10. Creatine phosphate had no effect on the rates of acetoacetate reduction but abolished the inhibition of respiration by 2:4-dinitrophenol.

11. The observations on the formation of β -hydroxybutyrate from acetoacetate summarized in paragraphs 1, 4, 5, 7 and 10 are in agreement with Scheme I, according to which the reduction takes place whenever reaction (1) is more rapid than reaction (2).

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Oligosaccharides in Dextran-Producing Cultures of *Streptococcus bovis*

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Species of *Leuconostoc mesenteroides* and *Streptococcus bovis* produce dextran and fructose when grown in media containing sucrose (Hehre, 1951; Tsuchiya *et al.* 1952; Bailey, Barker, Bourne & Stacey, 1957; Dain, Neal & Seely, 1956; Bailey & Oxford, 1958a). In addition, reducing disaccharides containing glucose and fructose units are formed. One of these, leucrose (5-O-D-glucopyranosyl-D-fructopyranose), has been isolated from cultures of

L. mesenteroides (NRRL B-512F) and characterized by Stodola, Sharpe & Koepsell (1956), who also mentioned the formation of isomaltulose (Sharpe, Stodola & Koepsell, 1954). Bailey & Bourne (1959) have isolated leucrose from dextran-producing cultures of *S. bovis* and noted that a second disaccharide was produced. The present paper reports a closer examination of the oligosaccharides produced in cultures of *S. bovis*.

EXPERIMENTAL

Organism. *Streptococcus bovis* (strain I; Bailey & Oxford, 1958a, b) was used.

Culture medium. The organism was grown in a medium containing yeast extract (1%, w/v), Bacto-tryptose (1.5%, w/v), sucrose (8.5%, w/v) and separately sterilized potassium carbonate (0.004M), pantothenic acid (20 mg./l.) and biotin (0.01 mg./l.). A 1 l. culture was incubated at 37° for 24 hr., when more sucrose (30 g.) and sodium bicarbonate (1 g.) were added. The pH of the medium was adjusted to 6.5 with N-NaOH and the culture incubated for a further 3 days.

Paper chromatography. Whatman no. 1 filter paper was used. The solvent systems were: (a) organic phase of ethyl acetate–water–pyridine (2:2:1, by vol.); (b) ethyl acetate–acetic acid–water (9:2:2, by vol.); (c) butanol–benzene–pyridine–water (5:1:3:2, by vol.); (d) water-saturated butanol.

Paper ionophoresis. Ionophoresis was conducted on Whatman no. 3 MM filter paper in 0.1M-borate, pH 10 (Foster, 1953), and 0.1M-molybdate, pH 5.5 (Bourne, Hutson & Weigel, 1959), at 80 v/cm. for about 1 hr.

Selective spray reagents. The spray reagents used were: (a) acetone–silver nitrate–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950), for the general detection of carbohydrates; (b) urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955), for the detection of ketoses, particularly compounds containing a leucrose unit, which stain a dull brown, strongly fluorescent in u.v. light; (c) aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956), found to give a specific green spot with 6-O-substituted glucose (e.g. isomaltose); (d) triphenyltetrazolium chloride (Feingold, Avigad & Hestrin, 1956), reaction with which was regarded as evidence that reducing glucose units were unsubstituted at the C-2 hydroxyl group.

Reduction of aldoses and ketoses. Aqueous solutions of the sugars (about 1%) were mixed with an equal volume of potassium borohydride solution (1%) and allowed to stand overnight (Bragg & Hough, 1957). The solutions were deionized by treatment with Amberlite resin IR-120 (H⁺) followed by evaporation to dryness and repeated distillation with dry methanol. Completion of reduction was checked by ionophoresis in molybdate solution (Bourne *et al.* 1959). The product was always purified by paper chromatography.

Acid hydrolysis. Hydrolysis was effected at 96° in 0.5N-sulphuric acid for 3 hr. (complete) or 1 hr. (partial). The solutions were deionized by treatment with Amberlite resin IRA-400 (carbonate).

RESULTS

Isolation of oligosaccharides

At the end of the incubation period dextran was removed from the culture fluid (1 l.) by precipitation with acetone (2 vol.). The supernatant liquid was evaporated to 200 ml. and fractionated on a charcoal–Celite column (40 cm. × 7 cm.; Whistler & Durso, 1950). Water eluted fructose and a small amount of glucose. Di- and tri-saccharides were

eluted with aqueous 1–6% and 6–10% ethanol respectively. A small quantity of material eluted with aqueous 15% ethanol was not examined further.

Leucrose (m.p. 156°, mixed m.p. 156°) was crystallized from the disaccharide fraction in ethyl acetate (Bailey & Bourne, 1959). The mother liquor was fractionated on a cellulose column (Whatman cellulose powder, standard grade; 60 cm. × 4.5 cm.) with solvent (c) as the irrigant. The fraction containing disaccharide A was concentrated, dissolved in water and freeze-dried. From the fraction containing leucrose a disaccharide B was isolated in trace quantity by paper chromatography with solvent (b).

Paper chromatography of the trisaccharide fraction (300 mg.) with solvent (a) revealed a trace quantity of a trisaccharide A, which was isolated by this method. The main trisaccharide fraction then appeared to be homogeneous in the four chromatography solvents, but 100 mg. was resolved into two components, trisaccharide B (about 50 mg.) and C (about 40 mg.), by paper ionophoresis in borate solution. The yields of oligosaccharides from a 1 l. culture are shown in Table 1.

Structural examination of the oligosaccharides

Disaccharide A. Disaccharide A stained blue with spray (b) (ketose-type) and had the characteristics shown in Table 2. Its high rate of mobility during ionophoresis in molybdate solution was indicative of a 6-O-substituted fructose (Bourne, Hutson & Weigel, 1960). Acid hydrolysis gave glucose and fructose (paper chromatography), shown by elution and determination with anthrone (Yemm & Willis, 1954) to be present in the ratio 1:0.75. Determination of glucose and fructose in an artificial mixture (ratio 1:1), treated with acid in the same way as the disaccharide, revealed a glucose/fructose ratio of 1:0.81.

Disaccharide A was reduced with potassium borohydride (Bragg & Hough, 1957). Ionophoresis in molybdate solution (pH 5.5) showed that the alcohol migrated as a single component, with

Table 1. Yields of oligosaccharides from a 1 l. culture of *Streptococcus bovis* grown on sucrose (115 g.)

The expected yield of dextran would be about 40 g.	
Compound	Yield
Leucrose	1.8 g.
Disaccharide A (isomaltulose)	250 mg.
Disaccharide B (isomaltose)	Trace
Trisaccharide A (isomaltotriulose)	Trace
Trisaccharide B (isomaltotriose)	150 mg.
Trisaccharide C (5-O- α -isomaltosyl-D-fructose)	120 mg.

mobility referred to sorbitol (M_s 0.8 (identical with that of a 5- or 6-*O*-glycosyl-sorbitol or -mannitol), and that on hydrolysis it gave glucose and hexitol but not fructose.

When estimated by the Shaffer & Hartmann (1921) method disaccharide A (4 mg.) had a reducing power equivalent to 1.84 mg. of fructose. This corresponded to 87.7% of the calculated value for a disaccharide.

Disaccharide A was incubated with almond β -glucosidase at 25° for 24 hr. Paper chromatography showed that no hydrolysis had occurred. Under the same conditions 1-*O*- β -D-glucopyranosyl-D-fructose was completely hydrolysed.

Disaccharide A (50 mg.) was converted into its phenylosazone (30 mg.) by the method of Avigad (1959). The recrystallized phenylosazone had m.p. 171–173°. Admixture with isomaltose phenylosazone, m.p. 173–175°, caused no depression in melting point. The phenylosazone behaved as a pure disaccharide phenylosazone during chromatography in solvents (a) and (d). Its infrared spectrum was identical with that of isomaltose phenylosazone. Thus disaccharide A was isomaltulose.

Disaccharide B. Disaccharide B could be detected on paper chromatograms and ionophoretograms with spray reagents (a), (c) and (d), and was indistinguishable from isomaltose (Table 2). Spray (c) gave a green spot characteristic for a 6-*O*-substituted glucose. Acid hydrolysis gave only glucose (paper chromatography). Thus the trace disaccharide B was almost certainly isomaltose.

Trisaccharide A. Trisaccharide A could be detected on paper chromatograms and ionophoretograms (Table 2) with the spray reagents (a) and (b). Chromatography and ionophoresis of the complete acid hydrolysate revealed the presence of com-

ponents identical with glucose and fructose (small amount), and a partial hydrolysate contained also components identical with isomaltose and isomaltulose.

Trisaccharide A alcohol, obtained by reduction of trisaccharide A with potassium borohydride (Bragg & Hough, 1957), had M_s 0.75, identical with that of 6-*O*-isomaltosylsorbitol and different from all but 5- or 6-*O*-biosyl-sorbitols or -mannitols. Complete hydrolysis of the alcohol gave components identical with glucose and a hexitol; a partial hydrolysate contained isomaltose and glucose as the only reducing components. Thus trisaccharide A was most probably isomaltotriulose.

Trisaccharide B. Trisaccharide B could be detected on paper chromatograms and ionophoretograms with spray reagents (a), (c) (characteristic green for a 6-*O*-substituted glucose) and (d). It was indistinguishable from isomaltotriose (Table 2). Complete acid hydrolysis gave only glucose, whereas a partial acid hydrolysis gave isomaltose and glucose. It was not hydrolysed by almond β -glucosidase.

Trisaccharide B was reduced with potassium borohydride (Bragg & Hough, 1957). Ionophoresis in molybdate solution (pH 5.5) showed that the alcohol migrated at the same rate as 6-*O*-isomaltosylsorbitol (M_s 0.75; see above). Complete hydrolysis of the alcohol gave glucose and a hexitol. A partial hydrolysate of the alcohol was shown, by chromatography in solvents (a) and (b) and ionophoresis in molybdate solution (pH 5.5), to contain two reducing components identical with glucose and isomaltose, and two non-reducing components identical with a hexitol and 6-*O*-glucopyranosyl-sorbitol, as well as the unchanged alcohol.

Table 2. *Properties of some oligosaccharides compared with those isolated from Streptococcus bovis cultures*

Solvents: (a) organic phase of ethyl acetate–water–pyridine (2:2:1, by vol.); (b) ethyl acetate–acetic acid–water (9:2:2, by vol.); (c) butanol–benzene–pyridine–water (5:1:3:2, by vol.); (d) water-saturated butanol.

Compound	Chromatography R_f				Ionophoresis		Phenylosazone (m.p.)
	Solvent (a)	Solvent (b)	Solvent (c)	Solvent (d)	M_g (borate)	M_s (molybdate)	
1- <i>O</i> - β -Glucopyranosyl-fructose	0.59	0.41	0.57	0.28	0.74	0.25	—
Sucrose	0.74	0.63	0.81	0.50	0.10	0	—
Turanose	0.75	0.54	0.81	0.51	0.69	0.1	202–204°
Maltulose	0.63	0.44	0.64	0.39	0.63	0.15	202–204
Leucrose	0.55	0.50	0.50	0.32	0.56	0.35	186–188
Isomaltulose	0.64	0.50	0.68	0.33	0.60	0.64	173–175
Isomaltose	0.58	0.27	0.50	0.31	0.70	0	173–175
Isomaltotriose	0.35	0.15	—	0.10	0.58	0	—
Disaccharide A	0.63	0.50	0.69	0.35	0.61	0.65	171–173
Disaccharide B	0.59	0.28	0.50	0.31	0.70	0	—
Trisaccharide A	0.41	0.24	—	0.15	0.50	0.47	—
Trisaccharide B	0.35	0.15	—	0.10	0.57	0	—
Trisaccharide C	0.35	0.15	—	0.10	0.44	0.30	—

Trisaccharide C. Trisaccharide C could be detected on paper chromatograms and ionograms (Table 2) with spray reagent (b) as a brown spot which fluoresced in u.v. light (as did leucrose). Acid hydrolysis yielded glucose and a smaller quantity of fructose (paper chromatography). A partial hydrolysate contained glucose, isomaltose and small amounts of leucrose and fructose, as well as the unchanged trisaccharide. Chromatography of an equimolar mixture of isomaltose and leucrose, after the same treatment with acid, gave a similar pattern.

Trisaccharide C was reduced with potassium borohydride (Bragg & Hough, 1957). During ionophoresis in molybdate solution (pH 5.5) the product migrated with M_s 0.75 (see above). Complete acid hydrolysis of the alcohol gave glucose as the only reducing hexose together with a hexitol. Chromatography and ionophoresis (molybdate) of a partial hydrolysate of the alcohol revealed the presence of components identical with glucose, a hexitol, isomaltose, 5- or 6-*O*-glycosylsorbitol and unchanged material.

Trisaccharide C was not hydrolysed by almond β -glycosidase; thus it was most probably *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 5)-D-fructose (5-*O*- α -isomaltosyl-D-fructose).

Action of partially purified Streptococcus bovis dextranucrase on leucrose in the presence of sucrose

Leucrose (50 mg.) and sucrose (50 mg.) were incubated at 37° with dextranucrase (2 mg.) in 0.05M-acetate buffer (pH 5.2, 2 ml.) for 24 hr. The dextranucrase preparation was supplied by Dr R. W. Bailey and prepared according to Bailey (1959). Chromatography of the digest revealed a small amount of a component with R_f 0.35, 0.15 and 0.10 in solvents (a), (b) and (d) respectively. It could be detected with spray reagents (a) and (b). The latter gave a brown spot which fluoresced strongly in u.v. light. Some of this component was isolated by paper chromatography and subjected to the same analysis as trisaccharide C. In all these tests the compound was identical with trisaccharide C. The enzyme did not produce detectable amounts of this trisaccharide from leucrose or sucrose separately. This confirmed the structure assigned to the trisaccharide.

DISCUSSION

The evidence presented here and by Bailey & Bourne (1959) characterizes three of the oligosaccharides in *S. bovis* cultures as isomaltulose, leucrose and *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 5)-D-fructose (5-*O*- α -isomalto-

syl-D-fructose); other products were almost certainly isomaltose, isomaltotriose and isomaltotriulose. A large quantity of fructose and a smaller amount of glucose were also present; all of the sucrose had been utilized.

It has been shown that certain simple sugars can act as receptors of transferred glucosyl units when dextranucrase preparations from *L. mesenteroides* (NRRL B-512) (Koepsell *et al.* 1953), *L. mesenteroides* (Birmingham) (Bailey *et al.* 1957) and *S. bovis* (Bailey, 1959) act on sucrose. Such sugars lead to the synthesis of oligosaccharides. Isomaltose and isomaltotriose were found, in the *Leuconostoc* system, to be good receptors whereas glucose, fructose and leucrose acted as poor receptors. The present work shows that the same is true for the *S. bovis* dextranucrase system.

Glucosyl transfer of the above type can occur during dextran synthesis and be responsible for by-products. The amount of such by-products will depend not only on the efficiency of the receptor molecules but also on their relative concentrations. Thus transfer to fructose, which is a poor receptor but present in large quantities, results in relatively large amounts of leucrose (mainly) and isomaltulose. Transfer to glucose would result in much less isomaltose. The concentrations of disaccharides could increase until they competed with fructose as receptors, thus leading to 5-*O*- α -isomaltosyl-D-fructose and isomaltotriulose. The production of isomaltotriose is almost certainly via isomaltose. That the latter was found only in very small amount is reasonable as it is a good receptor and would be soon converted into isomaltotriose. It is interesting that fructose appears to act as a receptor in both the pyranose and furanose forms, to give leucrose and isomaltulose respectively, the former being preferred.

After the present work had been completed, Sharpe, Stodola & Koepsell (1960) confirmed the formation of isomaltulose as a by-product during the synthesis of dextran by enzyme preparations of *L. mesenteroides* (NRRL B-512F).

SUMMARY

1. *Streptococcus bovis*, when grown in a medium containing sucrose, has been shown to produce leucrose, isomaltulose, isomaltose, isomaltotriose, 5-*O*- α -isomaltosyl-D-fructose and isomaltotriulose.
2. The mechanism of the formation of these oligosaccharides is discussed.

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The Effect of Hind-Limb Ischaemia on the Products and Intermediates of Glycolysis in Rat Tissues

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In small mammals the survival period after fatal limb ischaemia can be divided into two parts. In the first phase, under ordinary environmental conditions (air temperature 18–22°), the temperatures of the main heat-producing organs in the rat, the liver and brain, fall before their oxygenation is impaired (Stoner, 1958*a*). This has been interpreted as an effect of injury on the control of heat production (see Stoner & Threlfall, 1960; Stoner, 1960). In the second phase, when the animal is dying, the physiological and biochemical changes are dominated by the gradually increasing hypoxia of the tissues. Our interest has been focused on the first stage and attempts to elucidate the mechanism of its changes have so far yielded the following information.

The injured rat's condition cannot be explained by depletion of the stores of energy-rich phosphates (Goranson, Hamilton & Haist, 1948; Stoner & Threlfall, 1954; Threlfall & Stoner, 1957). After injury the catabolism of glucose is inhibited in the early stages of its breakdown and in the oxidation of the pyruvate formed from it (Stoner, Heath & Collins, 1960). At the same time it would appear

that ischaemic limb injury does not damage liver mitochondria, in so far as this can be judged from their behaviour *in vitro* after isolation (Aldridge & Stoner, 1960).

We have now investigated the concentrations of glycolytic products and intermediates in the tissues during the first part of the survival period, determining them chromatographically (except for lactate) since the methods used previously (LePage, 1946; Threlfall & Stoner, 1954) were inadequate. The effect of hind-limb ischaemia has been studied under different environmental and endocrinological conditions to determine the effect of varying the intensity of the response to injury. Some of the results have been mentioned in reviews (Stoner & Threlfall, 1960; Stoner, 1960).

METHODS

Animal techniques. Male albino rats (242 ± 36 g. body wt.) of the Porton strain were used. M.R.C. diet 41B (Bruce & Parkes, 1956) was available until the start of the experiment except when 'fasting' rats were used, when it was removed from the cages on the day before the experiment.