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Use of tartaric acid isomers and citric acid in the biotyping of *Salmonella typhimurium*

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SUMMARY

The colour-change and lead acetate tests for fermentation of *d*-, *l*- and *m*-tartaric acids and citric acid used in the Kristensen scheme for biotyping *Salmonella typhimurium* were found to be unreliable because, whatever the conditions of culture, they gave different results in replicate tests of the same strains. Many genotypically non-fermenting strains gave inconsistent reactions due to the emergence of fermenting mutant bacilli in some of their test cultures. No reliable test was found for the fermentation of citric acid.

A 'turbidity' test was found to give consistent and reliable results with the three tartaric acid isomers. It demonstrated fermentation by the significantly greater amount of growth obtained in a 24 hr. culture in Oxoid peptone water with added isomer than in a control culture without isomer. Lewis & Stocker's (1971) plate-inhibition test for fermentation of *m*-tartrate, which identifies *m*-tartrate-negative strains because *m*-tartrate inhibits their growth on citrate- or glycerol-containing minimal medium, was found to be as reliable as, and easier to read than, the turbidity test.

Use of the turbidity test for *d*- and *l*-tartrates and the plate-inhibition test for *m*-tartrate in biotyping 1435 strains of *S. typhimurium* showed that many strains had previously been mistyped by the lead acetate test and distinguished 16 new biotypes in addition to the 22 biotypes already recognized.

INTRODUCTION

Type differentiation within a pathogenic species such as *Salmonella typhimurium* is valuable for epidemiological studies and may be done with different kinds of discriminating tests. Bacteriophage typing gives reliable results. A system devised by Felix & Callow (1943, 1951) and extended by Callow (1959) distinguished 34 phage-types of *S. typhimurium*, and many further 'provisional' types in this scheme have been identified at the Enteric Reference Laboratory of the Public Health Laboratory Service, London (E. S. Anderson, unpublished). Another phage-typing scheme, that of Lilleengen (1948), subdivides *S. typhimurium* into 24 types.

Biochemical methods have been used to differentiate strains of *S. typhimurium* into epidemiologically significant 'fermentation types', or 'biotypes'. With tests on nine substrates, Kristensen, Bojlen & Faarup (1937) differentiated 361 strains

Table 1. *The Kristensen scheme for biotyping* Salmonella typhimurium *as used by Kallings & Laurell* (1957)

Biotype	Acid produced within 24 hr. in peptone water from: Xylose	Rhamnose	Inositol	Stern's glycerol reaction in 48 hr.	Lead acetate test for fermentation in peptone water: *d*-Tartrate (24 hr.)	*l*-Tartrate (24 hr.)	*m*-Tartrate (5 days)	Citrate (3 days)	Growth on glucose ammonium agar in 48 hr.	Acid in Bitter-xylose medium in 20 hr.
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	−
2 M	−	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	+	+	+	−	−
4	+	+	+	+	+	+	−	+	+	−
5	+	+	+	+	+	−	+	+	+	−
6	+	+	+	+	−	+	+	+	+	+
7	+	+	+	−	+	+	+	+	+	+
8	+	+	+	−	+	+	+	+	+	−
9	+	+	−	+	+	+	+	+	+	+
10	+	+	−	+	+	+	+	+	+	−
11	+	+	−	+	+	+	+	+	−	−
12	+	+	−	+	+	+	−	+	+	−
13	+	+	−	−	+	+	+	+	+	−
14	+	−	+	+	−	+	+	−	+	−
15	+	−	−	+	+	+	+	+	+	−
16	+	−	−	+	+	+	−	+	+	−
17	+	−	−	+	−	+	+	−	+	−
18	+	−	−	−	−	+	+	−	+	−
19	+	−	+	+	+	+	+	+	+	−
20	+	−	+	+	−	+	+	−	−	−

of *S. typhimurium* into 18 biotypes and their scheme was extended by the addition of type 20 by Hansen (1942) and types 2M and 19 by Harhoff (1948). The extended scheme is shown in Table 1. Kallings & Laurell (1957), Kallings, Laurell & Zetterberg (1959), Rische & Kretzschmar (1962) and Lewis & Stocker (1971) later showed that strains of the same phage-type can be differentiated into a number of different biotypes, so that the combination of biotyping with phage-typing gives greater precision in defining relationships between strains.

The successful application of biotyping depends on the use of *reliable* tests, i.e. tests in which each strain gives the same result on different occasions of testing and in which the result is not unduly influenced by minor or uncontrollable variations in the method. In a study of 1435 strains of *S. typhimurium* we have found that many strains are inconstant in their reactions with the four organic acids used in the scheme of Kristensen *et al.* (1937) when the tests are performed by the methods of these and later workers. The organic acids are the *dextro* (*d*), *laevo* (*l*), and *meso* (*m*) isomers of tartaric acid, and citric acid. The methods of testing for their fermentation were originally developed by Brown, Duncan & Henry (1924). Bacteria are cultured at 37° C. in Difco Bacto–Peptone water containing the organic acid and a positive reaction is read by the observation of (i) an increased amount of growth, (ii) a reduced production of precipitate on the

addition of lead acetate, or (iii) a change of colour in bromothymol blue indicator to yellow (acid). Different authors have used different periods of incubation from 24 hr. to 14 days and different methods of observation for the definitive readings, and there is no agreement on what methods and criteria are optimal for each organic acid.

In our preliminary work we found that many strains of *S. typhimurium* gave different results in replicate test cultures observed after the same period of incubation and that the proportion of positive reactions in sets of replicate tests increased with the period of incubation from 1 to 14 days. These 'variable' strains appeared to be genotypically non-fermenting but to give late fermentation by the emergence of fermenting mutants on prolonged culture, as has been shown for *d*-tartrate by Kristensen *et al.* (1937) and Kristensen (1944). We therefore investigated different methods of performing the tests in an attempt to determine a method of testing and a duration of incubation that would give the same results in all replicate tests of the same strain and would reliably distinguish genotypically fermenting ('positive') strains from genotypically non-fermenting ('negative') strains that are capable of giving late, mutative fermentation. We included among the methods to be evaluated the recently described plate-inhibition test of Lewis & Stocker (1971) for fermentation of *m*-tartrate.

MATERIALS AND METHODS

Bacteria

Strains (1435) of *Salmonella typhimurium* were mainly from a collection previously biotyped and examined for fimbriae by Duguid, Anderson & Campbell (1966). They were from a wide range of sources and places of origin and included representatives of all 21 biotypes, except type 14, in the extended Kristensen scheme and biotype 19Xd, which resembles type 6 except that it is rhamnose-negative (Morgenroth & Duguid, 1968).

We describe these strains and their biotypes as being 'positive' or 'negative' for the organic acid under test according to whether or not they utilized the acid for growth under either aerobic or anaerobic conditions. The biotypes of strains are given as ultimately determined by the tests recommended in this paper and in some cases differ from those determined by the methods of previous authors. Biotypes resembling previously recognized types, but differing from them in giving a negative reaction with one or more of the tartaric acid isomers, are designated by the number of the recognized type followed by the symbols *d*T−, *l*T− or *m*T−, e.g. 2*d*T−, 10*d*T−*l*T−.

Organic acids

Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and the three isomers of tartaric acid, namely *d*(+), $[(CH(OH).COOH)]_2$, *l*(−), $[(CH(OH).COOH)]_2$, and *m* (inactive), $[(CH(OH).COOH)]_2.H_2O$, were from British Drug Houses Ltd. *d*-Tartrate was used in some experiments as potassium (+) tartrate. The acids were prepared as 10% solutions in deionized water and neutralized with 5N-NaOH; the solutions were autoclaved and their pH was then 7·2.

Culture media

Nutrient broth was Oxoid Nutrient Broth no. 2, pH 7·5. Nutrient agar was Oxoid Nutrient Agar, pH 7·4; it was poured in *ca.* 20 ml. amounts in plastic Petri dishes, 8·5 cm. diameter.

Media for colour-change and lead acetate tests were 1% Difco 'certified' Bacto-Peptone with 1% *d*-tartrate (potassium salt), 0·5% *l*-tartaric acid, 0·5% *m*-tartaric acid or 1% sodium citrate (Kauffmann, 1966). The acids were neutralized with NaOH and the pH of the completed medium was 7·2. For colour-change tests, 12 ml. of 0·2% aqueous solution of bromothymol blue was added per litre. The medium was dispensed in 5 ml. amounts in screw-capped bijou bottles, capacity 6 ml.; the caps were closed tightly and the bottles autoclaved at 121° C. for 15 min.

Medium for turbidity tests was 1% Oxoid peptone water (pH 7·2), i.e. 0·67% Oxoid L37 peptone and 0·33% sodium chloride in deionized water; it was autoclaved at 121° C. for 15 min. A portion of each batch of peptone water without the addition of organic acid was kept for use for control cultures. The four organic acids were added as sterile (autoclaved) 10% solutions to four other portions of the peptone water, the *d*-, *l*- and *m*-tartrates to give final concentrations of 10 g./l. and the sodium citrate to give 2·5 g./l. The completed media (pH 7·2) were dispensed in 10 ml. amounts in cotton-wool stoppered test-tubes (1·5 × 15 cm.) and, for shaken cultures, in 20 ml. amounts in wide-mouthed glass jars, capacity 200 ml., with aluminium screw caps. Comparative tests were made with similar media in which 1% Difco Bacto-Peptone solution was used as the basic peptone water.

Minimal medium was that of Davis & Mingioli (1950). It contained per litre of deionized water, 7 g. K_2HPO_4, 3 g. KH_2PO_4, 1 g. $(NH_4)_2SO_4$ and 0·1 g. $MgSO_4 . 7H_2O$, pH 7·0. Minimal agar medium contained also 12 g./l. Oxoid Ionagar no. 2. Carbon sources were added in the following amounts (g./l.): sodium citrate 2·5; glycerol 5·0; *d*-, *l*- or *m*-tartrate 5·0; or sodium citrate 2·5 plus *m*-tartrate 5·0. The media were poured in amounts of 20 ml. in dishes 8·5 cm. diameter.

Preparation of inocula for tests

Cultures of *S. typhimurium* were maintained on Dorset's egg slants at ambient temperature. The test strain was plated on nutrient agar and after incubation for 20–24 hr. at 37° C. the bacterial inoculum was prepared by one of the following methods, which gave nearly similar results: (i) a single colony was inoculated into nutrient broth and incubated aerobically and statically for 24 hr., or (ii) several colonies were suspended in saline (0·85% NaCl) solution to a density of about 10^9 bacteria per ml. Test media were given inocula of *ca.* 0·05 ml. of the broth culture or saline suspension per 10 ml.

Colour change and lead acetate tests

In the definitive tests the inoculated bijou bottles were incubated for 24 hr. at 37° C. under aerobic and static conditions with their caps lying *loosely* on their tops. In comparative tests incubation was prolonged to 2 or more days, or the

bottles were tightly capped. The colour of the incubated test was noted, yellow being read as positive and blue or green as probably negative. A saturated aqueous solution of lead acetate was then added to the culture, or to a 1 ml. sample of it taken into a small tube, in the proportion of 0·1 ml. lead acetate per 1 ml. culture. After mixing and standing for 18–24 hr. to allow the precipitate to settle, the height of the precipitate in the bottle or tube was observed. If the precipitate occupied less than a half the height of the medium (generally less than a fifth) the test was read as positive and if more than a half (generally about three-quarters) it was read as negative.

Turbidity test for stimulation of growth

Cultures were inoculated and incubated in parallel in Oxoid peptone water containing the organic acid under test and in Oxoid peptone water without added organic acid. In the definitive tests incubation of tubes of 10 ml. medium was for 24 hr. at 37° C. under aerobic static conditions, i.e. in air, but without movement or artificial aeration, and the tubes were stoppered with cotton-wool. In special tests, 20 ml. cultures in wide jars were incubated aerobically for 24 hr. at 37° C. with shaking at 160 rev./min. in an incubator shaker. The cultures were killed by the addition of formaldehyde to 0·1 % and after uniform mixing the turbidity of the organic acid-containing culture was compared with that in the organic acid-free culture, first with the naked eye and then by measurement with a Spekker photoelectric absorptiometer with a no. 5 filter.

In tests for the utilization of *d*- or *l*-tartrate the result was read as positive if the turbidity in the tartrate-containing medium was more than 100 % greater than the turbidity in the tartrate-free (control) medium and as negative if it was less than 50 % greater than that in the control. The few tests in which the difference was 50–100 % were read as doubtful and repeated. In most positive tests the difference was so great, e.g. 500 %, that reliance could be placed on reading with the naked eye.

In tests for utilization of *m*-tartrate the result was read as positive if the turbidity of the *m*-tartrate-containing culture was more than 35 % *greater* than the turbidity in the control culture and as negative when it was more than 10 % *less* than that in the control, i.e. the growth of the culture had been inhibited by the presence of the *m*-tartrate. Tests in which the difference was intermediate, that is between + 35 % and − 10 %, were read as doubtful and repeated.

Test for growth on minimal agar medium

Saline suspensions of bacteria of twelve strains were inoculated with a twelve-pronged inoculator in spots in equivalent positions, 2 cm. apart, on a series of plates of minimal agar medium, each of which contained one of the organic acids or glycerol as sole carbon source. The plates were enclosed in plastic bags to minimize drying and were incubated aerobically at 37° C. They were read for the presence and amount of growth over each spot inoculation after 24 hr., 48 hr. and 7 days.

Plate-inhibition test for m-*tartrate*

Bacteria of twelve strains were cultured in parallel on two plates of minimal agar medium, the first of which contained sodium citrate as sole carbon source and the second, *m*-tartrate and sodium citrate together. The bacteria were inoculated and the plates incubated as in the test for growth on minimal agar. The amount of growth was observed after 24 and 48 hr., the definitive reading being at 48hr. A strain was read as positive with *m*-tartrate if it gave as much growth on the plate with *m*-tartrate and sodium citrate as on that with sodium citrate alone, and as negative if its growth was very much less on the former than on the latter plate, i.e. if it was *inhibited* by the presence of *m*-tartrate.

A few strains did not grow well on sodium citrate as sole carbon source and these strains were re-tested on plates containing glycerol in place of the sodium citrate. Auxotrophic strains were re-tested on sets of plates supplemented with the required growth factor at the concentration recommended by Meynell & Meynell (1965, pp. 36, 37).

RESULTS

Comparison of methods

Each of the 1435 strains of *S. typhimurium* was tested at least twice by the colour-change and lead acetate methods and once by the minimal agar growth method with each of the four organic acids, and at least once by the 24 hr. turbidity method with *d*- and *l*-tartrates and by the plate-inhibition method with *m*-tartrate. In addition, a number of strains selected from the principal biotypes were examined in series of 3–10 replicate tests by the lead acetate and turbidity methods and in tests by various modified methods.

The colour-change test proved to be very unreliable and frequently gave false-negative results. In tests in which the bromothymol blue indicator changed to the acid, yellow colour the positive result was generally confirmed by the lead acetate and turbidity methods, but in many of the tests that remained blue or green, and therefore were read as negative, the addition of lead acetate showed that the organic acid had in fact been utilized.

Lead acetate test for fermentation of d-tartrate

When done on cultures incubated in loosely capped bottles for 24 hr., the lead acetate test gave clear-cut results with the majority of strains, dividing them into two groups: (1) strains, mostly in biotypes 1, 2, 8, 9, 10, 11 and 12, that regularly gave positive results in replicate tests, and (2) strains, mostly in biotypes 6, 17, 18 and 19Xd, that regularly gave negative results in replicate tests. Strains of the latter, negative group gave positive results in a proportion of replicate tests if incubation was prolonged beyond 24 hr.; a few tests were positive at 2 days and an increasing proportion became positive after longer periods up to 14 days.

Unfortunately there was a minority of strains that did not give regular results in replicate tests made at 24 hr. Some strains of biotypes 15 and 16, which generally gave a positive result at 24 hr., and which turbidity tests at 24 hr. also

showed to be *d*-tartrate-positive, occasionally gave a negative lead acetate reaction at 24 hr. Conversely, some strains of biotypes 2*d*T−, 10*d*T− and 16*d*T−, which generally gave a negative lead acetate reaction at 24 hr., and which turbidity tests also showed to be *d*-tartrate-negative, occasionally gave a positive lead acetate reaction in replicate tests at 24 hr. Thus a single 24 hr. lead acetate test sometimes wrongly determined the *d*-tartrate genotype of a strain in these minority groups.

Mutative fermentation

The irregular production of positive results after periods of incubation greater than 24 hr., i.e. 'late' fermentation, appeared to be due to the emergence of *d*-tartrate-positive mutants in cultures of strains of a *d*-tartrate-negative genotype. This explanation was confirmed in the case of a few strains by plating out the late-fermented culture and growing separate subcultures from several of the colonies. A proportion of these subcultures were found to behave as typical *d*-tartrate-positive strains, regularly giving positive reactions in tests incubated for 24 hr.

There were marked differences in the frequency with which strains in the different *d*-tartrate-negative biotypes gave mutative fermentation in tests in loosely capped bottles. Strains of types 2*d*T−, 10*d*T− and 16*d*T− occasionally gave mutative fermentation within 1 day and generally did so within 2–3 days; strains of types 6 and 19Xd gave mutative fermentation sometime between 2 and 14 days in a large proportion of replicate tests, whilst strains of types 17 and 18 gave mutative fermentation in only a small proportion of 14-day tests.

The frequency of mutative fermentation was also influenced by the conditions of culture, particularly by the degree of aeration. Thus, *d*-tartrate-negative strains gave 'late' positive reactions in a larger proportion of replicate tests and after shorter periods of incubation when the culture bottles were incubated with their caps screwed on tightly to prevent access of air than when they were incubated with the caps applied loosely. For example, in several replicate series of ten tests of a strain of biotype 19Xd, all of ten tests in tightly capped bottles fermented on the sixth day, whereas only one of ten tests in loosely capped bottles fermented within 14 days. In a further series of tests incubated for 14 days all of 31 strains in biotypes 6, 17, 18 and 19Xd gave fermentation in each of two tightly capped bottles, but only four of the 31 strains gave fermentation in either of two loosely capped bottles.

Turbidity test for fermentation of d-*tartrate*

These tests were done by growing each strain for 24 hr. in two tubes of Oxoid peptone water, with and without 1% *d*-tartrate. The amount of growth of strains in the *d*-tartrate-positive biotypes was much greater in the medium with *d*-tartrate than in that without it; the difference in turbidity between the two media was generally very obvious to the naked eye and measurements with the absorptiometer showed that the degree of stimulation of growth by *d*-tartrate varied between +100 and +600%. Strains of *d*-tartrate-negative biotypes did not give noticeably greater growth in the medium containing *d*-tartrate than in that without it and

Table 2. *Results of representative turbidity tests showing the effect of* d-*tartrate on the amount of growth of strains of* Salmonella typhimurium *from* d-*tartrate positive (above) and* d-*tartrate negative (below) biotypes in two different liquid peptone media incubated for 24 hr. at 37° C. under aerobic static conditions*

		Results in Oxoid peptone medium			Results in Difco Bacto–Peptone medium		
		Amount of growth (turbidity) in medium		% stimulation of growth by *d*-tartrate	Amount of growth (turbidity) in medium		% stimulation of growth by *d*-tartrate
Strain	Biotype	Without *d*-tartrate	With 1% *d*-tartrate		Without *d*-tartrate	With 1% *d*-tartrate	
S2644	1*m*T−	0·14	1·00	+615	0·21	0·80	+280
S1827	10	0·15	1·02	+580	0·25	0·85	+240
S1464	12	0·21	0·90	+330	0·20	0·77	+285
S2294	16	0·19	0·80	+320	0·17	0·62	+264
S2327	16	0·15	0·84	+460	0·19	0·53	+177
S2332	16	0·14	0·53	+280	0·17	0·43	+153
S2633	16	0·15	0·47	+215	0·21	0·47	+121
S2348	6	0·19	0·19	0	0·20	0·27	+35
S1811	17	0·13	0·15	+15	0·15	0·22	+46
S2257	17	0·12	0·13	+8	0·13	0·22	+69
S2314	17	0·12	0·14	+17	0·13	0·23	+77
S2316	17	0·15	0·14	−7	0·17	0·27	+58
S2335	17	0·13	0·11	−15	0·13	0·23	+77
S2581	17	0·12	0·14	+17	0·14	0·19	+36

the turbidity measurements in the tartrate medium were generally between 20% less and 50% more than in the tartrate-free medium. The results for some representative strains are shown in Table 2.

The results of the turbidity tests read at 24 hr. were generally similar to, but more consistently reproducible than those of the 24 hr. lead acetate tests in loosely capped bottles. Thus they were uniform in replicate tests of the exceptional strains of biotypes 2*d*T−, 10*d*T−, 15 and 16, which gave variable results with lead acetate. The only strains giving irregular results by the 24 hr. turbidity method were some of biotype 16*d*T−, which occasionally gave a positive, mutative reaction. These strains might have been mistyped if only a single test had been done on them. When turbidity tests were done after periods of incubation longer than 24 hr., strains of all the *d*-tartrate-negative biotypes gave irregular results due to the occurrence of mutative fermentation.

Turbidity tests made in Difco Bacto-Peptone water gave generally similar results to those made in Oxoid peptone water, but the differences between positive and negative strains were less marked. The *d*-tartrate-positive strains showed rather less stimulation of growth by *d*-tartrate in the Difco than in the Oxoid medium and, surprisingly, the *d*-tartrate-negative strains regularly showed a small stimulation of growth, e.g. +50%, in the Difco medium (Table 2). The *d*-tartrate-negative strains, moreover, gave mutative fermentation earlier and oftener in the Difco than in the Oxoid medium. Strains of biotype 2*d*T−, for example, gave positive results due to the emergence of *d*-tartrate-positive mutants, confirmed by

plating and testing of colonies, in 3–9 out of 20 replicate 24 hr. turbidity tests in Bacto-Peptone but in none of 20 parallel tests in Oxoid peptone. The reason why the Difco medium favoured the emergence of mutants is unknown, though the amount of growth of the 2*d*T− strains was somewhat greater in the Difco than in the Oxoid medium.

The 24 hr. turbidity test in Oxoid peptone water classified our 1435 strains into 1045 that were *d*-tartrate-positive and 390 that were *d*-tartrate-negative. The latter group included 145, 174, 10, 15 and 1 strains, respectively, in the previously recognized *d*-tartrate-negative biotypes 6, 17, 18, 19Xd and 20, but also 15, 4, 1, 24 and 1 strains, respectively, in biotypes 2*d*T−, 10*d*T−, 10*d*T−*l*T−, 16*d*T− and 16*d*T−*l*T−, not recognized by previous workers.

Absence of utilization of d-*tartrate under fully aerobic conditions*

The conditions of culture in the static liquid media used for the lead acetate and turbidity tests were poorly aerobic, and we found that it was only under such conditions that *d*-tartrate could be utilized. Fourteen strains of the *d*-tartrate-positive biotypes 1, 2, 10, 12, 15 and 16 were cultured for 24 hr. in Oxoid peptone media with and without 1% *d*-tartrate (1) under poorly aerobic conditions in static tubes and (2) under highly aerobic conditions in continuously shaken flasks. In the static cultures the presence of *d*-tartrate stimulated the growth of every strain by more than 400%, whilst in the shaken cultures the difference in turbidity between the tartrate-containing and tartrate-free media was in all cases less than 10%.

The inability of *S. typhimurium* to utilize *d*-tartrate under fully aerobic conditions was also demonstrated in tests for growth on minimal agar medium containing *d*-tartrate as the sole source of carbon and energy. None of the 1045 positive and 390 negative strains gave visible growth within 7 days on this medium, though most of these strains grew well in 2 days on minimal media containing glucose or sodium citrate.

Fermentation of l-*tartrate*

The lead acetate and turbidity tests done at 24 hr. gave concordant results for the fermentation of *l*-tartrate with most strains and the turbidity test gave consistent results in replicate tests of representative strains. In the turbidity test, chosen as standard, the degree of stimulation of growth of the *l*-tartrate-positive strains in the medium containing *l*-tartrate was in all cases so great that the results could be read reliably with the naked eye; the turbidity measurements were mostly between 500 and 800% greater than those in the tartrate-free medium.

Only 34 of the 1435 strains were found to be *l*-tartrate-negative; 10 belonged to biotype 5, the only *l*-tartrate-negative type in the Kristensen scheme, and 24 belonged to the hitherto unrecognized biotypes 1*l*T− (3 strains), 10*l*T− (11 strains), 17*l*T− (2 strains) and 1*l*T−*m*T−, 3*l*T−, 6*l*T−, 8*l*T−, 9*l*T−, 10*d*T−*l*T−, 11*l*T− and 16*d*T−*l*T− (1 strain each). Several of these negative strains gave 'late' positive results in lead acetate and turbidity tests incubated for 3–4 days due to the emergence of *l*-tartrate-fermenting mutants; the remainder gave only negative results up to 14 days.

Table 3. *Results of representative lead acetate and turbidity tests for fermentation of* m-*tartrate by strains of* Salmonella typhimurium *of* m-*tartrate-positive (above) and* m-*tartrate-negative (below) biotypes*

		Results at 2 and 14 days in lead acetate test in bottles with caps				Amount of growth (turbidity) after 24 hr. in Oxoid peptone water with (+*m*T)* and without (−*m*T) 1 % *m*-tartrate when culture was			
		Loose		Tight		Static		Shaken	
Strain	Biotype	2 days	14 days	2 days	14 days	−*m*T	+*m*T	−*m*T	+*m*T
S712	1	+	+	+	+	0·21	0·38 (+81)*	0·90	1·95 (+117)*
S1446	2	−	+	+	+	0·16	0·30 (+88)	1·13	2·10 (+86)
S1535	6	+	+	+	+	0·18	0·46 (+155)	1·13	1·68 (+49)
S1444	8	+	+	+	+	0·22	0·43 (+95)	1·13	1·86 (+65)
S1542	9	−	+	−	+	0·17	0·30 (+77)	1·25	1·73 (+38)
S206	10	+	+	+	+	0·19	0·52 (+173)	0·85	1·74 (+105)
S576	15	+	+	+	+	0·13	0·33 (+153)	0·63	1·38 (+118)
S6624	17	+	+	+	+	0·16	0·30 (+87)	1·14	2·15 (+88)
S706	18	+	+	+	+	0·13	0·23 (+77)	1·03	2·15 (+108)
S1180	19Xd	+	+	+	+	0·15	0·34 (+127)	1·08	2·00 (+85)
S527	1*m*T−	−	−	−	+	0·20	0·10 (−50)	1·15	0·71 (−38)
S2317	1*m*T−	−	−	−	−	0·21	0·10 (−52)	1·15	0·71 (−38)
S1573	4	−	−	−	+	0·19	0·13 (−32)	1·08	0·82 (−24)
S2591	4	−	−	−	−	0·13	0·09 (−31)	1·10	0·46 (−58)
S747	12	−	−	−	+	0·24	0·10 (−58)	1·02	0·62 (−39)
S826	12	−	+	−	+	0·17	0·10 (−41)	1·16	0·94 (−19)
S1412	12	−	−	−	−	0·16	0·09 (−44)	1·04	0·76 (−27)
S1423	12	−	−	−	+	0·13	0·10 (−23)	1·11	0·62 (−44)
S2306	16	−	−	−	−	0·12	0·10 (−17)	1·17	0·70 (−40)
S2565	16	−	−	−	−	0·15	0·11 (−27)	1·12	0·76 (−32)

* Figure in parentheses shows percentage difference in amount of growth between *m*-tartrate-containing and *m*-tartrate-free medium, i.e. the degree of stimulation (+) or inhibition (−) of growth by *m*-tartrate.

Most of the strains that fermented *l*-tartrate in peptone water were also able to utilize this tartrate under the more highly aerobic conditions on an agar plate; thus they gave good growth within 7 days on minimal medium containing *l*-tartrate as sole source of carbon and energy.

Lead acetate test for fermentation of m-*tartrate*

The lead acetate test gave very irregular results for the fermentation of *m*-tartrate whatever period of incubation was used and regardless of whether the bottles were loosely or tightly capped. When replicate tests in loosely capped bottles were put up with any *m*-tartrate-positive strain, most of them became positive after 2, 3 or 4 days' incubation, but a few remained negative until 5–7 days. Conversely, when such tests were done with an *m*-tartrate-negative strain, most of them were negative at 4 days, but a few were positive at 3–4 days and an increasing proportion, up to 50 %, became positive after longer incubation up to 14 days. Thus, although readings made at 4 days generally distinguished the

positive from the negative strains, reliance could not be placed on a single 4-day test of a given strain (Table 3). It was necessary to inoculate a series of, say, five replicate tests of the strain and judge its status from the results in the majority of the tests.

In tests made after 2, 3 or 4 days in tightly capped bottles both the *m*-tartrate-positive and *m*-tartrate-negative strains gave a greater number of positive results than in parallel tests in loosely capped bottles, but all strains gave aberrant results in a proportion of replicate tests.

The irregular production of positive results between 2 and 14 days by most strains of all *m*-tartrate-negative biotypes was due to the ability of these strains to give rise to *m*-tartrate-positive mutants. Some of the 'late' positive tests were plated on nutrient agar, subcultures were made from separate colonies and a proportion of the subcultures from each test were shown to have *m*-tartrate-positive characters in the lead acetate, turbidity and plate-inhibition tests.

Turbidity test for fermentation of m-*tartrate*

Selected strains from different biotypes were tested in Oxoid peptone water or Difco Bacto-Peptone water with and without the addition of 1% *m*-tartrate in tubes incubated under aerobic static conditions for 24 or 48 hr. The results did not differ significantly with the type of peptone or the period of incubation, and the method using Oxoid peptone incubated for 24 hr., which was optimal for tests with *d*- and *l*-tartrates, was adopted also as standard for *m*-tartrate. Representative results are shown in Table 3.

The 24 hr. turbidity test drew a clear distinction between *m*-tartrate-positive and *m*-tartrate-negative strains and, with rare exceptions, results were consistent in series of replicate tests on a given strain. The growth of *m*-tartrate-positive strains was stimulated by the presence of *m*-tartrate by amounts ranging in different tests from 35 to 220%. That of the *m*-tartrate-negative strains was never stimulated, but instead was inhibited by the presence of *m*-tartrate and depressed by amounts ranging from 10 to 60% below those in the control, tartrate-free cultures. The differences in amount of growth in these tests were commonly too small for reliable reading with the naked eye and it was necessary to read them with the absorptiometer. In most of the few cases in which a single test of a strain showed neither stimulation greater than +35% nor inhibition greater than −10%, repeated tests showed clearly that the strain was either *m*-tartrate-positive or *m*-tartrate-negative. There were, however, three strains in our series of 1435 that were truly indifferent to *m*-tartrate, neither utilizing for increased growth nor being inhibited by it; the plate tests, described below, showed that these strains were *m*-tartrate-positive.

When turbidity tests were done in peptone water aerated by continuous shaking during incubation for 24 hr., the amounts of growth were five- to tenfold greater than in the static cultures, but positive and negative strains still showed comparable degrees of stimulation and inhibition of growth to those observed under static conditions (Table 3). Highly aerobic conditions, therefore, did not prevent the utilization of *m*-tartrate as they did that of *d*-tartrate.

Minimal medium and plate-inhibition tests with m-*tartrate*

The 1435 strains were tested in parallel cultures on three plates of ammonium-salts agar medium in which the carbon sources were: (1) citrate alone, (2) citrate and *m*-tartrate, and (3) *m*-tartrate alone. Most strains grew well on the medium with only citrate and formed heavy disks of growth within 48 hr. The few that did not were retested on, and gave good growth on, medium containing glycerol instead of citrate.

All the strains found to be *m*-tartrate-positive in the turbidity tests gave heavy growth within 7 days on the minimal medium containing *m*-tartrate as sole carbon source. They also gave good growth within 48 hr. on the medium containing both citrate (or glycerol) and *m*-tartrate. All strains found negative in the turbidity tests failed to give any growth within 7 days on the minimal medium containing *m*-tartrate or any growth within 48 hr. on the medium containing citrate (or glycerol) and *m*-tartrate (Pl. 1, figs. 1 and 2). The *m*-tartrate-negative strains, therefore, could be recognized either by their failure to utilize *m*-tartrate for growth on citrate-free medium or by their susceptibility to inhibition of growth by *m*-tartrate on citrate-containing medium. Since the former observation could be made only after 7 days, but the latter after 48 hr., the test for inhibition was preferred to that for growth. Since, moreover, the plate-inhibition test was as reliable as, and easier to read than, the turbidity test, it was chosen as the standard method for *m*-tartrate. It was applied to auxotrophic strains by using media supplemented with the required growth factor.

The 1435 strains of *S. typhimurium* were classified by the turbidity and plate-inhibition tests into 1181 that were *m*-tartrate-positive and 254 that were *m*-tartrate-negative. Of the latter, 5, 158 and 11, respectively, belonged to the recognized *m*-tartrate-negative types 4, 12 and 16, but 55, 1 and 24 belonged to the previously unrecognized types 1*m*T−, 9*m*T− and 16*d*T−.

Lead acetate test for fermentation of citrate

In the Kristensen scheme only biotypes 14, 17, 18 and 20 fail to ferment citrate. Our series of strains included none of type 14 and only one of type 20, and these two types were also rare in previous series. The main use of tests with citrate is therefore in confirming the distinction made by tests with *d*-tartrate between the inositol-negative rhamnose-negative biotypes that ferment citrate (types 15 and 16) and those that do not (types 17 and 18). We were unable, however, to find any conditions under which tests with citrate would give reliable results. In tests in loosely capped bottles, strains of biotypes 17 and 18 regularly failed to ferment citrate within 14 days, but strains of biotypes 15 and 16, identified by their reactions with *d*- and *m*- tartrates, sometimes fermented and sometimes failed to ferment citrate in this period.

Turbidity test for fermentation of citrate

Forty strains of the citrate-fermenting biotypes 1, 2, 10, 12, 15 and 16 and the non-fermenting biotypes 17 and 18 were grown for 24 hr. in Oxoid peptone media with and without the addition of 0·25 % sodium citrate. The difference of amount

of growth in the citrate-containing compared with the citrate-free medium ranged from −25% to +486% (mean +39%) with strains of the citrate-fermenting biotypes and from −29% to +10% (mean −8·3%) with those of the non-fermenting types. The test, therefore, did not reliably distinguish the fermenting from the non-fermenting types and no better differentiation was obtained by the substitution of Difco Bacto-Peptone for Oxoid peptone, the substitution of liquid ammonium-salts medium for peptone water, an extension of the incubation period to 48 hr., an increase in the concentration of sodium citrate to 1% or the substitution of potassium citrate for the sodium salt.

Growth on citrate minimal agar medium

The ability of a strain to grow on minimal agar medium with citrate as sole carbon source was unrelated to its ability to ferment citrate under the poorly aerobic conditions of static liquid cultures in the lead acetate and turbidity tests. The majority of strains of all biotypes, including the citrate-non-fermenting types 17 and 18, gave good growth within 2 days on citrate minimal medium or, in the case of auxotrophic strains, on this medium supplemented with the required growth factor. Among strains, e.g. in biotype 10, that fermented citrate readily in lead acetate tests, there were a few that could not utilize citrate for growth on minimal agar medium.

DISCUSSION

Our results show that the generally used methods of testing *S. typhimurium* for the fermentation of organic acids are unreliable. These methods gave different results in replicate tests of the same strain, so that an incorrect result must often be obtained in any single test. The method of observing an acid colour change was so unreliable with all four substrates that we abandoned its use entirely. The lead acetate test was highly unreliable for determining the fermentation of citrate and we were unable to develop any other test that was reliable with this substrate. Since reactions with citrate do not distinguish any biotypes in the Kristensen scheme that are not also distinguished by reactions with other substrates, the citrate-fermentation test should be omitted from the biotyping procedure.

The lead acetate test, which is done with cultures grown in Difco Bacto-Peptone water containing 0·5 or 1% of organic acid, was found to be unreliable if the culture was incubated for more than 24 hr. in tests with *d*-tartrate and *l*-tartrate and for more than 4 days in tests with *m*-tartrate. Genotypically fermenting ('positive') strains were rarely misidentified, but genotypically non-fermenting ('negative') strains were commonly read as positive in the prolonged tests due to the emergence of mutants which produced 'late' fermentation. Our observations indicate that most if not all of the strains genotypically non-fermenting with *d*- or *m*-tartrate and some of those non-fermenting with *l*-tartrate are capable of giving rise to fermenting mutants.

Mutants emerged earlier and more frequently in tests of negative strains in which access of air to the surface of the medium was restricted by tight capping of a nearly filled bottle. To identify the negative strains, therefore,

it was necessary to grow the cultures in loosely capped bottles and to read the results after only 24 hr. for *d*- and *l*-tartrates and 4 days for *m*-tartrate. Even so, a few discrepant results were obtained in sets of replicate tests. Thus a few strains genotypically negative for *d*- or *l*-tartrate (e.g. in biotypes 2*d*T− and 5) sometimes gave mutative fermentation within 24 hr., and many *m*-tartrate-positive and *m*-tartrate-negative strains were mistyped in a proportion of tests incubated for 4 days.

The evaluation of other methods established the 24 hr. turbidity test as being very reliable. It gave consistent results in replicate tests of any strain with any of the three tartaric acid isomers. The strain under test was grown for 24 hr. in two loosely stoppered tubes of Oxoid peptone water, one with and the other without the tartrate, and it was judged to be a fermenter if the amount of growth in the tartrate tube was significantly greater than that in the control tube. These tests did not give false-positive results due to the emergence of mutants even when applied to the exceptional strains, such as those of biotypes 2*d*T− and 5, which gave mutative fermentation within 24 hr. in the lead acetate test. The absence of mutative fermentation within 24 hr. in the turbidity test appeared to be due to the use of Oxoid peptone water in this test in the place of Difco Bacto-Peptone water used in the lead acetate test. Bacto-Peptone favoured the emergence of tartrate-fermenting mutants and when turbidity tests were done with Bacto-peptone water, strains of biotypes 2*d*T− and 5 gave some false-positive results due to mutative fermentation within 24 hr. It was not found practicable to avoid early mutative fermentation in the lead acetate test by using Oxoid peptone for that test, because Oxoid peptone differed from the Bacto-peptone in giving a considerable precipitate with lead acetate even when not containing added tartrate.

The turbidity readings indicative of fermentation differed between the *d*- and *l*-tartrates and *m*-tartrate. The degree of stimulation of the growth of positive strains by *d*- and *l*-tartrates over the amount of growth in tartrate-free medium was always more than 100% and generally about 500%; the growth of negative strains was not stimulated by these tartrates by more than 50% and was not inhibited by them. *m*-Tartrate, on the other hand, stimulated positive strains generally only by between 35 and 100%, and it inhibited *m*-tartrate-negative strains so that they gave 10–60% less growth than in tartrate-free medium. A strain was therefore judged to ferment *d*- or *l*-tartrate if its growth was stimulated at least 100% by the tartrate and to ferment *m*-tartrate if its growth was stimulated at least 35%.

The ability of strains to ferment *l*- or *m*-tartrate in static peptone water was associated with an ability to utilize that tartrate for growth within 7 days on ammonium-salts minimal agar medium. There was no such association in the case of *d*-tartrate and citrate. None of the many strains that fermented *d*-tartrate in the poorly aerobic, static peptone water was able to utilize it for growth under the highly aerobic conditions on plates of minimal agar medium, whilst many of the strains that failed to ferment citrate in static peptone water utilized it for growth on minimal medium. Growth tests on minimal medium were therefore valueless for demonstrating the ability to ferment these two substrates.

Lewis & Stocker's (1971) plate-inhibition test for fermenting ability towards *m*-tartrate depends on the peculiar effect of *m*-tartrate in inhibiting the growth of *m*-tartrate-negative strains of *S. typhimurium* on minimal agar containing citrate or glycerol. We found that this test, read at 48 hr., was as reliable as the 24 hr. turbidity test and, since its results were clearer and more easily read than those of the turbidity test, recommend it as the method of choice for *m*-tartrate.

Application of the turbidity and plate-inhibition tests in biotyping 1435 strains previously typed by the lead acetate method of Brown *et al.* (1924) as used by Kallings & Laurell (1957), showed that many strains had been mistyped by the latter methods. Most of the mistyped strains belonged to types that were genotypically negative for one of the tartrates but gave false-positive, mutative reactions in the lead acetate test. The relative scarcity of strains allotted to *d*-tartrate-negative and *m*-tartrate-negative biotypes in some previously published series may have been due to such mistyping. Use of the turbidity and plate-inhibition tests, moreover, distinguished 16 new biotypes, additional to the 21 in the extended Kristensen scheme (Table 1) and type 19Xd of Morgenroth & Duguid (1968). The new types, which differed from existing ones by giving a negative reaction with one or more of the tartrates, were: 1*l*T−, 1*l*T−*m*T−, 1*m*T−, 2*d*T−, 3*l*T−, 6*l*T−, 8*l*T−, 9*l*T−, 9*m*T−, 10*d*T−, 10*l*T−, 10*d*T−*l*T−, 11*l*T−, 16*d*T−, 16*d*T−*l*T−, and 17*l*T−.

Thus the new tests are not only much more reliable than those previously used, but also subdivide the serotype *S. typhimurium* into a larger number of biotypes.

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EXPLANATION OF PLATE

Fig. 1. Growth of twelve strains of *Salmonella typhimurium* from spot inoculations on citrate minimal agar medium. Six strains (above) from biotype 10 and six (last in second row and five in bottom two rows) from biotype 12 have given good growth in 2 days.

Fig. 2. Growth of same twelve strains as in Fig. 1 from spot inoculations in corresponding positions on minimal agar containing *m*-tartrate in addition to citrate. The six *m*-tartrate-fermenting strains of biotype 10 (above) have given good growth in 2 days, whilst the six *m*-tartrate-non-fermenting strains of biotype 12 (below) have been inhibited and show no growth.

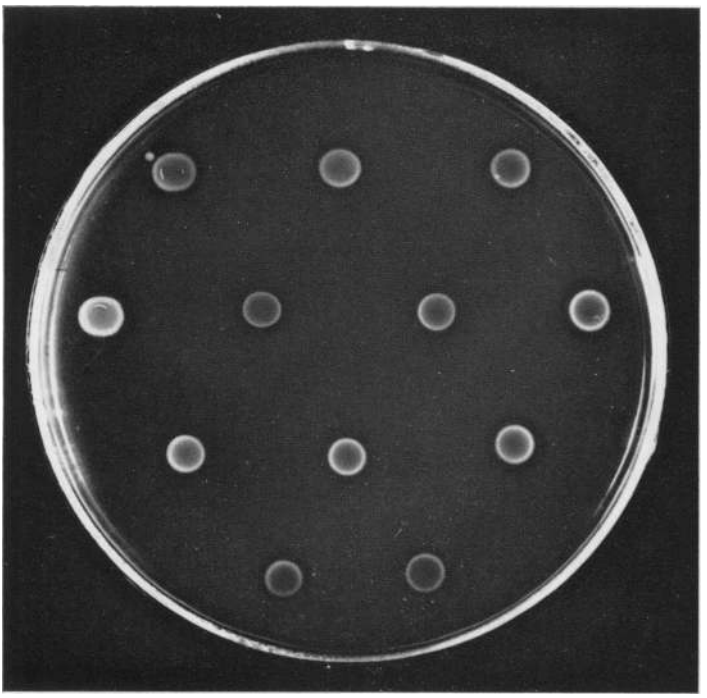

Fig. 1

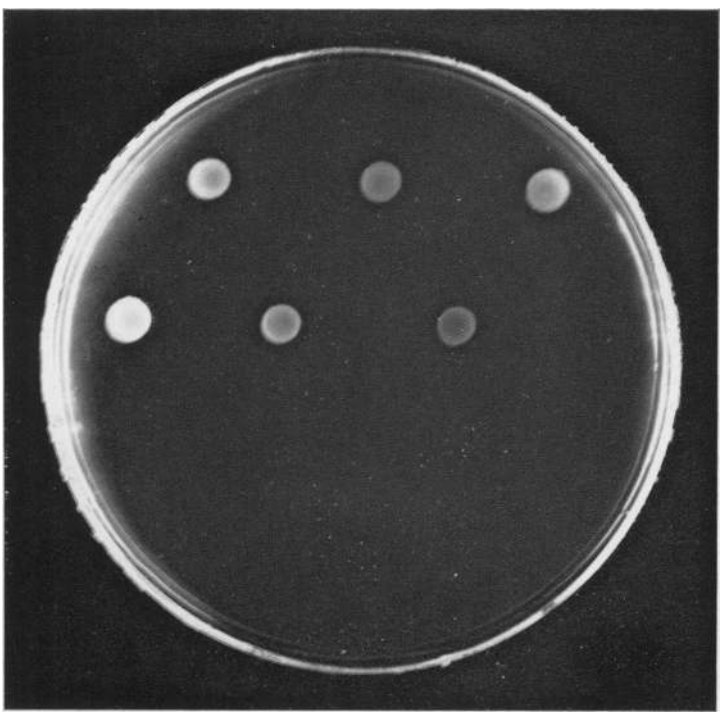

Fig. 2