USE OF CARBOHYDRATE FERMENTATION REACTIONS FOR DIFFERENTIATING STRAINS OF GROUP D STREPTOCOCCI

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SUMMARY

Thirty-two strains of Lancefield Group D streptococci isolated from fowls gave variable results for acid production from sorbitol and arabinose in media containing 1 per cent. carbohydrate and Andrade's indicator, in tests using inocula derived from cultures kept on agar slopes and in broth. In media containing raffinose, two different brands gave similar results.

Intermediate colour changes gave rise to difficulties in reading results using either bromcresol purple or Andrade's solution as an indicator. Results of electrometric pH determination with 115 strains in arabinose and sorbitol after 7 days' incubation showed that they could be divided into two distinct groups, with few strains producing intermediate results in arabinose and far greater numbers giving intermediate results in sorbitol.

I. INTRODUCTION

Most authors who have studied the "enterococci" or Group D streptococci have included fermentation of carbohydrates in the criteria used for defining species within the group. The ability to ferment raffinose has been suggested as a test to differentiate *Streptococcus bovis* from the other species (Shattock 1945), and the ability to ferment sorbitol and arabinose to differentiate *Str. faecalis* and its varieties from *Str. faecium* (Barnes and Ingram 1955).

During the study of Group D streptococci isolated from animal tissues and fluids, the results obtained for the fermentation tests with raffinose, arabinose and sorbitol were not as expected (Elder and Simmons 1963*d*). For this reason tests were done with two different brands of raffinose, and with Andrade's and

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bromcresol purple indicators. Also, the influence of age of culture was examined, and the value of solid media incorporating carbohydrate and indicator was investigated. The final pH values in liquid media were also determined electrometrically.

II. MATERIAL AND METHODS

Sixty-seven Queensland and 43 overseas strains described previously (Elder and Simmons 1963*a*, 1963*b*) were used. The Queensland strains were stored on blood agar slopes, which were inoculated with the strain either when it was first isolated or with freeze-dried cultures of subsequent subcultures. The overseas strains were received as freeze-dried cultures and were examined immediately after regeneration. For testing, all strains were inoculated into tryptone soya broth and onto a blood plate to check purity. After 24 hours' incubation at 37°C the tryptone soya broth culture was used to inoculate the carbohydrate test media for both colour and electrometric pH determination.

The 32 fowl strains included in the 67 Queensland strains were also stored in tryptone soya broth at 4°C. They were inoculated into another bottle of tryptone soya broth and plated on blood agar to check for purity. After 24 hours' incubation at 37°C, the second tryptone soya broth culture was used to inoculate the test media. The fowl strains stored in tryptone soya broth were examined in raffinose, sorbitol and arabinose, twice using Andrade's indicator and once using bromcresol purple, and the same strains stored on blood agar slopes were examined three times in raffinose, sorbitol and arabinose, using Andrade's indicator.

The pH produced by all strains after 7 days' incubation in arabinose and sorbitol was determined in duplicate electrometrically, using a Cambridge pH meter and an inoculum derived from blood agar slopes. The first time they were examined in two groups, the Queensland strains at one time and the overseas strains at another, and the second time they were examined together. The pH of the Queensland strains in raffinose was also determined electrometrically. (Uninoculated media were included, and the pH of these was 7·2).

The electrometric pH values of Strain 7 in arabinose and sorbitol and Strain 13 in arabinose were determined daily. Seven tubes of each were inoculated and one tube of each used daily.

The fermentation reactions were studied in peptone water containing 1 per cent. of indicator and 1 per cent. of carbohydrate made in the following way:—

10 g Difco proteose peptone
2 g Difco Bacto tryptone
5 g NaCl (AR)
1 l distilled water
10 ml Andrade's reagent.

pH adjusted to 7·2, filtered (DO Horman-Ekwip filter), dispensed in 90-ml quantities, and autoclaved at 15 lb for 15 min.

Ten millilitres of filtered (D9 Horman-Ekwip filter) 10 per cent. solution of carbohydrate were added to each 90-ml quantity of medium, which was dispensed in 2·5-ml quantities for indicator assessment and 15-ml quantities for pH determination. The L (+) isomer of arabinose was always used. The arabinose and sorbitol were British Drug House brand. Two brands of raffinose, Kerfoot's and British Drug House, were used.

Samples of uninoculated peptone water and sorbitol (B.D.H.) peptone water were submitted to the University of Queensland Microbiology Department for quantitative measurement of reducing sugars by a chromatographic method. The results were:

Peptone water	0·11 mg reducing substance/ml
Sorbitol peptone water	0·10 mg reducing substance/ml

These results lie within the limits specified on the batch of sorbitol by B.D.H. The indicator-containing media were examined daily for seven days.

The fowl strains were tested on agar medium incorporating a carbohydrate and phenol red indicator. The agar was prepared in the following way:—

NaCl	5 g
Peptone	10 g
Bacto agar	20 g
Meat extract	5 g
Distilled water	1 l.

The ingredients were dissolved, filtered through a DO Horman-Ekwip filter, and the pH adjusted to 7·4. About 10 ml of a 1 per cent. solution of phenol red was added. In later batches the pH was altered to 7·6, 7·8 and 8·0 in an attempt to increase the contrast between the colour of the colonies and the colour of the medium. The medium was autoclaved at 15 lb for 15 min.

III. RESULTS

Fermentation reactions of 32 fowl strains in sorbitol, arabinose and raffinose are given in Tables 1-3. Fourteen strains gave consistent results each time they were tested in sorbitol (Table 1) and 9 strains gave consistent results in arabinose (Table 2). With Andrade's indicator there was a gradation of colour from deep pink to a very pale pink. The same effect was found with bromcresol purple; the colour varied from deep purple to almost colourless mauve and pale yellow.

TABLE 1

ACID PRODUCTION FROM SORBITOL (B.D.H.)

Strain	Agar Slope Cultures			Broth Cultures			No. Postive	No. Negative	pH	
	Andrade's Indicator Test number			Andrade's Indicator		Brom-cresol purple				
	1	2	3	1	2	3			1	2
1	$+^{1*}$	—	..	$+^{1}$	$+^{1}$	$+^{1}$	4	1	4·6	4·4
2	—	—	—	$+^{2}$	$+^{1}$	$+^{1}$	3	3	5·6	4·8
3	$+^{1}$	$+^{2}$	—	—	$+^{1}$	$+^{1}$	4	2	5·4	5·7
4	—	—	—	$+^{1}$	$+^{1}$	$+^{1}$	3	3	5·9	5·6
5	$+^{1}$	—	—	—	$+^{1}$	—	2	4	5·6	4·5
6	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	4·4	4·6
7	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	7·3	7·4
8	—	—	—	$+^{2}$	—	—	1	5	4·3	4·4
9	$+^{2}$	—	—	$+^{1}$	$+^{1}$	$+^{1}$	4	2	4·3	4·5
10	—	—	—	$+^{2}$	$+^{1}$	$+^{1}$	3	3	7·5	6·2
11	$+^{2}$	—	—	—	$+^{1}$	$+^{1}$	3	3	5·8	5·1
12	—	—	—	—	$+^{1}$	$+^{1}$	2	4	4·5	4·5
13	—	—	—	$+^{1}$	$+^{1}$	$+^{1}$	3	3	7·4	7·4
14	$+^{1}$	—	—	$+^{2}$	$+^{1}$	$+^{2}$	4	2	4·2	4·4
15	—	—	—	$+^{1}$	—	$+^{1}$	2	4	4·5	4·5
16	—	—	—	$+^{2}$	$+^{1}$	$+^{1}$	3	3	4·3	4·5
17	$+^{1}$	$+^{4}$	—	$+^{1}$	$+^{1}$	$+^{1}$	5	1	4·3	4·5
18	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	5·3	4·3
19	—	—	—	$+^{1}$	$+^{1}$	$+^{1}$	3	3	7·2	7·4
20	—	—	—	$+^{2}$	$+^{1}$	$+^{1}$	3	3	4·3	4·5
22	$+^{1}$	$+^{2}$	$+^{2}$	$+^{1}$	$+^{2}$	$+^{1}$	6	0	4·4	..
23	$+^{1}$	$+^{6}$	$+^{2}$	$+^{1}$	$+^{1}$	$+^{2}$	6	0	4·1	4·6
24	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	5·2	4·5
25	—	—	—	$+^{1}$	$+^{1}$	$+^{1}$	3	3	4·3	4·5
26	—	—	—	$+^{1}$	$+^{1}$	$+^{1}$	3	3	6·6	7·3
27	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	4·2	4·4
28	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	4·3	4·4
29	—	—	—	$+^{1}$	$+^{1}$	$+^{1}$	3	3	4·3	4·4
30	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	4·2	4·5
31	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	4·3	4·2
32	$+^{1}$	—	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	5	1	5·2	5·5
33	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	6	0	4·4	4·2

* No. of days incubation at which indicator gave acid colour reaction

— Indicates no acid reaction after 7 days' incubation

TABLE 2

ACID PRODUCTION FROM ARABINOSE (B.D.H.)

Strain	Agar Slope Cultures			Broth Cultures			No. Positive	No. Negative	pH	
	Andrade's Indicator Test number			Andrade's Indicator		Brom-cresol purple				
	1	2	3	1	2	3			1	2
1	+1*	+1	+1	+1	+1	+1	6	0	4·3	4·2
2	+1	—	—	+1	+1	+1	4	2	4·2	4·2
3	+1	+1	+1	+1	+1	+1	6	0	4·4	4·4
4	+1	—	—	+1	+1	+1	4	2	4·2	4·2
5	+1	+1	+1	+1	+1	+1	6	0	4·3	4·2
6	+1	+1	—	+1	+1	+1	5	1	4·3	4·2
7	+1	—	—	—	+2	+2	3	3	7·1	7·4
8	+1	—	—	+1	+1	+1	4	2	4·2	4·2
9	+1	—	+1	+1	+1	+1	5	1	4·3	4·2
10	+1	—	—	+1	+1	+1	4	2	4·0	4·2
11	—	—	—	+1	+1	+1	3	3	4·4	4·3
12	—	—	—	+1	+1	+1	3	3	6·5	6·9
13	+1	—	—	+1	+1	+1	4	2	7·4	6·7
14	+1	—	—	+1	+1	+1	4	2	4·2	4·2
15	—	—	—	+1	+1	+1	3	3	5·7	4·2
16	—	—	—	+1	+1	+1	3	3	4·3	4·2
17	—	—	..	+1	+1	+1	3	2	4·9	4·2
18	—	+1	+1	+2	—	+2	4	2	4·4	4·2
19	—	—	—	+1	+1	+1	3	3	4·1	4·3
20	—	—	—	+1	+1	+1	3	3	4·2	4·2
22	+1	+1	+1	+1	+1	+1	6	0	6·7	7·4
23	+1	+1	+1	+1	—	+1	5	1	4·1	4·2
24	—	—	—	+1	+1	+1	3	3	7·0	7·4
25	—	—	—	+1	+1	+1	3	3	4·6	4·2
26	—	—	—	+1	+1	+1	3	3	7·2	6·8
27	—	—	—	—	—	—	0	6	6·4	7·2
28	—	—	—	—	—	—	0	6	6·8	7·1
29	—	—	—	+1	+1	+1	3	3	7·2	7·2
30	—	+1	—	+1	+1	+1	4	2	4·1	4·2
31	—	—	..	—	—	—	0	5	6·9	7·1
32	+1	+1	+1	+1	+1	+1	6	0	4·1	4·3
33	—	+2	—	+1	+1	+1	4	2	4·1	4·2

* No. of days incubation at which indicator gave acid colour reaction

— Indicates no acid reaction after 7 days' incubation

Table 3 shows that the two different brands of raffinose gave similar results, and from all three tables it can be seen that bromcresol purple and Andrade's indicators gave equivalent readings. Acid production from raffinose usually occurred only after several days' incubation, although Strain 3 consistently fermented this carbohydrate after 24 hours' incubation.

TABLE 3

ACID PRODUCTION FROM RAFFINOSE

Strain	Agar Slope Cultures, Andrade's Indicator		
	Kerfoot's Raffinose	B.D.H. Raffinose	
	1	2	3
1	—	—	—
2	+5*	+6	+5
3	+1	+1	+1
4	+1	—	+5
5	—	—	—
6	+1	+1	+1
7	—	—	—
8	+5	+5	+5
9	+5	+5	+5
10	+2	+5	+5
11	+1	+6	+5
12	+2	+6	+5
13	+2	+6	+5
14	+5	+7	+5
15	+5	+7	+5
16	+5	+6	+5
17	+5	+3	+5
18	—	—	—
19	+2	+6	+5
20	+5	+3	+5
22	+5	+6	+5
23	+5	—	+5
24	—	—	—
25	+1	+2	+5
26	+5	+6	+5
27	—	—	—
28	—	—	+5
29	+5	+6	—
30	—	—	—
31	—	—	—
32	+2	+6	+5
33	—	—	+6

* No. of days incubation at which indicator gave acid colour reaction

— Indicates no acid reaction after 7 days' incubation

Tables 1 and 2 indicate that strains produced acid more often if they had been previously stored in broth than if stored on agar.

Media containing arabinose gave pH values mainly in the region pH 4·0–5·0 and 6·2–7·4 (Figure 1), whereas media containing sorbitol gave a greater number of intermediate values over the whole range of pH 4·0–7·6 (Figure 2).

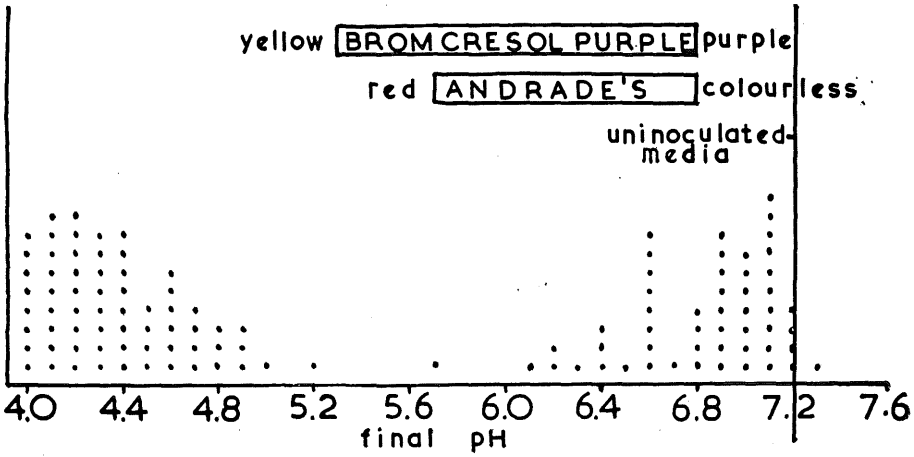

Fig. 1.—pH values in arabinose.

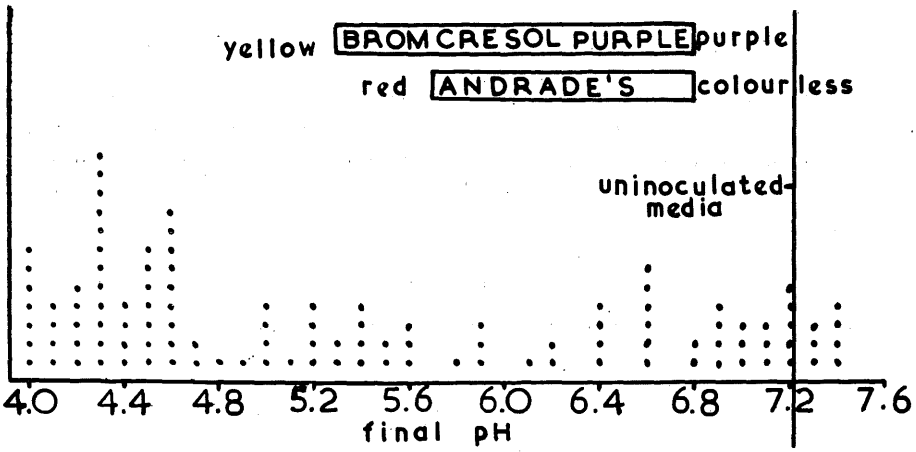

Fig. 2.—pH values in sorbitol.

The electrometric pH values of the fermentation of arabinose and sorbitol by Strain 7, and the fermentation of arabinose by Strain 13, did not show alkali reversion during incubation up to 7 days.

On solid media of pH 7·2 the colonies of the fowl strains were very small and it was impossible to distinguish between a pink colony and a colourless one. The media of pH 8·0 did not increase the red colour of the acid-producing colonies sufficiently to allow mutant colonies to be distinguished with any certainty. However, there did appear to be differences in colour between separate colonies and even in a single colony.

IV. DISCUSSION

From Tables 1–3 and Figures 1 and 2 it can be seen that variations in the fermentation results of particular strains in particular carbohydrates were frequent. For most positive fermentation reactions the decrease in pH was to between 4·0 and 4·6, and for most negative reactions the decrease was slight, to between 6·6 to 7·2. However, the pH decreased for several strains to between 4·6 and 6·6, and this may give rise to difficulties in reading changes in the colour of indicators incorporated in the media.

Results of examining sorbitol peptone water for reducing sugar (glucose) indicated that the presence of glucose is not a factor in the variation of results for sorbitol.

The varying results obtained with cultures stored either on slopes or in broth would indicate that fermentation of sorbitol and arabinose is not a stable characteristic and therefore would appear to be unsuitable for taxonomic purposes unless the conditions of tests are rigidly defined.

Raibaud *et al.* (1961) also noticed variations in results of a single strain in carbohydrates. They found that strains obtained from a single colony may be heterogeneous with regard to their behaviour towards certain carbohydrates.

From the distribution of pH values obtained by the electrometric method, it is apparent that few strains will lower the pH to the vicinity of pH 6·0 and that the majority either cause only a small reduction in pH or reduce it within the pH values of 4·0–5·0. pH 6·0 could therefore be used as an arbitrary point.

REFERENCES

BARNES, E. M., and INGRAM, M. (1955).—Ann. Inst. Pasteur Lille 7:115.

ELDER, JEAN K., and SIMMONS, G. C. (1963*a*).—*Qd J. Agric. Sci.* 20:257.

ELDER, JEAN K., and SIMMONS, G. C. (1963*b*).—*Qd J. Agric. Sci.* 20:263.

ELDER, JEAN K., and SIMMONS, G. C. (1963*d*).—*Qd J. Agric. Sci.* 20:279.

RAIBAUD, P., CAULET, M., GALPIN, J. V., and MOCQUOT, G. (1961).—*J. Appl. Bact.* 24:285.

SHATTOCK, P. M. F. (1945).—*Proc. Soc. Appl. Bact.* p. 18.

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