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FINGERPRINTING OF CARBOHYDRATES OF *STREPTOCOCCUS MUTANS* BY COMBINED GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A modified method is described for the analysis, by gas–liquid chromatography, of various sugars as the trimethylsilyl derivatives of their methyl glycosides. The technique was employed for the analysis of the cellular carbohydrate of *Streptococcus mutans* NCTC 10832 and provided reproducible fingerprints, consisting of peaks due to glycerol, rhamnose, xylose, galactofuranose, glucose, N-acetylglucosamine and N-acetylmuramic acid. Absolute identification of the latter was by combined gas–liquid chromatography–mass spectrometry.

INTRODUCTION

Cell wall polysaccharides of streptococci have played an important role in classification since the work of Lancefield[1] in 1933. The polysaccharides have traditionally been extracted from whole cells by mild acid hydrolysis or hot formamide treatment[2]. Analysis of polysaccharide used to be achieved serologically but more recently analysis of individual sugars has been attempted[3,4]. Gas–liquid chromatography (GLC) is now an established technique in bacterial chemotaxonomy[5]. GLC techniques for sugar analysis are now well established[6–8] and have been applied both to glycoproteins and to bacterial polysaccharides[9–11] with success.

The object of the present study was to adapt the methods of Clamp *et al.*[8] to the analysis of cellular carbohydrate of *Streptococcus mutans*, and to determine the identity of trimethylsilyl (TMS) derivatives of individual monosaccharides by on-line mass spectrometry (MS).

EXPERIMENTAL

Chromatographic conditions

A PYE 104 gas chromatograph equipped with dual flame ionisation detectors was employed. Samples (1 μl) were injected into a coiled glass column 1.83 m × 4 mm packed with 3% (w/w) SE-30 on 100–120 mesh Chromosorb W HP (Phase Separations, Queensferry, Great Britain). Chromatographic parameters were: column

temperature from 80° to 250° at 2°/min; detector oven temperature, 220°; nitrogen carrier gas flow-rate, 25 ml/min; hydrogen and air flow-rates, 40 and 600 ml/min, respectively. The temperature programme chosen was the best of several programmes tested for the monosaccharides of interest.

Chromatograms were recorded on a Techman chart recorder using a chart speed of 30 cm/h, after peak integration by Spectra-Physics computing integrator.

Reagents

Only analytical-grade reagents were used. Glycerol, arabinose, ribose, xylose, fructose, galactose, glucose, mannose, rhamnose, specially dried methanol, hydrogen chloride, dry pyridine, and acetic anhydride were supplied by BDH (Poole, Great Britain). Arabitol, ribitol, sorbose, fucose, perseitol, N-acetylgalactosamine, N-acetylglucosamine and N-acetylmuramic acid were purchased from Sigma (St. Louis, Mo., U.S.A.). Hexamethyldisilazane (HMDS), and trimethylchlorosilane (TMCS), were obtained from Pierce & Warriner (Chester, Great Britain).

The organism used had been isolated by one of the authors[12] and was grown at 37° for 48 h with shaking brain-heart infusion supplied by Difco Labs. (West Molesey, Great Britain).

Derivatization of standards

Aliquots (100 μl) of an equilibrium solution of monosaccharides (5 μmole/ml of each) were dried in 2-ml ampoules over phosphorus pentoxide *in vacuo*. Methanolysis was carried out with methanolic hydrogen chloride at 87° for 24 h in nitrogen-filled ampoules. As alternatives to silver carbonate, barium carbonate, calcium carbonate and ammonia were each tried as neutralizing agents. Only ammonia gave similar molar response factors to silver carbonate. Neutralised samples were acetylated with acetic anhydride (50 μl) at 22° for 12 h, then dried *in vacuo*. Dried methanolysates were silylated with 100 μl of pyridine–TMCS–HMDS (5:1:1, v/v/v) at 22°. Analysis of silylated samples stored in a desiccator indicated that maximum silylation of all monosaccharides requires rather longer times than are sometimes employed. A minimum of 30 min was indicated.

Application to bacterial sample

Pure cultures in broth were harvested, washed twice in 0.067 *M* Sorensen's phosphate buffer and once with distilled water, then freeze-dried. Dried cells (5 mg) were weighed into 2-ml ampoules. Monosaccharides were extracted and methylated in a single step, using 1.0 m methanolic hydrogen chloride *ut supra*; in addition, hydrochloric acid, of molarities ranging from 0.5–5.0 *M*, was tested, for varying lengths of time up to 4.0 h. Hydrolyses were performed in sealed ampoules at 100°. Each ampoule contained perseitol (250 nmole) as internal standard. Acetylation and silylation were carried out as for standards (see above).

Gas–liquid chromatography–mass spectrometry

Mass spectra were obtained with an AEI MS30 mass spectrometer–Pye 104 gas chromatograph combination. Samples were analysed on-line, using a scan time of 10 sec. Other parameters were: ionization potential, 70 eV; ionisation current, 100 μA; emission current, 2 mA; ion-source block temperature, 200°; molecular separator temperature, 200°; peak matching against perfluorokeresene (PFK).

RESULTS

Retention data

Retention characteristics of TMS ethers of monosaccharides are tabulated in Table I. The resolution of some of these monosaccharides obtained by methanolysis is shown in Fig. 1. Most of the monosaccharides, except for alditols, yielded multiple peaks, corresponding to proportions of anomers in equilibrium mixture. Rhamnose and ribose had similar retentions but could be separately identified by combined GLC–MS.

TMS ethers of acid-hydrolysed monosaccharides are less well separated (Fig. 2) than those of methanolysed samples but did enable ketoses to be detected and avoided an anomalous peak retention for deoxyribose. Methanolysis caused extensive deacetylation of N-acetylaminosugars so that reacetylation was essential. It was found that a minimum of 12 h is required for quantitative reacetylation. The proportions of pyridine solvent and the two silylating reagents were occasionally varied; since a minority of bacterial samples yielded larger peaks with a smaller volume of more concentrated reagent consisting of pyridine–HMDS–TMCS (2:1:1, v/v/v). In this case extraneous peaks appeared on chromatograms but could be removed by a benzene extraction prior to silylation.

MS characteristics of standard peaks

TMS ethers had characteristic mass spectra (Table II). Fragmentation of monosaccharide derivatives followed a similar pattern in all cases with cleavage of C–C bonds and stepwise loss of trimethylsilyoxy (TMSO), trimethylsilanol (TMSOH), methoxy (CH_3O), and methanol in addition to ion-rearrangement. Differing molecular structures of monosaccharides did ,however, effect modification of fragmentation pattern resulting in characteristic spectra for individual monosaccharides. Several spectra were of particular interest. Galactose peak 1 had a base peak of *m/e* 217 with a large peak at *m/e* 204 (90.6%); in contrast galactose peak 2 had a base peak of *m/e* 204 and a much smaller peak at *m/e* 217, together with a much reduced peak at *m/e* 319 (2.8%). These differences are due to the galactofuranose (peak 1) and galactopyranose (peak 2) forms. The peaks of glucose and the major peak of mannose resembled galactopyranose in their mass spectra. The minor peak due to mannose appeared to fragment as a furanose sugar. The mass spectra of ribose and rhammose were readily distinguishable, and permitted detection of the pentose and deoxyhexose despite their poor chromatographic separation. The pentoses were characterised by a peak at *m/e* 275. The acyclic nature of the alditols yielded different fragmentation patterns with a base peak of *m/e* 205, and characteristic minor peaks at *m/e* 277 and 307. In N-acetylated hexoses, the substitution of the acetamido-group at C-2 shifted the 2-C fragment from *m/e* 204 to *m/e* 173, forming the base peak of the spectrum. Unlike neutral sugars, the N-acetylamino-sugars had an $M^+ - 15$ peak due to loss of a methyl group. Further loss of methanol and trimethylsilanol yielded fragments of *m/e* 404 and 314, respectively. N-Acetylmuramic acid, which as a C-3 O-lactyl substituent, differed from the other acetamido sugars in having an intense peak at *m/e* 89. This corresponded to a lactate fragment. Another characteristic peak was of *m/e* 187. The identities of some MS fragments are depicted in Fig. 3.

TABLE I

RETENTION DATE FOR TMS ETHERS OF SOME MONOSACCHARIDES

Monosaccharides		*Methyl glycoside*		*Free monosaccharides*	
		Peak area proportion	*Relative retention*	*Peak area proportion*	*Relative retention*
Glycerol		—	0.203	—	0.209
Deoxyribose	1	—	0.766	47.1	0.342
	2	—	—	52.9	0.398
Arabinose	1	63.9	0.446	43.2	0.517
	2	27.4	0.455	56.8	0.590
	3	9.3	0.472	—	—
Ribose	1	—	0.473	68.3	0.545
	2	—	—	23.6	0.561
Rhamnose	1	—	0.475	51.6	0.528
	2	—	—	48.4	0.570
Fucose	1	63.9	0.491	6.7	0.539
	2	36.1	0.510	38.8	0.559
	3	—	—	54.5	0.587
Xylose	1	65.0	0.528	44.9	0.587
	2	35.0	0.545	55.1	0.634
Ribitol		—	0.622	—	0.623
Mannose	1	94.1	0.653	56.9	0.684
	2	5.9	0.669	43.1	0.757
Galactose	1	28.6	0.665	2.4	0.701
	2	49.5	0.686	11.6	0.724
	3	21.9	0.709	26.4	0.743
	4	—	—	58.9	0.787
Glucose	1	72.6	0.724	47.8	0.757
	2	27.4	0.743	4.5	0.787
	3	—	—	47.6	0.824
Fructose	1	—	—	0.8	0.734
	2	—	—	1.6	0.753
	3	—	—	97.2	0.782
Sorbose	1	—	—	1.8	0.689
	2	—	—	3.4	0.741
	3	—	—	92.2	0.796
	4	—	—	2.6	0.814
Mannitol		—	0.788	—	0.789
N-Acetylgalactosamine	1	40.0	0.825	9.0	0.854
	2	60.0	0.854	11.0	0.874
N-Acetylglucosamine	1	9.4	0.806	9.4	0.857
	2	12.2	0.824	90.6	0.893
	3	3.7	0.841	—	—
	4	71.1	0.871	—	—
	5	3.1	0.875	—	—
N-Acetylmuramic acid	1	4.3	0.776	—	—
	2	3.6	0.825	—	—
	3	2.9	0.874	—	—
	4	89.2	0.975	—	—
Perseitol		—	1.000	—	1.000

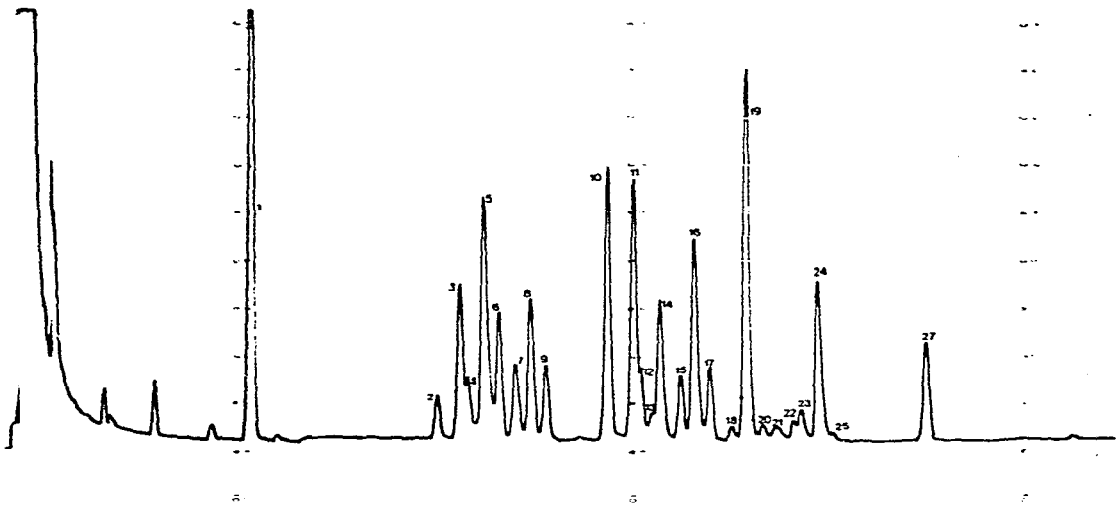


Fig. 1. Chromatogram of a standard mixture of TMS-methyl glycosides on SE-30 columns temperature programmed from 80° to 250° at 2°/min. Peaks: 1 = glycerol; 2, 10 = ribitol; 3, 4 = arabinose; 5 = rhamnose/ribose; 6,7 = fucose; 8, 9 = xylose; 11, 13 = mannose; 12, 14, 15 = galactose; 16, 17 = glucose; 18 = mannitol; 19, 20, 21, 24, 25 = N-acetylglucosamine; 21, 23 = N-acetylgalactosamine; 27 = perseitol.

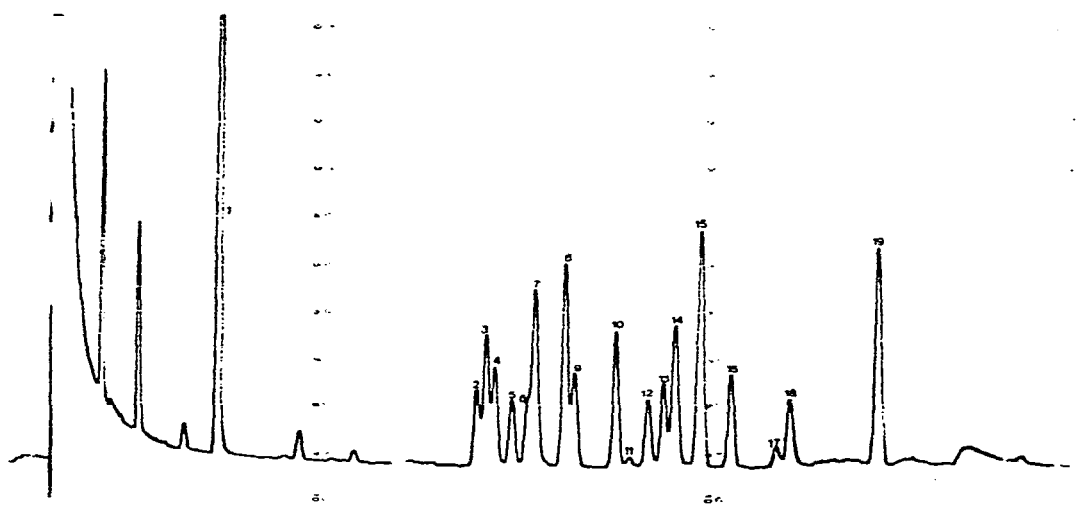


Fig. 2. Chromatogram of a standard mixture of TMS ethers of free monosaccharides on SE-30 columns temperature programmed from 80° to 250° at 2°/min. Peaks: 1 = glycerol; 2, 4 = arabinose; 3, 6 = rhamnose; 4, 5, 7 = fucose; 7, 9 = xylose; 8 = ribitol; 10, 14 = mannose; 12, 12, 13 15 = galactose; 14, 15, 16 = glucose; 15 = mannitol; 17 = N-acetylgalactosamine; 18 = N-acetylglucosamine; 19 = perseitol.

TMS-sugar fingerprints of Streptococcus mutans

Combined GLC–MS data are listed in Table II. A typical gas chromatogram is shown in Fig. 4. With the aid of mass spectral data, it was possible to positively identify at least seven monosaccharides derived from bacterial cells, *viz.* glycerol, xylose, galactose, glucose, rhamnose, N-acetylglucosamine and N-acetylmuramic

TABLE II

PARTIAL MASS SPECTRA OF SOME TMS ETHERS OF METHYL GLYCOSIDES, ALTIDOLS AND AMINO SUGARS

Compound	*Base (m/e)**	*m/e 103 (%)*	*m/e 131 (%)*	*m/e 133 (%)*	*m/e 161 (%)*	*m/e 173 (%)*	*m/e 187 (%)*	*m/e 204 (%)*	*m/e 205 (%)*	*m/e 217 (%)*	*m/e 218 (%)*	*m/e 275 (%)*	*m/e 277 (%)*	*m/e 305 (%)*	*m/e 307 (%)*	*m/e 314 (%)*	*m/e 319 (%)*	*m/e 330 (%)*
Arabinose	217	9	0	24	0	0	0	33	7	100	0	2	0	0	0	0	1	0
Ribose	217	6	0	31	0	0	0	37	8	100	0	1	0	0	0	0	1	0
Xylose	204	10	0	40	0	0	0	100	27	85	0	2	0	0	0	0	1	0
Fucose	204	3	0	23	0	0	0	100	22	35	0	0	0	0	0	0	4	0
Rhamnose	204	5	0	21	0	0	0	100	19	34	0	0	0	0	0	0	2	0
Galactose**	217	11	0	33	0	0	0	91	31	100	0	0	0	0	0	0	12	0
	204	9	0	21	0	0	0	100	24	39	0	0	0	0	0	0	3	0
Glucose	204	7	0	25	0	0	0	100	24	32	0	0	0	0	0	0	2	0
Mannose	204	6	0	21	0	0	0	100	21	21	0	0	0	0	0	0	2	0
Ribitol	205	85	0	7	0	0	0	26	100	66	0	0	7	0	49	0	46	0
Mannitol	205	50	0	4	0	0	0	12	100	40	0	0	5	0	27	0	87	0
Perseitol	205	53	0	3	0	0	0	10	100	45	0	0	6	0	52	0	84	0
N-acetyl-galactosamine	173	7	18	8	0	100	0	20	9	12	0	0	0	0	0	3	2	0
N-acetyl-glucosamine	173	12	29	11	0	100	0	23	10	20	0	0	0	0	0	6	6	0
N-acetyl-muramic acid	187	17	5	3	26	0	100	3	10	26	33	0	0	0	2	0	4	3

* Base peak is not due to M^+ ion which rapidly fragments.

** Different spectra due to galactofuranose and galactopyranose.

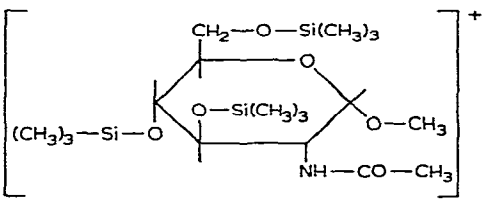
Fragment m/e	Structure of ion
89	$^{+}CH_2—CH(OH)—COOH$
103	$^{+}CH_2—OSi(CH_3)_3$
173	$[(CH_3)_3Si—O—CH=CH—NH—CO—CH_3]^{+}$
204	$[(CH_3)_3Si—O—CH=CH—O—Si(CH_3)_3]^{+}$
205	$(CH_3)_3Si—O—CH^{+}—CH_2—O—Si(CH_3)_3$
217	$(CH_3)_3Si—O—CH=CH—^{+}CH—O—Si—(CH_3)_3$
307	$(CH_3)_3Si—O—^{+}CH—CH(O—Si(CH_3)_3)—CH_2—O—Si(CH_3)_3$
451	

Fig. 3. Some fragmentation ions formed during MS of TMS derivatives of monosaccharides derived from *S. mutans*.

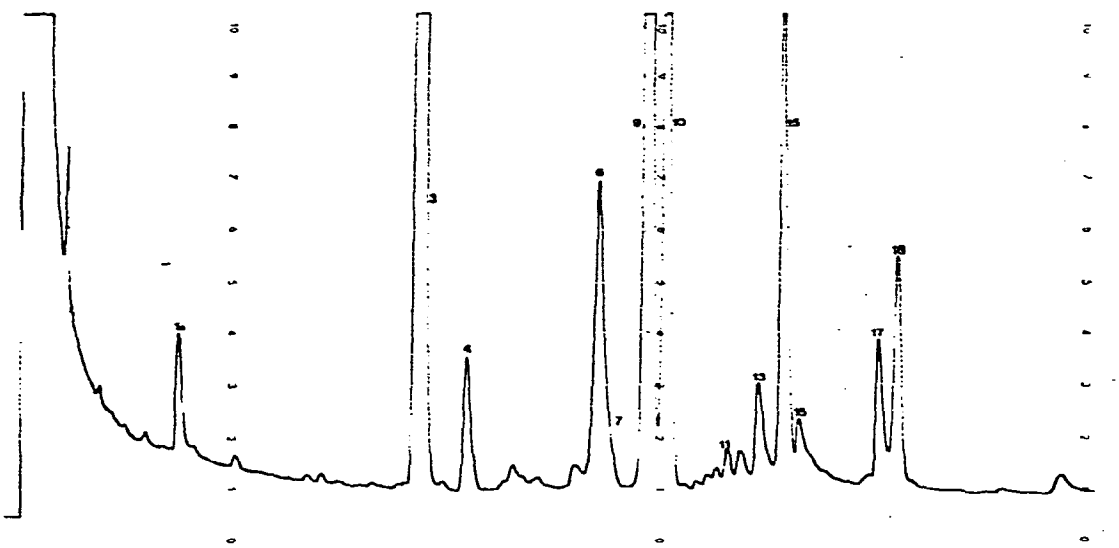


Fig. 4. Chromatogram of a TMS-sugar fingerprint of *S. mutans* NCTC 10823 on SE -30 columns temperature programmed from 80° to 250° at 2°/min. Cellular monosaccharides were extracted by methanolysis. Peaks: 1a = glycerol; 3 = rhamnose; 4 = xylose; 6, 7 = galactose; 9, 10 = glucose; 11, 13, 15, 16 = N-acetylglucosamine; 17 = N-acetylmuramic acid; 18 = perseitol.

acid. Other sugars were either absent or below the limit of detection. The galactose appeared mainly to be in the furanose form. The quantitative sugar fingerprint, showing total peak area ratios of each sugar component relative to that of perseitol is shown in Table III. Reproducibility was assessed in terms of the coefficient of varia-

TABLE III

REPRODUCIBILITY OF CARBOHYDRATE FINGERPRINTS OF *STREPTOCOCCUS MUTANS* NCTC 10832, GROWN IN BRAIN-HEART INFUSION

Total peak area ratio: the ratio of total peak area of each monosaccharide to that of perseitol. Sample: triplicate batch cultures of the strain, inoculated from the same culture and grown at the same time. $\bar{x}$ = arithmetic mean value; σ = standard deviation; C.V. = coefficient of variation, which is the standard deviation taken as a percentage of the mean, *i.e.* C.V. = $100\sigma/\bar{x}$.

Sample	*Total Peak Area Ratio*						
	Glycerol	*Rhamnose*	*Xylose*	*Galactose*	*Glucose*	*N-acetyl-glucosamine*	*N-acetyl-muramic acid*
1	1.66	9.42	0.28	0.25	6.81	2.42	0.71
	1.60	9.44	0.28	0.24	6.81	2.42	0.71
2	1.50	9.26	0.30	0.22	6.58	2.14	0.70
	1.51	9.26	0.30	0.21	6.59	2.20	0.70
3	1.58	9.34	0.29	0.24	6.70	2.28	0.71
	1.57	9.35	0.29	0.24	6.70	2.27	0.71
$\bar{x}$	1.57	9.35	0.29	0.23	6.70	2.29	0.71
σ	0.06	0.08	0.01	0.02	0.10	0.11	0.01
C.V.	3.70	0.80	3.09	6.47	1.52	4.90	0.71

tion. Based on six sets of analyses, the coefficient ranged from 0.8 to 6.4 depending on the peak. The coefficient increased slightly when cells were harvested following differing incubation times or after different growth media had been used. The galactose component appeared to be especially liable to variation.

The rate at which monosaccharides could be released from bacterial cells by 1.0 *M* methanolic hydrogen chloride was determined. Maximum yield of each sugar was attained within 4.0 h. Depolymerisation and extraction of cellular sugars with solutions of hydrochloric acid ranging in molarity from 0.5–5.0 *M* revealed slight decrease in yields of galactose, glucose and N-acetyl-glucosamine, as acid molarity increased; whereas, extraction of xylose and N-acetylmuramic acid improved. Optimum yield of sugars on acid hydrolysis required 2.0 *M* hydrochloric acid. No artefactual chromatogram peaks, indicating degradation, were observed.

DISCUSSION

The results indicate that monosaccharide fingerprints of *S. mutans* may be easily and reproducibly obtained by the technique described. The methanolysis method appears unsuitable for organisms containing either ketoses or deoxypentoses; acid hydrolysis would overcome this difficulty. The production of multiple peaks appears to offer additional chemotaxonomic information, especially in the case of galactose, and aids identification of monosaccharides. The mass spectra appear to be particularly valuable for identifying individual monosachcharides, for each monosaccharides has its own characteristic mass spectrum (*cf.* Tables II and IV). Galactofuranose has been described elsewhere[13] and is a sugar known to partly comprise the type antigen of this strain. The major monosaccharide, rhamnose, is a characteristic cell wall sugar of streptococci and was originally detected in related bacterial species

TABLE IV

PARTIAL MASS SPECTRA OF SOME TMS ETHERS OF METHYL GLYCOSIDES, ALDITOLS AND AMINO SUGARS OF *STREPTOCOCCUS MUTANS* NCTC 10832

Compound	*Relative retention*[*]	*Base (m/e)*	*m/e 89 (%)*	*m/e 103 (%)*	*m/e 131 (%)*	*m/e 133 (%)*	*m/e 147 (%)*	*m/e 161 (%)*	*m/e 173 (%)*	*m/e 187 (%)*	*m/e 204 (%)*	*m/e 205 (%)*	*m/e 217 (%)*	*m/e 218 (%)*	*m/e 240 (%)*	*m/e 299 (%)*	*m/e 305 (%)*	*m/e 307 (%)*	*m/e 314 (%)*	*m/e 319 (%)*
?	0.197	299	0	10	3	4	22	0	0	0	3	12	0	3	0	100	0	0	22	0
Glycerol	0.208	205	0	35	5	9	73	0	0	0	11	100	0	20	0	13	0	0	4	7
Ribose	0.460	217	5	11	0	29	26	0	0	0	50	0	100	0	0	0		0	0	3
Rhamnose	0.476	204	2	3	9	17	19	0	0	0	100	19	19	0	0	0	2	0	0	1
Xylose	0.529	204	6	6	0	32	49	0	0	0	100	24	98	0	0	0	2	0	0	3
?	0.636	299	0	32	9	9	56	0	0	0	32	10	18	15	0	100	0	0	0	0
Galactofuranose	0.671	217	5	14	12	28	26	0	0	0	38	16	100	0	0	3	0	0	0	13
Galactopyranose	0.685	204	4	9	11	22	19	0	0	0	100	24	32	0	0	0	4	0	0	3
Glucopyranose	0.724 0.744	204	3	7	7	25	19	0	0	0	100	10	25	0	0	0	2	0	0	1
N-acetyl-glucosamine	0.822 0.841 0.872	173	2	8	16	11	15	0	100	2	35	8	13	0	0	0	0	0	2	2
N-Acetyl-muramic acid	0.976	187	10	8	4	4	23	28	0	100	4	2	19	36	7	0	0	0	0	2

[*] Relative to perseitol.

by paper chromatography[14]. Glycerol is known to exist in streptococci both as a component of glycerides[15] and as its polymerised phosphodiester, glycerol teichoic acid and the more complex lipoteichuronic acid. Xylose has never been absolutely identified in streptococci[16] previously, although an anisaldehyde colour reaction similar to that of xylose has been described for thin-layer chromatographic analysis. Glucose is the major constituent of soluble and insoluble glucans produced by *S. mutans* and large amounts would be expected. N-acetylaminoglucosamine and N-acetylmuramic acid comprise the carbohydrate backbone of the cell wall murein molecule[17,18], the ratios of peak areas, if directly related to ratio of molar concentrations, would indicate that for each N-acetylmuramyl residue, three of N-acetylglucosaminyl must be present. Alternatively, some of the N-acetylglucosamine could be incorporated into additional polysaccharides. The absence of deoxyribose, derived from deoxyribonucleic acid, is interesting; presumably the amount present is comparatively small compared to the much larger quantities of cell wall and extramural sugars. The unknown peaks 1 and 6 are currently being investigated.

The data presented in this study indicate that as little as 5 mg of freeze-dried material is sufficient for analysis. This amount is less than the quantity required for other studies[4,18], in which similar proportions of monosaccharides were found in purified cell walls of *S. mutans* serotype c, analysed by colorimetric methods[19].

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REFERENCES

1 R. C. Lancefield, *J. Exp. Med.*, 57 (1933) 571.
2 A. T. Fuller, *Brit. J. Exp Pathol*, 19 (1938) 130.
3 G. Colman and R. E. O. Williams, *J. Gen. Microbiol.*, 41 (1965) 375.
4 A. S. Bleiweis, R. A. Craig, D. D. Zinner and J. M. Jablon, *Infect. Immun.*, 3 (1971) 189.
5 D. B. Drucker, in J. R. Norris (Editor), *Methods in Microbiology*, Vol. IX, Academic Press, London, 1976, Ch. 3, p. 52.
6 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Oil Chem. Soc.*, 85 (1963) 2497.
7 G. A. Levvy, A. J. Hay, J. Conchie and J. Strachan, *Biochim. Biophys. Acta*, 222 (1970) 333.
8 J. R. Clamp, T. Bhatti and R. E Chambers, *Methods Biochem. Anal.*, 19 (1971) 229.
9 M. Wurst, V. Vančura and L. Kalachová, *J. Chromatogr*, 91 (1974) 469.
10 W. F. Dudman, *Carbohydr. Res.*, 46 (1976) 97.
11 L. D. Melton, L. Mindt, D. A. Rees and G. R. Sanderson, *Carbohydr. Res.*, 46 (1976) 245.
12 D. B. Drucker, R. M. Green and D. K. Blackmore, *J. Dent. Res.*, 50 (1972) 1510.
13 D. C. De Hongh, T. Radford, J. D. Hribar, S. Hannessian, M. Bigber, G. Dawson and C. C. Sweeley, *J. Amer. Chem. Soc.*, 91 (1969) 1728.
14 C. S. Cummins and H. Harris, *J. Gen. Microbiol.*, 14 (1956) 583.
15 E. I. Szabo, B. H. Amdur and S. S. Socransky, *Caries Res.*, 12 (1978) 21.
16 D. B. Drucker, *Lab. Pract.*, 18 (1969) 1288.
17 H. J. Rogers, *Bact. Rev.*, 34 (1970) 194.
18 H. R. Cooper, F. W. Chorpenning and S. Rosen, *Infect. Immun.*, 11 (1975) 823.
19 R. M. Vaught and A. S. Bleiweis, *Infect. Immun.*, 9 (1974) 60.