Sucrose Utilization by *Zymomonas mobilis*: Formation of a Levan

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1. Molar growth-yield coefficients of *Zymomonas mobilis* for glucose, fructose, glucose plus fructose, and sucrose are reported. Yield coefficients for sucrose are appreciably lower than those for the equivalent concentrations of glucose plus fructose.
2. Only 2.6% of [U-¹⁴C]glucose supplied in the growth medium is incorporated into cell substance by *Z. mobilis* utilizing glucose as the energy source.
3. During growth on sucrose a levan is formed. It has been characterized and shown to resemble other bacterial levans.
4. Levan formation from sucrose could be demonstrated with both washed cell suspensions and cell extracts of *Z. mobilis*.
5. Sucrose phosphorylase could not be demonstrated in extracts of the organism.

Tracer studies indicated that *Zymomonas mobilis* ferments glucose via an anaerobic Entner–Doudoroff pathway (Gibbs & DeMoss, 1954) and we have reported that the key enzymes are present in extracts of these cells (Ribbons & Dawes, 1961; Dawes, Ribbons & Large, 1966). As Bauchop & Elsdon (1960) pointed out, this is an inefficient pathway when compared with the glycolytic route of ethanol formation in yeast. They demonstrated that the growth yield of *Z. mobilis* per mole of glucose was 8.3, a value which is approximately one-half of that obtained for *Saccharomyces cerevisiae*, but which is accounted for by the provision of only 1 mole of ATP/mole of glucose fermented by an Entner–Doudoroff pathway. Our studies included determination of molar growth yields for *Z. mobilis* when glucose, fructose and sucrose were provided as sources of energy in peptone–yeast extract media, so that we might see how efficiently sucrose is utilized and perhaps distinguish whether hydrolysis or phosphorylation occurred. Molar growth yields on sucrose were, however, erratic and low and this we have shown to be due to the formation of a levan. The isolation, analysis and synthesis of the levan are described together with the molar-growth-yield experiments that led to its discovery. A preliminary account of this work has been presented to The Biochemical Society (Ribbons, Dawes & Rees, 1962).

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EXPERIMENTAL

Organism, cultural conditions and preparation of cell extracts. *Z. mobilis* (N.C.I.B. 8938) was maintained, cultured and extracts were prepared from it as described in the preceding paper (Dawes *et al.* 1966) except that, where required, sucrose was added (2%, w/v) to the growth medium as an alternative energy source.

Chemicals and buffer solutions. These were as described in the preceding paper (Dawes *et al.* 1966). [U-¹⁴C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks.

Analytical methods

Chemical estimations. Fructose was determined with the resorcinol reagent (Umbreit, Burris & Stauffer, 1957). Total carbohydrate was estimated by the anthrone method of Trevelyan & Harrison (1952). For other estimations see Dawes *et al.* (1966). Elemental analyses were performed by Mr J. M. L. Cameron, Chemistry Department, University of Glasgow.

Levan determination. (a) Levan was estimated turbidimetrically in 1 cm. cells with a Spekker absorptiometer by using filters H503 and 603; linearity of levan concentration against drum reading was observed up to 2.5 mg. (15.43 μ -moles of fructose equivalent) of levan/ml., when the value was 0.615. Thereafter increments of drum readings decreased as the levan concentration was further raised, e.g. 4.25 mg. of levan/ml. gives a reading of 0.91. (b) Levan was estimated by measurement of the amount of total fructose that is precipitated by 75% ethanol, after washing the precipitate in 75% ethanol. Results have been expressed as μ -moles of fructose equivalent, assuming a mean residue weight of 162.

Cell fractionation. The procedures of Roberts, Abelson, Cowie, Bolton & Britten (1957) were used.

Isotopic procedures. The radioactivity of the fermentation CO_2 trapped in alkali was measured by precipitation of replicate portions as BaCO_3 by the methods described by Sakami (1955). The radioactivity of cells was determined by spreading portions (0.1 and 0.2 ml.) of suitably diluted suspensions on stainless-steel planchets having an effective raised area of 1.5 cm.² (Dawes & Holms, 1958). Cell fractions were plated in a similar manner. Ethanol was steam-distilled from supernatants at neutral pH and collected in dichromate (Neish, 1952). The acetate formed was distilled, titrated with 0.05 N-NaOH and portions (0.1 and 0.2 ml.) of the resulting sodium acetate solution were spread on planchets.

Fig. 1. Relationship between concentration of energy source and cell yield of *Z. mobilis*. Growth was measured turbidimetrically and the experimental protocol was as described by Bauchop & Elsdén (1960). (a) Glucose; (b) fructose; (c) equimolar mixture of glucose and fructose; (d) sucrose. The points are the average values of six individual determinations for each concentration.

All estimations were made at or corrected to infinite thinness by using an end-window Geiger-Müller tube and an Ekco automatic scaler type N530 G. Counts were collected for a period sufficient to ensure that the counting error was less than $\pm 2\%$. Corrections were applied for background and coincidence.

Paper chromatography. Descending paper chromatograms were developed in the solvent systems: (a) water-satd. phenol + 1% of aq. NH_3 (sp.gr. 0.88); (b) butan-1-ol-acetic acid-water (4:1:5, by vol.); (c) propan-1-ol-ethyl acetate-water (7:1:2, by vol.) (Feingold, Avigad & Hestrin, 1956); (d) butan-1-ol-ethanol-water (5:1:4, by vol.; upper phase). Carbohydrates were detected with ammoniacal AgNO_3 (Partridge, 1948; Trevelyan, Procter & Harrison, 1950), aniline oxalate (Horrocks & Manning, 1949), urea-phosphate (Wise, Dimler, Davis & Rist, 1955) and triphenyl-tetrazolium chloride (Wallenfels, 1950).

Gas-phase chromatography. This was carried out with a Pye Argon Gas Chromatograph with a ^{90}Sr detector.

Molar growth yields. The yields of *Z. mobilis* on glucose, fructose and sucrose were generally determined turbidimetrically in a manner identical with that employed by Bauchop & Elsdén (1960). Occasionally direct weight measurements were made on centrifuged and washed cultures as described by the same authors.

Spectrophotometry. Ultraviolet and visible spectra were established as before (Dawes *et al.* 1966). Infrared spectra were determined with a Perkin-Elmer Infracord with liquid paraffin (Nujol) mulls.

Ultracentrifuge. A Spinco model E ultracentrifuge was used. The polysaccharide concentration was 2.7 mg./ml. (water, solvent) and the rotor speed 11573 rev./min. A schlieren angle of 55° and exposures of 15 sec. at 4 min. intervals were employed. The temperature was 17.4° .

RESULTS

Molar growth-yield coefficients

The relationship between total bacterial growth and the concentration of energy source provided is shown in Fig. 1. Glucose and fructose and an equimolar mixture of glucose and fructose allow growth yields (g. dry wt. of bacterial cells/mole of hexose equivalent) of 9.32, 9.21 and 9.16, whereas

Table 1. *Growth-yield coefficients of Zymomonas mobilis with glucose and sucrose as sources of energy*

Cultures were grown in 1% Bacto-Peptide-1% yeast-extract media supplemented with the energy source in 250 ml. conical flasks. The culture volume was 155 ml., and was inoculated with a loop of an exponential-phase culture developing on media of similar energy source which was limiting total growth. Cell densities were determined turbidimetrically and by direct weighing.

Energy source	Concn. (mm)	Total cell yield (mg.)		Yield coefficient	
		Turbidimetric	Direct weighing	Turbidimetric	Direct weighing
Glucose	29.0	37.2	39.2	8.30	8.71
Sucrose	14.5	28.1	31.0	6.22	6.90
Sucrose	14.5	28.5	34.5	6.32	7.45

sucrose gives a yield of only 7.36, which varies from experiment to experiment between 5.35 and 8.0. A direct determination of the weight of bacterial cells is illustrated in Table 1, where a consistently lower yield of cells is obtained on sucrose than on glucose. The yield of ethanol from these substrates was 1.68 (glucose) and 1.35 (sucrose) moles/mole of hexose equivalent.

We had previously demonstrated that the energy source (with glucose) is not incorporated appreciably into cellular materials (Table 2); hence it was considered likely that other metabolic products were formed from sucrose as no sucrose could be detected after fermentation. The values which trace the fate of the carbon from [$U-^{14}C$]glucose after fermentation agree favourably with those obtained recently by Belaïch & Senez (1965) and demonstrate that, even under conditions of excess of energy source, there is little incorporation into cellular material. Assuming a carbon content of cells of 50%, then the ratio of the specific activity of cellular carbon to the substrate glucose carbon is 0.83:1.0, i.e.

considerable dilution of the glucose into cellular material has not occurred. The distribution of the ^{14}C in the cells was also fairly uniform, indicating that it is not accounted for by a specific storage material or by structural carbohydrate as in *Zymosarcina ventriculi* (E. A. Dawes & D. W. Ribbons, unpublished work) (Table 3). The carbohydrate content of the organism is, in fact, very low, representing only some 3% of the dry weight.

Isolation and analysis of the levan

Isolation. Centrifugation of sucrose cultures of *Z. mobilis* for 1–2 min. at 15000g yielded a cell pellet and an opalescent supernatant fluid which could be clarified by centrifugation at 25000–30000g for 30 min. The pellets obtained were translucent gels, and were easily resuspended and precipitated by further centrifugation. Isolation of sufficient material for its chemical characterization was achieved as follows.

Four cultures (81. each) of *Z. mobilis* on 2%

Table 2. *Fate of [$U-^{14}C$]glucose during growth of Zymomonas mobilis in glucose-limiting cultures*

A culture medium (53 ml., 2% glucose, 1% Bacto-Peptone and 1% yeast extract) containing [$U-^{14}C$]glucose (3×10^6 counts/min.) was inoculated with a stationary-phase culture of *Z. mobilis* (5 ml.). A sample (5 ml.) was immediately withdrawn for chemical and radiochemical analysis and the rest of the culture harvested after 16.5 hr., washed twice with the same volume of water and then with 25 ml. of water. The cells were suspended in water and made up to 50 ml., giving a cell density 581 $\mu g.$ dry wt./ml. The culture supernatant (1 ml.) was steam-distilled and excess of potassium dichromate oxidizing mixture (Neish, 1952) added to the distillate (4 ml.). After standing for 1 hr. at 22° the acetic acid formed was collected by steam-distillation in a volume of 25 ml. and portions were titrated with CO_2 -free 0.05 N-NaOH and plated and counted as sodium acetate. The radiochemical yield of CO_2 was calculated from the yield of ethanol in the fermentation, i.e. 1.6 moles/mole of hexose.

Fermentation yields			Radiochemical distribution (counts/min.)			
Glucose fermented (m-moles)	Ethanol formed (m-moles)	Cell yield (mg.)	Glucose utilized	Ethanol	CO_2	Cells
4.67	7.45	20.91	2.6×10^6 (100%)	1.32×10^6 (50.8%)	7.6×10^5 (26.7%)	6.7×10^4 (2.6%)

Table 3. *Distribution of ^{14}C in cellular fractions after growth on [$U-^{14}C$]glucose*

Cells from the experiment of Table 2 were used. Duplicate portions (5 ml., 5.8 mg. dry wt.) of a cell suspension in water were centrifuged and subjected to the fractionation procedure of Roberts *et al.* (1957).

Fraction	Radioactivity (counts/min.)		Percentage of total radioactivity	
	27000		100	
Whole cells				
Cold trichloroacetic acid (5%)	1920	1699	7.1	6.25
Ethanol (75%, v/v)	4280	5210	15.8	19.2
Hot trichloroacetic acid (5%)	5600	6240	20.8	23.0
Residue	9788	10080	36.0	37.2
Recovery			79.7	85.65

sucrose–Bacto–Peptone–yeast–extract medium were grown into the stationary phase. Centrifugation at 50000g with a fast flow rate (500ml./min.) on the Sharples Super-Centrifuge yielded a cloudy supernatant which was almost devoid of bacterial cells. The supernatants were cooled to 0–2° and centrifuged in batches at 27000g for 30 min. on an MSE angle 13 centrifuge. Supernatants were discarded and the sedimented gel was taken up into water (1l.) and resedimented at 27000g for 90 min.; the process was repeated. Suspension of the pellets in 600ml. of water yielded a grey–brown opalescent solution which was treated with 1800ml. of industrial spirit. A brown gum immediately separated to the bottom of the beaker, the cloudy white supernatant was clarified at 20000g for 5 min. and the pellets were combined with the gum. Solution of the solid in 300ml. of water and addition of ethanol to 75% (v/v) reprecipitated the levan, which was taken up in 250–300ml. of water, filtered and dialysed for 24 hr. against distilled water at room temperature. Filtration of the dialysed preparation and freeze-drying produced a fluffy white material which was dried over phosphorus pentoxide. The yield was 4.5g.

Characterization of the polysaccharide as a fructosan. Fructose was the only sugar that could be detected in this hydrolysate by paper chromatography with solvents (a) and (b). Polysaccharide (300mg.) was heated in 0.02N-sulphuric acid (20ml.) at 100° for 45 min. The solution was neutralized with barium carbonate and concentrated to dryness under diminished pressure. The resulting syrup was thoroughly dried in a vacuum desiccator over phosphorus pentoxide and treated with acetone in the presence of conc. sulphuric acid as described by Bell (1947) to give 2,3:4,5-di-isopropylidene-D-fructose: 250mg.; m.p. and mixed m.p. with authentic material, 93.5–95°, $[\alpha]_D - 34.5^\circ$ (c 0.8 in water). Analysis of a sample of levan which had been pre-dried at 60° *in vacuo* for 24 hr. by the method of Roe (Umbreit *et al.* 1957) showed 94.5% of anhydrofructose residues. Nitrogen analysis gave < 0.01%.

Periodate oxidation. Fructosan (15.3mg.) was dissolved in sodium metaperiodate (0.07M; 3ml.) and the solution left at room temperature, the periodate uptake being determined from time to time by Aspinall & Ferrier's (1957) method. There was an initial rapid consumption of 0.96 mole of oxidant (within 5 hr.), rising finally to a value of 1.00 mole/mole of anhydrofructose (24 hr.). These results are consistent with a formula for the fructosan consisting entirely of fructofuranose residues linked 2,1 or 2,6 or both.

Partial acidic hydrolysis. Fructosan (20mg.) was partially hydrolysed in 0.01N-sulphuric acid (2ml.) at 60° for 1 hr. After neutralization on IR-4B resin

and concentration the hydrolysate was examined by paper chromatography with solvent (c). For comparison, samples of dahlia inulin and levan of perennial rye-grass tops were treated in exactly the same way. *Zymomonas fructosan* gave a series of oligosaccharides (detected up to the pentasaccharide) which resembled in R_F value and in behaviour towards triphenyltetrazolium chloride spray (cf. Feingold *et al.* 1956) the series from perennial rye-grass levan. In contrast the oligosaccharides from dahlia inulin had different R_F values and could not be detected with triphenyltetrazolium chloride, though they were readily detected with alkaline silver nitrate (Trevelyan *et al.* 1950).

Methylation of levan. Levan (1.0g.) was stirred with water (25ml.) until it dissolved and the flask was then cooled in an ice bath. Sodium hydroxide (30%, w/v; 45ml.) and dimethyl sulphate (15ml.) were added simultaneously in portions with vigorous stirring over 5 hr. The reaction mixture was allowed to reach room temperature and stirred overnight. The same reagents were added similarly the following day (at room temperature) and the procedure was repeated a further three times. Acetone was added from time to time to maintain complete solution. Finally the mixture was stirred at 60° for 1 hr., cooled, neutralized with 10N-sulphuric acid, and the methylated polysaccharide extracted with chloroform. The combined extracts were washed once with water, evaporated under diminished pressure to small volume and poured into excess of light petroleum (b.p. 60–80°). The precipitate was removed on the centrifuge, washed thoroughly with light petroleum, and dried *in vacuo* at 60° to give a buff amorphous solid (0.9g.), which showed no hydroxyl group absorption in the infrared spectrum [Found: OCH₃, 45.6; fully methylated fructosan requires OCH₃, 45.5%. $[\alpha]_D - 59.4 \pm 1^\circ$ (c 0.6 in chloroform)].

Paper chromatography of hydrolysed methylated levan. The material was hydrolysed by the method of Arni & Percival (1951) and the resulting syrup compared with authentic specimens of methylated fructoses by chromatography in solvent (d) with urea–phosphate spray. The hydrolysate contained three components. The first (R_F relative to tetramethylfructose, 0.65) moved at the same rate as authentic 3,4-di-O-methylfructose. The second component (relative R_F 0.84) moved at the same rate as authentic 1,3,4-tri-O-methylfructose. Authentic 3,4,6-tri-O-methylfructose on the same chromatogram could be distinguished in that (i) it travelled a little faster, and (ii) it gave a very strong reaction with triphenyltetrazolium spray, whereas the tri-O-methylfructose in the hydrolysate, like the authentic 1,3,4-tri-O-methylfructose, gave no reaction. The third component moved at

the same rate as authentic 1,3,4,6-tetra-*O*-methylfructose and gave a similar inky blue-green colour with urea-phosphate spray. Similar amounts of di- and tetra-*O*-methylfructoses were present, as judged from spot intensities, with larger amounts (six to ten times as much) of the tri-*O*-methylfructose.

Gas-phase chromatography of methanolysed methylated levan. The material (10mg.) was treated in a sealed Pyrex tube with methanolic hydrogen chloride (2.5%; 1.5ml.) at 60° for 3hr. After neutralization with silver carbonate the filtrate was concentrated to a syrup and compared by gas-phase chromatography with authentic di-, tri- and tetra-*O*-methylfructoses which had been treated in the same way. The systems used were (i) 15% butanediol succinate polyester on Celite at 175° and (ii) 10% polyphenol on Celite at 200° (Aspinall, 1963). The two tri-*O*-methylfructoses could be readily distinguished (Aspinall, 1963) and peaks corresponding to those given by the authentic 1,3,4-isomer were found to be present. No 3,4,6-tri-*O*-methylfructose was detected. Other peaks on

the chart indicated the presence of 3,4-di- and 1,3,4,6-tetra-*O*-methylfructose derivatives.

Ultracentrifugal analysis. Concentrated solutions of the levan showed one major and one slower minor peak. Results obtained gave $S_{20,w}$ as 390×10^{-13} sec.

Formation of levan

Formation during growth. Yields of levan from 2% sucrose cultures varied between 100 and 200mg./l. Neither glucose nor fructose supported levan synthesis. Raffinose was not tested as a growth substrate.

Formation by washed cell suspension. Fig. 2 demonstrates the course of sucrose degradation and levan formation by non-growing cells. This experiment clearly shows that glucose is utilized faster than the fructose portion, and that levan synthesis stops when, at the most, the sucrose concentration is 500 μ g. (1.46 μ moles)/ml. (i.e. the difference between total carbohydrate and fructose present). After about 4hr. incubation only free fructose is left and this continues to be metabolized by the cells without levan formation.

Enzymic synthesis. Levan is readily synthesized by crude extracts of *Z. mobilis*, without added primer (Fig. 3). Approximately 10% of the sucrose

Fig. 2. Synthesis of levan by washed cell suspensions. A washed cell suspension (15ml., 11.7mg./ml.) was obtained from a 350ml. sucrose culture and added to phosphate-citrate buffer, pH 5.6 (15ml.), and 15% (w/v) sucrose solution (15ml.), all previously incubated at 30°, and samples (2ml.) were taken as indicated. Samples were immediately centrifuged for 1–2min. on a bench centrifuge (2000g) to remove cells and subjected to analysis of levan (turbidimetrically) (●), total carbohydrate (including levan) (▲) and total fructose (■). Note that the levan scale differs by a factor of 10.

Fig. 3. Enzymic formation of levan. The reaction mixture contained: citrate-phosphate buffer, pH 5.0 (3ml.); 15% (w/v) sucrose (3ml.); enzyme solution (crude extract, 3ml., containing 74mg. of protein). Temperature, 32°. Samples (1ml.) were taken for analysis as indicated. ●, Levan formation (turbidimetric); ○, total non-levan carbohydrate (anthrone), i.e. carbohydrate soluble in 75% ethanol; Δ, reducing sugar formed. Controls containing 75% of ethanol were included in the anthrone assays (Binnie, Dawes & Holms, 1960). Reducing sugar was determined before ethanol treatment on the supernatant after deproteinization by the method of Somogyi (1930).

Table 4. *Levan formation by extracts of Zymomonas mobilis*

Each test tube contained: citrate-phosphate buffer of the indicated pH (3 ml.); sucrose or raffinose, 15%, as indicated (3 ml.); cell-free extract (3 ml.; 21 mg. of protein/ml.). Controls without added sugars were run at the three pH values so that increases in turbidity due to protein denaturation could be followed, and subtracted from the reaction tubes. Turbidity due to protein denaturation amounted to approx. 25% of the values obtained in reaction tubes. Figures in the columns represent (a) levan formed (measured turbidimetrically), (b) reducing sugar formed and (c) total fructose soluble in 75% ethanol.

Time (min.)	Sucrose, pH 4.6				Sucrose, pH 6.0				Raffinose, pH 5.0			
	Levan formed (μ moles/ ml.)	Reducing sugar (μ moles/ ml.)	Total fructose (μ moles/ ml.)	Levan formed (μ moles/ ml.)	Reducing sugar (μ moles/ ml.)	Total fructose (μ moles/ ml.)	Levan formed (μ moles/ ml.)	Reducing sugar (μ moles/ ml.)	Total fructose (μ moles/ ml.)	Levan formed (μ moles/ ml.)	Reducing sugar (μ moles/ ml.)	Total fructose (μ moles/ ml.)
2		16.2	179.0		14.1	160.0		4.5	164.5		7.1	73.7
25	16.2			11.5			1.2			4.3		
30		83.7	154.0		91.2	148.0		62.5	163.0		31.0	67.5
50	23.1			19.3			6.2			10.8		
60		131.0	136.0		142.0	157.0		115.5	168.0		55.3	62.5
80	42.9			34.3			12.0			29.0		
90		175.0	136.0		181.2			155.0	156.0		71.2	65.0
110	66.4			43.5			13.9			30.9		
120		180.0	126.2		185.0	145.0		169.0	152.5		82.5	60.0
190	117.3			83.3			26.2			46.3		
300	123.5	218.5	122.0	115.4	225.0	141.0	48.8	204.0		40.7	106.5	61.7
360	118.5	205.0	118.0	118.5	226.0	136.0	52.5	231.0	156.0	40.1	125.0	

utilized by extracts is converted into levan, the remainder of the sucrose being hydrolysed to glucose and fructose. As might be expected, levan formation does not occur at a linear rate, as judged by the methods we have employed to assay its formation, namely increased turbidity during incubation, analysis of total carbohydrate that is insoluble in 75% ethanol (not shown in this experiment), and also by difference from the analysis of total carbohydrate that is soluble in 75% ethanol (Fig. 3). This latter value also shows a lag before it starts to decline. The molecular size which must be attained before these turbidimetric or precipitation methods of analysis are accurate is not known. The reaction mixtures were not investigated further for other products such as fructosylsucrose. However, there seems little reason to doubt that this levan sucrose differs from those previously studied in *Acetobacter levanicum* (Hestrin, Feingold & Avigad, 1956) and *Bacillus subtilis* (Dedonder, 1960). The pH optimum is about 5 and the speci-

ficity of reaction extends to other fructofuranosyl- α -D-aldosides, e.g. raffinose (Table 4).

In crude unsupplemented extracts the main products of sucrose metabolism are glucose and fructose. Addition of inorganic phosphate (or its use in buffer systems) neither stimulates nor inhibits the rate of sucrose metabolism, nor do the products alter appreciably. It seems therefore that phosphorylation plays little part in sucrose degradation by *Z. mobilis*. Only when ATP is added to extracts does further metabolism of the hexoses occur. Table 5 records the formation of pyruvate from glucose, fructose and sucrose by extracts supplemented with ATP and NAD. The rate of sucrose degradation is relatively low, irrespective of the energy source used in the growth media, a fact noted earlier when washed suspensions of *Z. mobilis* were allowed to ferment glucose, fructose or sucrose (Table 6). However, this is a property which may easily vary with pH value. The rates of oxygen consumption by whole cells metabolizing

Table 5. *Formation of pyruvate from sucrose, glucose and fructose*

Reaction mixtures contained: 0.2M-glycylglycine, pH 7.3 (2.0 ml.); 20mM-carbohydrate as indicated (1.0 ml.); 20mM-NAD (0.1 ml.); 40mM-MgCl₂ (0.3 ml.); 50mM-ATP (1.0 ml.); 0.56M-hydrazine (1.2 ml.); cell-free extract (0.5 ml., 12.5 mg. of protein), the last-named addition initiating the reactions. Temperature, 30°. Samples (1 ml.) were taken at intervals for analysis of pyruvate and carbohydrate (anthrone method).

Time (min.)	Carbohydrate substrate					
	Glucose		Fructose		Sucrose	
	Pyruvate (μ moles/ml.)	Carbohydrate (μ moles/ml.)	Pyruvate (μ moles/ml.)	Carbohydrate (μ moles/ml.)	Pyruvate (μ moles/ml.)	Carbohydrate (μ moles/ml.)
1.0	0.02	3.73	0.10	3.32	0.08	9.40
2.5	0.08	3.21	0.20	3.28		9.00
5	0.39	2.52	0.33	2.60		9.00
10	0.52	1.74	0.52	1.64	0.19	8.95
20	0.76	0.53	0.79	0.39	0.25	
30	1.26	0.58	1.16	0.40	0.36	8.60

Table 6. *Relative rates of carbohydrate utilization by washed suspensions of Zymomonas mobilis grown with different carbohydrates*

Carbon dioxide formation was measured manometrically under an N₂+CO₂ (95:5) atmosphere. Each flask contained: 0.1M-citrate buffer, pH 6.6 (1.0 ml.); substrate (1.0 ml., 10 μ moles); cell suspension (1.0 ml.). Temperature, 30°. Dry wt. (mg.) of cells per flask: (a) 3.3; (b) 5.5; (c) 7.2; (d) 4.2; (e) 4.2; (f) 8.6. Rates are expressed as μ l. of CO₂ evolved/hr./mg. bacterial dry wt. (Q_{CO_2}).

Carbohydrate in growth medium ... Fermentation substrate	Fermentation rates (Q_{CO_2})					
	Glucose		Fructose		Sucrose	
	(a)	(b)	(c)	(d)	(e)	(f)
Glucose	319	317	346	358	293	206
Fructose	54.5	60.0	154	178	137	77
Sucrose	3.6	3.6	14.7	6.6	12.3	24.4

sucrose were also much slower than for glucose or fructose.

DISCUSSION

The observed low values for the molar growth yield of *Z. mobilis* utilizing sucrose, which led to the discovery of the levan, can be explained by the diversion of some of the energy source to fructan formation. Variations in the quantity of levan formed would account for the differences in yield coefficient recorded. Furthermore, the determination of yield coefficient was subject to variation due to (a) the light-scattering properties of the levan and (b) the ease with which it is centrifuged when bacterial dry weights are measured.

Utilization of sucrose could conceivably occur by hydrolysis or by phosphorysis. A sucrose phosphorylase which catalyses reaction (1) is present in

Pseudomonas saccharophila and *Leuconostoc mesenteroides* (Doudoroff, 1955), but no evidence for the activity of this enzyme in *Z. mobilis* could be obtained. An organism which possesses sucrose phosphorylase is energetically more efficient in its utilization of sucrose than one which does not, for phosphate is introduced into the glucose molecule without the expenditure of ATP. Consequently, where the Entner-Doudoroff pathway operates, the ATP yield by phosphorysis would be 3, as opposed to 2 moles/mole of sucrose by hydrolysis. The molar growth yield on sucrose should thus be greater than that on the equivalent concentration of glucose plus fructose by a factor of 1.5 if phosphorysis occurs. The observed yields were, in fact, substantially lower and, together with the enzymic evidence, it is concluded that *Z. mobilis* utilizes sucrose by a hydrolytic mechanism.

Our results show clearly that levan formation is accompanied by hydrolysis to glucose and fructose, but we have not established whether an invertase is present. Levan sucrose catalyses the reaction (2),

where the acceptor is fructose and the donor either sucrose or raffinose; if water is the acceptor, then the products will be fructose and glucose. With crude extracts the products of sucrose metabolism are glucose, fructose and levan; the hexoses are not further metabolized unless ATP is added. The levan sucrose alone could thus account for our findings.

Bacterial levans are a family of high-molecular-

weight fructans in which D-fructofuranosyl units are joined mainly by 2,6-linkages but with about every ninth unit carrying a 2,1-branch-point (Hestrin, 1959), the glycosidic configuration probably being β throughout. *Z. mobilis* levan would appear to be a typical member of this family in every respect. Periodate oxidation revealed that if the sugar units were in the furanose ring form, then the glycosidic linkages were attached exclusively to primary hydroxyl groups. Partial hydrolysis showed that the major linkage was 2,6, that large numbers of such linkages were consecutive and that many (probably all) of the units were indeed in the furanose ring form. Methylation analysis gave results that agreed with these conclusions and showed further that 2,1-linkages were present in about the usual proportions for bacterial levans, and that all these linkages were present at branch-points.

The specific optical rotation of the methylation polysaccharide confirmed the general similarity to other bacterial levans. The results of the partial hydrolysis study, together with the value for the optical rotation of the methylated derivative, suggest that most, if not all, of the glycosidic linkages have the β -configuration. The sedimentation coefficient (390s) was similar to the values quoted (Feingold & Gehatia, 1957) for levans with molecular weights of the order of 10^7 .

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