centrifuged solid was inoculated into the sucrose + thioglycollate liquid medium (10 ml., above). Two successive serial dilutions were prepared by transferring 2 large loopfuls (0·02 ml.) through 2 samples (10 ml.) of sucrose + thioglycollate liquid medium containing 0·075% (w/v) agar. After incubation at 37° with CO_2 as gas phase the final dilution almost invariably showed a few discrete growths of *Streptococcus bovis* which were purified by plating as above. Plating on the sucrose + proteose peptone agar in CO_2 always produced mucoid colonies whereas with sucrose + Brewer's anaerobic agar (Difco, no glucose or methylene blue) the colonies were often non-mucoid. No explanation is offered for this fact, and buffering the medium with phosphate did not alter it.

Nine new strains of *Streptococcus bovis* were thus isolated, denoted I (cow I); A1, A2, 293C, 30 and 34 (cow 293); and IV, 40 and 44 (cow 294). Strains I, A1, A2, and IV were obtained when the cows were on a diet of pure rye grass (*Lolium* species) and the other strains when they were on a diet of pure red

clover (*Trifolium pratense*). For the main quantitative experiments *S. bovis* strains 1010Y (old) and I (new) were used. All cultures were maintained in Difco tomato juice agar or in 0·25% sucrose+thioglycollate (Difco) agar stabs kept at 0°.

Leuconostoc strains. For comparative purposes a culture of *Leuconostoc mesenteroides* (NCIB 8590) was obtained from the National Collection of Industrial Bacteria (D.S.I.R.), Teddington, Middlesex, England.

Liquid media

For dextran production. Cultures of *Streptococcus bovis* in 3% (w/v) sucrose +3% (w/v) proteose-peptone no. 3 (Difco) liquid medium without agar did not always yield dextran in a CO_2 atmosphere; an exception with *S. bovis* strain IV is described below. The following modified Bacto-thioglycollate liquid medium was invariably successful. Its composition at three concentrations ($a<b<c$) of phosphate buffer was (%, w/v): Bacto-yeast extract, 0·5; Bacto-casitone, 1·1, L-cystine (dissolved separately in a little boiling 0·1 N-HCl), 0·025; sodium thioglycollate, 0·03; K_2HPO_4, (*a*) 0·45; (*b*) 0·54; Na_2HPO_4 $12H_2O$, (*c*) 1·2; KH_2PO_4, (*a*) 0·15; (*b*) 0·18; (*c*) 0·20; $(NH_4)_2HPO_4$, 0·03; sucrose, usually 8; pH 7·0. All salts (except K_2HPO_4, British Drug Houses Ltd) were of Analar quality.

For an initial pH value between 7·0 and 8·0, ammonia solution (sp.gr. 0·91) was added, drop by drop, to give the desired value. Sucrose could safely be sterilized in solution in this basal medium; other sugars to replace sucrose were sterilized separately in concentrated aqueous solution. Basal medium free from CO_2 and HCO_3^- was obtained sterile by three successive steamings, not by autoclaving. In this case the medium nearly filled a conical flask (1 l.) carrying a rubber bung pierced with a piece of open glass tubing. After each steaming the flask was removed, cooled rapidly, and the outlet tube closed. After the final steaming the medium was rapidly dispensed into small sterile conical flasks (50 or 100 ml.) containing any required sterile supplements (see below), inoculated, and plugged. The inoculum was 1 loopful/50 ml. medium, from an 18 hr. culture at 37° in liquid 2% (w/v) sucrose+Bacto-thioglycollate medium.

Capsulation of the bacteria. The presence of capsules was demonstrated by negative staining, with 0·25% (w/v) nigrosine, of the washed bacteria obtained by centrifuging cultures (5 ml.) at 15,000 *g*.

Cultural techniques

Incubation techniques were designed to cover: (*a*) growth in the absence of CO_2 or HCO_3^- but in the presence of oxygen or hydrogen; (*b*) anaerobic growth in the presence of controlled amounts of HCO_3^-; (*c*) growth with a CO_2 gas phase, with or without control of pH value; (*d*) growth in the presence of $CaCO_3$ giving continuous CO_2 and pH control. All cultures were incubated at 37° for a specified time. This incubation temperature was below that of the rumen (40°). Leuconostoc dextransucrase is rapidly destroyed at 40° (Koepsell & Tsuchiya, 1952) and it was therefore decided to incubate the

Streptococcus bovis strains a little below this temperature. In cultures incubated for more than 1 day where it was judged that good dextran production would occur, more sucrose was added every 24 hr. as a sterile solution (4 ml. of 25 % (w/v) solution/50 ml. of culture).

Growth in the absence of CO_2 *or* HCO_3^-. Conical flasks (50 ml.) nearly filled with media were used. They were either closed with a rubber bung or fitted with a bung carrying an open tube filled with self-indicating soda-lime. Cultures were also grown in shallow layers of medium in cottonwool-plugged flasks. Growth under a hydrogen gas phase was done in cottonwool-plugged flasks within a metal anaerobic jar (see below). Tween 80 (Atlas Powder Co. N.Z.) at 1 % (v/v) was added to several of these cultures.

Growth in the presence of controlled amounts of HCO_3^-. The cultures were grown in the conical flasks closed with rubber bungs. Before inoculation the calculated amount of heat-sterilized K_2CO_3 solution (5·4 % w/v) was added.

Growth in a CO_2 *atmosphere.* This was carried out by placing the inoculated cultures, in cottonwool-plugged flasks, in a 4 l. metal anaerobic jar (Messrs Gallenkamp Ltd). Not more than 1 l. of liquid was incubated at a time so that the weight of CO_2 in the jar (> 6 g.) was sufficient, if all had dissolved in the liquid medium during incubation, to give a concentration at least 0·1 M in the medium. Air was displaced by a stream of water-washed CO_2, from a cylinder, for at least 30 min. No special effort was made to free the CO_2 from traces of oxygen.

Growth in the presence of $CaCO_3$. The sterile medium was added to sterile finely divided $CaCO_3$ (3 g./50 ml.) just before inoculation and the flask loosely plugged with cottonwool.

Control of the pH *value of the cultures*

The pH value of the growing cultures fell from 7·0–7·5 to 4·0–4·5 in 24 hr. When required the pH value was adjusted, with sterile aqueous ammonia (sp.gr. 0·95), to 7·0 (or 6·7 if the buffering capacity was high) every 24 hr. This was done by the cautious addition of ammonia whilst checking the pH value with narrow range indicator papers (British Drug Houses Ltd) with aseptic precautions. The ammonia solution was previously sterilized by heating it to the boiling point, rapidly cooling, and repeating the process 3–4 times during 30 min. Inoculation of this solution into sterile nutrient broth never showed growth when incubated at 37° for 3 days. When it was desired to introduce CO_2 or HCO_3^- into the growing culture sterile K_2CO_3 (20 %, w/v) was used to adjust the pH value.

Dextran estimation

Qualitative detection. Soluble polysaccharide was detected by the addition of ethanol (4 vol.) to the supernatant fluid obtained after centrifuging the culture (5 ml.) for 30 min. at 13,000 *g*. When dextran was present the solution yielded a heavy precipitate or a turbidity which readily flocculated after shaking for a few minutes. Cultures containing dextran were always opalescent. Uninoculated medium or clear culture fluid containing no dextran

yielded, on adding ethanol, a turbidity which did not flocculate even after prolonged shaking.

Quantitative measurement of dextran. A volume (50 ml.) of culture was centrifuged for 30 min. at 13,000 *g* to remove bacteria. Very viscous cultures were diluted with an equal volume of water before centrifuging, whilst cultures containing $CaCO_3$ were acidified with 2 N-H_2SO_4 to remove calcium and to avoid the precipitation of calcium lactate by the ethanol. Dextran was precipitated from the centrifuged fluid by the slow addition of ethanol (2 vol.) and then standing the mixture at 0° overnight. The clear supernatant fluid was decanted and kept for paper chromatographic analysis. After draining, the precipitate was dissolved in water (100 ml.), boiled for 3 min., recentrifuged, and reprecipitated with ethanol (2 vol.). The final precipitate was dissolved in water, centrifuged, and made up to volume (100 or 250 ml.) for analysis. After diluting a portion of the solution (1–5 ml.) to 100 ml. with water, dextran was determined by the anthrone method of Roe (1954), a carefully purified sample of the streptococcus dextran being used as a standard. The extinction value of the developed colour was measured, at 625 μ., on a Beckman model DU spectrophotometer with 1 cm. thick optical cells. The yield of dextran was expressed as % conversion of the available anhydroglucose of the sucrose to polymerized anhydroglucose (i.e. dextran). The medium itself, when submitted to the above analysis, gave no measurable amount of polysaccharide.

Yield of bacteria

The solid residue obtained from the first centrifugation of the culture (50 ml.) was suspended in water (70 ml.), thoroughly shaken and centrifuged for 30 min. at 13,000 *g*. The washed organisms were freeze-dried, weighed, and analysed for total-N by the micro-Kjeldahl method.

Structural investigations on the dextran

Purification of the dextran. Dextran solution obtained in the analytical procedure (above), was further purified to remove traces of associated protein. This was achieved by shaking the solution four times with an equal volume of chloroform + amyl alcohol (2·5 : 1, v/v) and centrifuging to remove the emulsion formed (Sevag, Lackman & Smolens, 1938). The purified dextran was precipitated with ethanol (2 vol.), dissolved in water, centrifuged, dialysed overnight, freeze dried and finally dried under vacuum at 60°.

Hydrolysis of the dextran. Dextran (20 mg.) dissolved in 1·5 N-H_2SO_4 (10 ml.) was heated for 9 hr. at 100°, neutralized (3 N-NaOH) and diluted to volume (100 ml.). Reducing sugars were measured in the hydrolysate, as glucose, by the method of Shaffer & Hartmann (1921). The results obtained were corrected by the factor given by Pirt & Whelan (1951) for these conditions. For paper chromatographic analyses the hydrolysate was neutralized with solid $BaCO_3$, filtered, and concentrated to 0·2 ml. Partial hydrolyses were carried out by heating as above for 1·5 hr.

Paper chromatographic analyses. These were prepared from the hydrolysates

and ethanol supernatants, by the usual techniques and developed with either of the following solvents:

(*a*) The top layer of a mixture consisting of *n*-butanol, ethanol, water and ammonia (49, 10, 40 and 1 %, v/v; Barker *et al.* 1954).

(*b*) The top layer of a mixture containing ethyl acetate, water and pyridine (2:2:1 v/v; Jermyn & Isherwood, 1949). Sugars were detected with the following sprays: silver nitrate (Trevelyan, Proctor & Harrison, 1950), aniline hydrogen phthalate (Partridge, 1949), naphthoresorcinol (Partridge, 1948), β-indolylacetic acid (Heyrovsky, 1956), benzylamine+ninhydrin (Bayly & Bourne, 1953).

Periodate oxidation and optical rotation of the dextran

Dextran (100 mg.) was oxidized and periodate consumption and formic acid production measured by the methods referred to by Bailey, Barker, Bourne & Stacey (1957). Optical rotations were measured on solutions of dextran (30 mg.) dissolved in N-NaOH (10 ml.) with a Hilger polarimeter and a 1 dm. tube. Because of the opalescence of dextran solutions exact readings were difficult to obtain.

RESULTS

Fermentation and other reactions of the Streptococcus bovis *strains*

All 16 strains of *Streptococcus bovis* were catalase-negative, morphologically similar, Gram-positive cocci and were typical *S. bovis* in the sense of Mann & Oxford (1955) in that all were amylolytic, and fermented raffinose and inulin but not mannitol. Only one strain (293 C) fermented arabinose, causing a fall in pH to 4·5. No strain produced dextran, in the presence of CO_2, from any simple sugar or oligosaccharide save sucrose. Fructose was fermented by strain I at least. All strains produced very similar mucoid colonies on 3 % (w/v) sucrose + 3 % (w/v) proteose peptone No. 3 (Difco) agar on incubation for 1 day in a CO_2 atmosphere at 37° or 40°. All were homofermentative towards glucose even in the presence of Tween 80 (1–3 %) and brought about a decrease of pH to 4 or less in 1 day at 37°.

Capsulation of the bacteria. All of the strains of *Streptococcus bovis*, particularly in 3-day cultures with pH control, produced zoogleal masses of capsulated cocci in all of the cultures including those producing dextran. *Leuconostoc mesenteroides* also produced capsulated organisms, indistinguishable from those of *S. bovis*, in sucrose-containing cultures.

Qualitative observations on the need for CO_2 *to obtain good dextran production in liquid media*

Streptococcus bovis. All strains gave obvious dextran production in the 8 % (w/v) sucrose + phosphates liquid medium (cf. p. 133) when incubated for 1 day in a CO_2 atmosphere. It seemed to make little difference in this respect whether the surface/volume ratio of the liquid culture was large or small. The cultures became viscous and opalescent and a clean supernatant fluid could not easily be obtained by centrifuging. Another way of providing CO_2 with-

out using a CO_2 atmosphere was to neutralize with K_2CO_3 after some growth had taken place with a consequent fall in pH.

The preliminary results are summarized in Table 1 from which it will be seen that yields of dextran of over 50% of the theoretical were obtained and that phosphate buffering could be dispensed with (see strain IV, Table 1); presumably ammonium lactate acted as the buffer in the sucrose + proteose-peptone medium after some growth had taken place.

Table 1. *Yields of dextran produced by various strains of* Streptococcus bovis, *in liquid media, in the presence of* CO_2

All media contained 8% (w/v) sucrose unless otherwise stated. Incubation was for 3 days at 37°. See p. 133 for composition of the medium.

S. bovis strain	Medium	Volume (ml.)	Cultural conditions	Dextran yield; g. (% conversion of available anhydroglucose)
A_1	Phosphate (*a*)	100	CO_2 atm.; no neutralization	0·36 (9·49)
A_1	Phosphate (*b*)	100	CO_2 atm.; neutralized with K_2CO_3 after 1 day	0·68 (17·94)
A_2	Phosphate (*a*)	100	CO_2 atm.; no neutralization	2·55 (67·28)
A_2	Phosphate (*a*)	100	No CO_2 atm.; neutralized with K_2CO_3 after 1 and 2 days	1·75 (46·17)
18/M2	Phosphate (*b*)	75	CO_2 atm.; neutralized with K_2CO_3 after 1 day	2·12 (83·46)
293C	Phosphate (*c*)	65	CO_2 atm.; no neutralization	0·67 (27·23)
I	Phosphate (*b*)	75	CO_2 atm.; no neutralization	1·29 (50·78)
1010Y	Phosphate (*a*)	100	CO_2 atm.; neutralized with K_2CO_3 after 1 and 2 days	1·44 (37·99)
IV	3% sucrose + 3% (w/v) proteose-peptone No. 3 (no phosphate)	200	2 days CO_2 atm.; neutralized with NH_4OH and more sucrose (8·5 g.) added after 2 days; 3rd day in air	4·00 (50·16)

In the complete absence of CO_2, either in the closed full flask or in air under soda-lime, the results were not so clear cut. Some strains of *Streptococcus bovis*, e.g. I, 18/M2 consistently gave practically no dextran in 3 days (see, however, Table 3 for the effect of Tween 80 in substitution for CO_2 with strain I), even though obvious growth had occurred in 6 hr. Other strains sometimes gave a good dextran production in the absence of CO_2, even in a closed system with oxygen excluded, e.g. strains 293C, 34, 40. The results were not consistent, strain 293C for example giving no dextran in a closed system on another occasion. In every instance, however, the presence of CO_2 either in the gas phase or in solution at 0·10 M did increase the yield of dextran. Shallow layer cultures in a CO_2-free medium, incubated in air, invariably produced some dextran. All *S. bovis* strains can probably utilize oxygen to some small extent since even freshly isolated strains are facultative anaerobes. It was noted that cultures, even in nearly full flasks under soda-lime sometimes produced enough CO_2 to change the colour of the bottom few mm. of the soda-lime layer.

Leuconostoc mesenteroides (*NCIB* 8590). This organism differs from *Streptococcus bovis* in being markedly heterofermentative. In our hands it gave a quicker growth and dextran production from 4% (w/v) sucrose medium, with half the phosphate buffering of (*a*) (p. 133) at 29° in either a closed system or CO_2-free air, than in air or in a CO_2 atmosphere not freed from oxygen. This may merely mean that the particular strain used was microaerophilic. The results were similar in all instances after 2–3 days of incubation. Gas production in the closed system cultures was very obvious.

Minimum concentration of HCO_3^- *required for good dextran production with* Streptococcus bovis *strain I incubated in a closed system*

The results summarized in Table 2 indicate that, with *Streptococcus bovis*, strain I, an initial CO_2 concentration of at least 0·005 M (as HCO_3^-) was necessary for good dextran production when neither CO_2 gas nor oxygen was provided. Results from a similar experiment with *S. bovis* strain 1010 Y were much

Table 2. *Effect of* HCO_3^- *concentration on the yields of dextran produced by* Streptococcus bovis, *strain I*

Medium (*c*) p. 133; 50 ml. (containing 4 g. sucrose) in a closed system; incubated for 2 days at 37°.

Initial K_2CO_3 (M)	Yield of organism: Dry wt. (mg.)	Yield of organism: Protein (N × 6·25) (mg.)	Dextran as % conversion of available anhydro-glucose	Sugars in supernatant fluid at end: Fructose	Sucrose	Reducing disaccharide
None	58	29·00	4·76	−	++++	−
0·0004	71	35·50	8·73	+	++++	−
0·0017	89	41·00	16·40	++	++++	+
0·005	87	46·00	39·15	+++	+++	+
0·014	90	42·10	50·27	++++	++	++
0·028	No growth		—	−	−	−

more erratic. This is one of the strains that does not seem invariably to need CO_2 in some form for dextran production. Neither strain grew in phosphate medium (*c*) in the presence of 0·028 M-HCO_3^-, even though the initial pH was below 8; presumably the salt concentration was too high.

It was obviously necessary to show that the extra buffering power of the medium due to the addition of K_2CO_3 was not the cause of dextran production. Medium (*c*) was therefore made a stronger phosphate buffer by the addition of Na_2HPO_4, $12H_2O$ (0·3%; 0·008 M) and KH_2PO_4 (0·05%; 0·004 M) without change of pH value. Parallel cultures of *Streptococcus bovis* strain I in medium (*c*) modified and unmodified (each neutralized with ammonia after 1 and 2 days) in the absence of CO_2 in a closed system gave no qualitative reaction for dextran in either instance although growth was profuse. When the third day of incubation was in a CO_2 atmosphere, however, there was good dextran production in each instance.

Minimum sucrose concentration necessary for dextran production in a CO_2 atmosphere

Five strains of *Streptococcus bovis* (A1, I, 293C, 1010Y, IV) all behaved similarly in that no dextran was detected qualitatively in liquid thioglycollate medium (*c*) cultures containing initially 0·25% (w/v) sucrose (final pH 6·1). The polysaccharide was, however, definitely produced from 0·5 (w/v) sucrose medium (final pH 5·5–5·7). The final pH value was always below 5 when the initial sucrose concentration exceeded 0·75%.

The effect of various cultural conditions on growth and dextran production

The results obtained, with *Streptococcus bovis* strains 1010Y and I are detailed in Table 3. It is evident that the presence of CO_2 increased dextran yield, with or without pH control, although it seems to have had little effect on yields of organism. Maintaining the pH at 6·5–7 improved dextran yields in CO_2 and gave an increased yield of organism. The best way so far found for fulfilling the above conditions, i.e. addition of $CaCO_3$, gave the highest yields (nearly 80% conversion of available anhydro-glucose with strain I) obtained for each strain. The results however (Tables 1 and 3) do show considerable strain differences in the effect of pH control and CO_2 on dextran production. Tween 80 did replace CO_2 to some extent in stimulating dextran formation. Un-neutralized cultures incubated in CO_2 for 1 or 3 days gave closely similar yields of organism and dextran. These results emphasize the need for pH control, as well as for CO_2 in order to obtain optimum dextran production and cell multiplication.

Chromatographic examination of the cell-free supernatant fluid showed that, as with *Leuconostoc mesenteroides* NCIB 8590, fructose appeared concurrently with dextran formation. The occurrence of other oligosaccharides in the cultures is discussed below.

Structure of the dextran

Structural studies were confined to the dextrans produced by the two strains of *Streptococcus bovis*, 1010Y and I, used in the experiments described in Tables 2 and 3 and isolated from the cultures described in Table 1. Chromatograms of total hydrolysates showed a single reducing component chromatographically identical with glucose. There was no sign of any of the component sugars (galactose and rhamnose) of the capsular polysaccharide or of fructose. Chromatograms of partial hydrolysates, developed with solvent (*a*), showed a single reducing component, $R_{glucose}$: 0·40, chromatographically identical with *iso*-maltose produced by the partial hydrolysis of *Leuconostoc* (*Betacoccus*) *arabinosaceous* dextran (Barker *et al.* 1954): $R_{glucose}$: isomaltose 0·40; maltose 0·50; cellobiose 0·47.

When sprayed with aniline hydrogen phthalate the disaccharide component gave the same brownish yellow colour as isomaltose. The component was also identical with isomaltose on chromatograms developed with solvent (*b*) and by the benzylamine technique with solvent (*a*). In this latter case the

Table 3. *Yields of dextran, and bacterial protein, produced by* Streptococcus bovis, *strains I and 1010Y under various cultural conditions*

Medium (*c*) p. 133 (50 ml. containing 4 g. sucrose, initially) in all cases; incubation for 2 days at 37° unless otherwise stated.

Gas phase	Neutralization during incubation	Supplements added after 1 and 2 days	Bacterial cell yields: Dry wt. (mg.)	Bacterial cell yields: Protein (N × 6·25) (mg.)	Dextran as % conversion of available anhydroglucose	Sugars in supernatant at end of growth: Fructose	Glucose	Sucrose	Reducing disaccharide
			Strain I						
None; closed system	None	None	48·0	22·4	7·14	0	0	++++	0
None; closed system	None	Tween 80 (1 % at start)	54·0	26·8	16·40	+++	0	+++	+
Hydrogen	None	None	68·0	20·8	0	0	0	++++	0
CO_2-free air; (1 day)	None	None	36·7	21·2	6·5	+	0	++++	+
CO_2-free air; (3 days)	None	None	47·7	23·0	6·7	+	0	++++	0
CO_2-free air; (2 days)	NH_4OH (once)	Sucrose (1 g.)	92·3	36·4	16·4	++	0	+++	+
CO_2-free air; (3 days)	NH_4OH (twice)	Sucrose (1 g.)	171·0	67·6	18·6	++++	++++	++	+++*
CO_2 (1 day)	None	None	52·5	27·2	32·2	+++	0	+++	+
CO_2 (3 days)	None	None	66·1	22·2	34·7	++++	0	+++	+
CO_2 (2 days)	NH_4OH (once)	Sucrose (1 g.)	102·3	48·3	50·0	++++	0	0	+++
CO_2 (3 days)	NH_4OH (twice)	Sucrose (1+1 g.)	116·9	54·0	59·0	++++	0	0	+++
Air	$CaCO_3$ continuously	Sucrose (1+1 g.)	—	—	79·2	++	0	0	+++
			Strain 1010Y						
None; closed system	None	None	—	—	9·0	+	0	++++	0
None; closed system	NH_4OH (once)	Sucrose (1 g.)	64·5	32·9	7·1	+	0	++++	0
Hydrogen	None	None	74·9	34·0	1·5	0	0	++++	0
CO_2-free air; (1 day)	None	None	47·6	27·6	20·2	++++	0	+++	0
CO_2-free air; (3 days)	None	None	52·8	25·6	19·0	++	0	+++	+
CO_2-free air; (2 days)	NH_4OH (once)	Sucrose (1 g.)	30·3	16·4	11·6	++	0	+++	+
CO_2-free air; (3 days)	NH_4OH (twice)	Sucrose (1+1 g.)	137·0	50·5	38·3	++++	+++	++	+++*
CO_2 (1 day)	None	None	45·0	23·4	29·4	++++	0	+++	+
CO_2 (3 days)	None	None	50·2	26·4	30·3	+++	0	+++	+
CO_2 (2 days)	NH_4OH (once)	Sucrose (1 g.)	50·2	19·0	54·0	++++	+	++	+++*
CO_2 (3 days)	NH_4OH (twice)	Sucrose (1+1 g.)	72·8	21·4	46·3	+++	0	+	++
Air	$CaCO_3$ continuously	Sucrose (1+1 g.)	—	—	57·2	++++	0	0	++++

* Also homologous series of oligosaccharides; probably the isomaltodextrins (see Turvey & Whelan, 1956).

disaccharide had an R_F value of 0·37, cf. isomaltose 0·37, maltose 0·44, cellobiose 0·42 and glucose 0·65. A series of higher oligosaccharides was also present in the chromatograms from the partial hydrolysates and appeared to be identical with the series produced from the leuconostoc dextran (cf. Turvey & Whelan, 1957). Using the benzylamine technique the trisaccharide of the series had an R_F value of 0·20 (cf. isomaltotriose 0·20).

Total acid hydrolyses of the dextrans from *Streptococcus bovis* strains I and 1010Y gave 97·0 and 96·0% conversion to glucose, respectively. Their optical rotations were: I dextran, $[\alpha]_D^{20} + 190°$ (C. 0·30) and 1010Y dextran, $[\alpha]_D^{20} + 187°$ (C. 0·36). During 96 hr. of oxidation, at 29°, with sodium *meta*periodate dextrans I and 1010Y consumed 2·00 and 1·94 mole periodate and produced 0·97 and 0·96 mole of formic acid/mole anhydro-glucose, respectively.

Other oligosaccharides in the cultures

In cultures producing dextran, in addition to fructose, a second reducing disaccharide was detected with the silver nitrate spray (see Table 3). This component had a value of $R_{glucose}$ in solvent (*a*); 0·56 (cf. sucrose 0·70; isomaltose 0·44). It gave a positive ketose test with naphthoresorcinol and β-indolyl acetic acid sprays when they contained 2 N-HCl, but not when the acid was trichloroacetic acid. A small portion of this sugar was isolated on paper chromatograms and hydrolysed with oxalic acid (1%, w/v) at 100° for 2 hr. Paper chromatograms of the hydrolysate showed the presence of glucose and fructose and much unhydrolysed disaccharide.

Normally no sign of glucose could be detected on chromatograms of the culture fluid. *Leuconostoc* spp. produce definite traces of glucose because of their secretion of small amounts of a levansucrase. The complete absence of glucose suggests that *Streptococcus bovis* does not secrete such a levansucrase. In a few 3-day neutralized cultures, however (Table 3), a strong glucose component together with components corresponding to the isomaltose series of oligosaccharides (cf. Turvey & Whelan, 1957) was present on the chromatograms.

DISCUSSION

The chromatographic identification of isomaltose as the sole disaccharide in the polysaccharide hydrolysates indicates that the polyglucosan is in fact dextran; this is confirmed by the positive optical rotation of the polysaccharide. The $[\alpha]_D$ values, together with the periodate oxidation figures, are in agreement with the results that would be expected for a polymer closely resembling unbranched leuconostoc dextran (Barker *et al.* 1955). There is a real need for CO_2 for maximum dextran production and presumably for dextransucrase secretion.

The appearance of an oligosaccharide concurrently with dextran production is not altogether unexpected as such sugars have been reported to occur in leuconostoc cultures (Stodola, Koepsell & Sharpe, 1952). In this latter case they are believed to arise through fructose acting as an alternative glucosyl acceptor. Whether this is the case in *Streptococcus bovis* cultures awaits identification

of the sugar. Detection of large amounts of glucose and the isomaltose series of oligosaccharides in occasional cultures was unexpected and an exception to the general complete absence of glucose. It may be suggested that in these cultures the organism had died and autolysis of the cells had released an invertase which had produced sufficient glucose to act as an alternative glucosyl acceptor. Glucose does act as such an acceptor to give oligosaccharides when added to leuconostoc cultures (Bailey, Barker, Bourne & Stacey, 1955). As *S. bovis* is capable of fermenting sucrose without producing dextran presumably it contains an invertase.

Given the right conditions there can be little doubt that *Streptococcus bovis* is practically as good a dextran producer as *Leuconostoc mesenteroides*. Since *S. bovis* has a higher optimum growth temperature than *L. mesenteroides* the former produces dextran at a faster rate. It is tempting to relate the almost invariable CO_2 requirement of *S. bovis* for good dextran production to the fact that this organism is in the main homofermentative (see Smith & Sherman, 1942), whereas *L. mesenteroides* is heterofermentative; i.e. produces its own CO_2 by hexose fermentation. One of our *S. bovis* strains (I), which had an absolute CO_2 requirement for dextran production, required only 0·005 M-HCO_3^- to produce a good yield of dextran. It may well be that *S. bovis* strains with no such absolute CO_2 requirement are ordinarily sufficiently heterofermentative to provide enough CO_2 for limited dextran formation. The data of Smith & Sherman (1942) show that *S. bovis* on the whole is not quite as good a lactic acid producer as *S. lactis*. In the above cases, however, so little gas is produced that they would be reported as homofermentative in ordinary bacteriological tests with Durham tubes. Since most *S. bovis* strains seem able to use oxygen to some extent, it is essential to exclude air as far as possible if a real CO_2 requirement for dextran production is to be demonstrated. It is worth noting that a closed culture flask half full of medium contains enough oxygen, in the air space, to yield 0·01 M-HCO_3^- in the medium, provided that all of the oxygen is used for the complete oxidation of a carbohydrate to CO_2.

If *Streptococcus bovis* dextransucrase has the same properties as leuconostoc dextransucrase, i.e. a pH optimum of 4·0–4·5 and a temperature optimum of 25–29° with destruction at 37° (Bailey *et al.* 1957) then good dextran yields can only be obtained when the enzyme is continuously produced. This can only occur during continuous growth and all of the evidence (Table 3) indicates that pH control between 6 and 7 is necessary to ensure this. The properties of cell-free enzyme preparations are being investigated in order to clarify these points.

An important question is whether the effect of CO_2 on dextran yield is due to CO_2 gas in solution, the bicarbonate ion, or both. According to Umbreit, Burris & Stauffer (1945) HCO_3^- cannot exist in solution at pH values below 5, nor can CO_3^{--} really exist below pH 8. Further, H_2CO_3 hardly exists at any pH value. Therefore in the pH range under study either or both pCO_2 and HCO_3^- may be implicated (cf. Loomis, 1957; Salisbury & Vandemark, 1957; Whitehead, Jones & Robertson, 1958; for instances of the control of biological

phenomena by pCO_2). We have not yet been able to settle this question. *Streptococcus bovis* (strain I) would not grow in a sucrose-containing nutrient medium containing HCO_3^- through which a slow stream of CO_2-free and O_2-free hydrogen was bubbled for 1 day. The same batch of medium did however give good growth and dextran production in the same time in an anaerobic jar filled with H_2. The culture which failed to grow in a stream of H_2 later grew when air was admitted to the culture flask. Presumably the medium in the latter instance had an initial pCO_2 of about 0·02 % by volume (0·00001 M) as have all undisturbed aqueous liquids in air at 30–40° regardless of pH and HCO_3^- concentration (cf. Loomis, 1957). It will obviously need much careful work to disentangle the effects of trace amounts of CO_2 in initiating growth on the one hand and of the larger amounts which encourage dextran production on the other hand.

It must be granted that the role of CO_2 in dextransucrase formation, if such a role exists, is not obvious at first sight. It may be a physical one since we have confirmed the observations of Dain *et al.* (1956) that Tween 80 can replace CO_2 in anaerobic conditions in encouraging dextran formation. A second possibility is that CO_2, at the higher concentration, inhibits all modes of sucrose fermentation operating with *Streptococcus bovis* save one, the dextransucrase pathway, which may be stimulated. This latter possibility seems to be the best working hypothesis at the moment (cf. Salisbury & Vandemark, 1957). Although traces of CO_2 are apparently necessary to initiate growth, higher concentrations do not increase it (see Table 3). An explanation of why CO_2 stimulates the secretion of dextransucrase by *S. bovis* might throw considerable light on the mechanism of the secretion of trans-glucosidases of bacterial cells.

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REFERENCES

Bailey, R. W., Barker, S. A., Bourne, E. J. & Stacey, M. (1955). Synthesis of oligosaccharides by growing cultures of *Betacoccus arabinosaceous*. *Nature, Lond.* 175, 635.

Bailey, R. W., Barker, S. A., Bourne, E. J. & Stacey, M. (1957). The isolation and properties of the dextransucrase of *Betacoccus arabinosaceous*. *J. chem. Soc.* p. 3530.

Barker, S. A., Bourne, E. J., Bruce, G. T., Neely, W. G. & Stacey, M. (1954). The structure of a *Betacoccus arabinosaceous* dextran. *J. chem. Soc.* p. 2395.

Barker, S. A., Bourne, E. J., James, A. E., Neely, W. B. & Stacey, M. (1955). The structure of a modified *Betacoccus arabinosaceous* dextran. *J. chem. Soc.* p. 2096.

BAYLY, R. J. & BOURNE, E. J. (1953). A new method for the paper chromatography of oligosaccharides. *Nature, Lond.* **171**, 385.

DAIN, J. A., NEAL, A. L. & SEELEY, H. W. (1956). The effect of carbon dioxide on polysaccharide production by *Streptococcus bovis*. *J. Bact.* **72**, 209.

HEHRE, E. J. & NEILL, J. M. (1946). Formation of serologically active dextran by streptococci from subacute bacterial endocarditis. *J. exp. Med.* **83**, 145.

HEYROVSKY, A. (1956). A new detection method for ketose sugars. *Biochim. Biophys. Acta*, **21**, 180.

HOBSON, P. N. & MACPHERSON, M. J. (1954). Some serological and chemical studies on materials extracted from an amylolytic streptococcus from the rumen of the sheep. *Biochem. J.* **57**, 145.

HUNGATE, R. E. (1957). Micro-organisms in the rumen of cattle fed on a constant ration. *Canad. J. Microbiol.* **3**, 289.

HUNGATE, R. E., FLETCHER, D. W., DOUGHERTY, R. W. & BARRENTINE, B. F. (1955). Microbial activity in the bovine rumen: its measurement and relation to bloat. *Appl. Microbiol.* **3**, 161.

JEANES, A. R. & WILHAM, C. A. (1952). Periodate oxidation of dextrans. *J. Amer. chem. Soc.* **74**, 5339.

JERMYN, M. A. & ISHERWOOD, F. A. (1949). Improved separation of the sugars on the paper partition chromatogram. *Biochem. J.* **44**, 402.

KOEPSELL, H. J. & TSUCHIYA, H. M. (1952). Enzymatic synthesis of dextran. *J. Bact.* **63**, 293.

LOOMIS, W. F. (1957). Sexual differentiation in Hydra. *Science*, **126**, 735.

MACPHERSON, M. J. (1953). Isolation and identification of amylolytic streptococci from the rumen of the sheep. *J. Path. Bact.* **66**, 95.

MANN, S. O., MASSON, F. M. & OXFORD, A. E. (1954). Facultative anaerobic bacteria from the sheep's rumen. *J. gen. Microbiol.* **10**, 142.

MANN, S. O. & OXFORD, A. E. (1955). Relationships between viable saccharolytic bacteria in rumen and abomasum of the young calf and kid. *J. gen. Microbiol.* **12**, 140.

NIVEN, C. F., SMILEY, K. L. & SHERMAN, J. M. (1941). The polysaccharide synthesized by *Streptococcus salivarius* and *Streptococcus bovis*. *J. biol. Chem.* **140**, 105.

PARTRIDGE, S. M. (1948). Filter paper partition chromatography of sugars. *Biochem. J.* **42**, 238.

PARTRIDGE, S. M. (1949). Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* **166**, 443.

PIRT, S. J. & WHELAN, W. J. (1951). The determination of starch by acid hydrolysis. *J. Sci. Fd Agric.* **2**, 224.

ROE, J. H. (1954). The determination of dextran in blood and urine with anthrone reagent. *J. biol. Chem.* **208**, 889.

SALISBURY, G. W. & VANDEMARK, N. L. (1957). Sulfa compounds in reversible inhibition of sperm metabolism by carbon dioxide. *Science*, **126**, 1118.

SEVAG, M. G., LACKMAN, D. B. & SMOLENS, J. (1938). The isolation of the components of streptococcal nucleoproteins in serologically active form. *J. biol. Chem.* **124**, 425.

SHAFFER, P. A. & HARTMANN, A. F. (1921). The iodometric determination of copper and its use in sugar analyses. *J. biol. Chem.* **45**, 365.

SMITH, P. A. & SHERMAN, J. M. (1942). The lactic acid fermentation of streptococci. *J. Bact.* **43**, 725.

STODOLA, F. H., KOEPSELL, H. J. & SHARPE, E. S. (1952). A new disaccharide produced by *L. mesenteroides*. *J. Amer. chem. Soc.* **74**, 3202.

Topley & Wilson's Principles of Bacteriology and Immunity (1946). 3rd ed. p. 600. Edited by Wilson, G. S. & Miles, A. A. London: Edward Arnold and Co.

TREVELYAN, W. E., PROCTOR, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, Lond.* **166**, 444.

TURVEY, J. R. & WHELAN, W. J. (1957). Preparation and characterization of the isomaltodextrins. *Biochem. J.* **67**, 49.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Publishing Co.

WHITEHEAD, H. R., JONES, P. A. & ROBERTSON, P. S. (1958). The influence of carbon dioxide on the growth of lactic streptococci. *J. Dairy Res.* (in Press).

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