SOME FERMENTATIVE VARIETIES OF *BACILLUS PARATYPHOSUS* B

By S. H. WARREN and J. L. G. IREDALE

From the Public Health Laboratory, The College of Medicine, Newcastle-on-Tyne

I. Anaerogenic strains

One of the oldest and most definite divisions of the Salmonella group is that into aerogenic and anaerogenic types as shown on growth in media containing "sugar." The existence of anaerogenic strains of normally aerogenic types has nevertheless been long recognised; Ledingham and Penfold (1915), for example, remark that "many paratyphoid strains give little or no gas in the sugars they normally ferment," and Bruce White (1929) states that "anaerogenic strains of all the commonly occurring aerogenic types have been reported with varying frequency." Less attention, however, has been paid to these exceptional strains in this country than on the continent (cf. Oette, 1913 and Hermann, 1929), and a recent series of cases of paratyphoid fever in the Newcastle area in 1931–2 from which anaerogenic strains of *B. paratyphosus* B were regularly isolated is of interest.

The nine patients were all children and all fell ill between November 1931 and February 1932, though no epidemiological connection between them could be established. Each of eight in fact, came from a different ward of the city of Newcastle and one from a neighbouring borough, though the last had been in the city prior to the onset of the illness. Clinically all were typical cases of paratyphoid fever, but in some the onset of the disease was obscured by pre-existing infections (1 scarlatina, 3 dysentery) and in these the laboratory examination was alone responsible for detecting the condition. In eight of the nine children a Widal test was done during the illness, and agglutination of *B. paratyphosus* B (Oxford Standard Suspension) was found to titres varying in the different cases from 1 in 250 to 1 in 10,000. In one child, anaerogenic, but serologically typical *B. paratyphosus* B was found in the blood and in the faeces, but a typical aerogenic strain was also isolated once from the faeces. In two other children anaerogenic strains were found on two or more occasions in the faeces accompanied on one occasion by an aerogenic strain. The other six children gave only anaerogenic strains, isolated from the faeces on from one to five occasions; in five of these children these strains were found 6 weeks to 3 months after the onset of the illness; the seventh child died in the third week.

As the anaerogenic type of *B. paratyphosus* B was new in our experience, we decided to examine these strains to see if they differed in any other cultural

or fermentative reaction from the common aerogenic type. For this purpose a set of thirty strains was studied; twenty-five were anaerogenic, isolated on different occasions from the cases noted above; three were aerogenic strains also isolated from three of the same cases; one was an anaerogenic strain isolated by Dr W. M. Scott from the sewage of Consett (Co. Durham) and one was the type (aerogenic) strain from the National Collection (No. 15 Lister Institute, Schottmuller-Bainbridge original 1920).

Cultural characters (30 *strains*)

(1) Motility; in young broth cultures all were actively motile.

(2) Gelatine liquefaction; all were negative after several weeks' incubation at 20° C.

(3) "Slime-wall" formation; on ordinary agar all strains produced colonies with the characteristic ring of mucoid material when incubated for 24 hours at 37° C. followed by 24–48 hours at 20° C.

(4) "Rough and smooth" colonies; all strains, after 3–7 months of sub-culture at irregular intervals, gave plates showing both "smooth" and "rough" or "semi-rough" colonies, except the type culture of the National Collection all the colonies of which were "rough," the strain being an old one long kept in the laboratory.

(5) Secondary papillae on 1 per cent. raffinose agar; six strains were tested and all produced these, the flat rough colonies in particular.

(6) Fermentations; all the strains attacked the following carbohydrates, dextrose, maltose, mannitol, dulcitol, arabinose, trehalose, xylose, rhamnose and inositol; all those which produced acid without gas in dextrose, *i.e.* the anaerogenics, produced acid without gas in the other "sugars" also: all the strains failed to attack lactose, sucrose and salicin; a slight acid reaction appeared in 24 hours with raffinose but disappeared on subsequent incubation. In all cases the reactions reached the maximum in 24–48 hours, but the cultures were kept under observation for 10 days at 37° C.

(7) Litmus milk; all gave the characteristic acid to alkaline change after 24 hours' incubation, and all gave strongly alkaline milk by the end of 7 days.

(8) Production of hydrogen sulphide; stab cultures in agar containing 1 per cent. Witte's peptone and 0·05 per cent. lead acetate were alike with all thirty strains, a brown colour developing round the needle track after 7–8 hours' incubation and gradually becoming darker thereafter.

(9) Indole production; all gave negative results with Ehrlich's reagent in peptone water (Fairchild's) after incubation at 37° C. for 4 days.

(10) Fermentation of salts of organic acids; all the thirty strains fermented citrate and laevo-tartrate and failed to ferment dextro-tartrate. The tests were done in liquid medium (1 per cent. peptone (Difco) and 1 per cent. organic salt) and on the agar media of Jordon and Harmon (1928)—dextro-tartrate with phenol-red, and of Simmons (1926)—citrate with brom-thymol-blue.

No cultural difference was, therefore, observable among the thirty strains under test with the exception of anaerogenesis with the twenty-six strains mentioned above.

Serological characters

The preliminary agglutination tests were done with young broth cultures of all the strains and the Oxford Standard H-specific sera. All agglutinated to titre (1 in 250) after having been in subculture for some time, though the primary culture of nineteen out of twenty-nine of the strains (*i.e.* excluding the type culture) agglutinated only to half-titre (1 in 125). All gave partial agglutination with the Oxford "Salmonella Group" serum. Some were tested, in addition, with the Oxford "H-specific" *aertrycke*, *gaertner*, *newport* and paratyphoid C sera; no agglutination was found.

With one of the anaerogenic strains a rabbit serum was prepared giving an agglutination titre of 1 in 15,000. This serum agglutinated all the thirty strains to titres varying from 10,000 to 15,000. A serum prepared with the stock type strain (Lister 15) had a titre of 5000 and agglutinated all the other strains to titres varying from 2500 to 5000.

Absorption of agglutinin was complete when the anaerogenic serum above mentioned was saturated with culture of one of the three aerogenic strains, the absorbed serum producing no agglutination at 1 in 100 dilution with the homologous strain, with the Oxford Standard paratyphoid B suspension and with two aerogenic and two anaerogenic strains out of the set of thirty. The aerogenic serum absorbed with an anaerogenic strain was similarly robbed of its agglutinin for the same set of cultures.

With the tests above described there was no indication of antigenic difference between the anaerogenic and the aerogenic strains.

Permanence of anaerogenesis

We have made cultures in a variety of "sugar" containing media, especially in liquid medium containing dextrose or mannite with different specimens of peptone: Witte peptone, Fairchild's peptone, Parke Davis peptone, bacto-peptone (Difco) and the Lemco peptone (Witte) of Dudgeon and Pulvertaft (1927) have all given the same results with the set of thirty strains; the twenty-six anaerogenics produced acid in 24 hours but no gas during this and the further 14 days' incubation. The tests were repeated after the strains had been in culture for 6–9 months, and again all the anaerogenics produced acid in dextrose containing media. Seventeen strains, including the four aerogenics, were tested, finally, at the end of the period of maintenance in culture, on a variety of liquid media all containing 0·5 per cent. dextrose: these were (1) peptone water (Witte) with 0·5 per cent. bile salt, (2) casein digest broth, (3) Douglas broth, (4) peptone water (bacto-peptone), with sodium citrate 1 per cent., (5) peptone water with (human) serum 1 per cent., (6) McLeod broth (1931), the Durham's tube being emptied by vacuum pump, and (7) the same medium as (6) but sterilised by steaming once. No variation was

observed with any strain in any of these media; the four aerogenic strains produced gas, though the amounts varied a good deal in the different media, and the thirteen anaerogenic strains all failed to produce gas as before. Growth in peptone water with 1 per cent. citrate but without dextrose failed to differentiate between the aerogenics and anaerogenics, the gas production being absent or minimal and irregular in distribution; *à propos* of this Brown, Duncan and Henry (1924) state that "with citrate...gas formation was unusual and no bacterial strain was found to yield gas constantly."

The anaerogenic property of these strains was thus found to be constant during artificial culture. The mechanism of aerogenesis and the reasons for its absence are discussed in a further paper.

II. Fermentative types according to Kristensen and Bojlèn

It will be remembered that Kristensen and Bojlèn (1929) were able to divide a large collection of Danish strains of *B. paratyphosus* B into different types by a set of fermentation tests. They found that these "fermentative types" remained constant in subculture and had an epidemiological value, all the sufferers in a particular outbreak yielding one particular type only. We thought that it would be of interest to apply this set of fermentation tests to our collection of anaerogenic strains to see if they gave results indicating epidemiological unity or not. For comparison, we have applied the same Kristensen and Bojlèn tests to as many other strains of *B. paratyphosus* B as we could collect, ninety-six in all; these strains were of diverse geographical origin and included twenty-eight received from Scott and stated to be isolated each from a different outbreak (with a few exceptions), thirty-eight strains recently isolated in our laboratory each from a different patient in six different outbreaks; twenty-three other strains sent to us—from Copenhagen (two), Glasgow (five), Bradford (seven), and Wakefield (nine), besides five strains from the National Collection of type cultures. We have also added to the set of fermentation tests those using various organic salts as described by Brown, Duncan and Henry (1924) which were also done by Kristensen and Bojlèn.

Technique for Kristensen and Bojlèn typing

Three tests are used:
(1) Fermentation of rhamnose.
(2) The Bitter test.
(3) Fermentation of inositol.

As in all these tests the only point considered is the rate of acid production by the various strains, the production of gas is ignored; thus it was possible to apply this method to our series of anaerogenic strains.

For the sugar media they use small tubes of Jena glass of 9 mm. bore, which are filled to a height of 4–5 cm.

The medium (titred to *p*H 7·5–7·6) consists of:
1 per cent. peptone (Witte),
1 per cent. Liebig's meat extract,
0·5 per cent. sodium chloride.
Indicator brom-thymol-blue 12 c.c. per litre (1 g. B.T.B. to 500 c.c. *N*/200 NaOH).

Sugar 0·5 per cent. (rhamnose or inositol), with a minimum amount of sterilisation, then testing for sterility by incubation for 24 hours at 37° C.

For the Bitter test (Bitter, Weigmann and Habs, 1926) the following medium is used:

0·5 g. disodium phosphate,	5·0 g. sodium chloride,
1·0 g. ammonium sulphate,	0·05 g. peptone (Witte),
2·0 g. sodium citrate,	1000 c.c. distilled water.

i.e. Seitz's whey without grape or milk sugar or indicator. To this 0·5 per cent. rhamnose is added; the *p*H is about 7·0. After the tubes have been inoculated and incubated for 15 hours at 37° C., two drops of methyl-red solution (0·5 per cent. in 96 per cent. alcohol) are added and the result read off.

We carried out the technique of the typing as follows:

We collect a series of cultures of *B. paratyphosus* B to be typed, usually between thirty and fifty. At about 4 o'clock in the afternoon from each agar slope (fairly recent culture) a broth tube is inoculated (using the same batch of broth and tubed in about the same amount) and these are incubated at 37° C.; at 10 o'clock next morning, using a 5 mm. platinum loop, a rhamnose tube is inoculated from each broth. The sugar tubes are put into the 37° C. incubator and the broths are kept at room temperature. During the day (about 4 p.m.), it is convenient to inoculate the organic salt tubes for the Brown, Duncan and Henry test, using a 3 mm. loop for inoculating from the broths. At 6 p.m. the same day inoculate a Bitter tube from each broth tube, using again a 3 mm. loop and at the same time inoculate an inositol tube in the same way.

These broth tubes are then formolised and later diluted with saline and were used for the agglutination reactions.

At 10 o'clock the same evening (that is after 12 hours' incubation) the rhamnose tubes are read off. All tubes which are yellow (acid) are positive and called R_1 or R_2, and tubes which are still green are negatives or R_3. These tubes are incubated further and all ultimately become yellow. It is only necessary to inoculate the tubes which are yellow at 12 hours' incubation for a Bitter test, but to fit in with the time we find it more convenient to inoculate all the cultures to the Bitter tubes. Next morning at 9 o'clock (after 15 hours' incubation) methyl red is added to each Bitter tube and the colour change noted.

Bitter positives, or R_1 tubes, give a colour range from orange red to purple.

Bitter negatives, or R_2 tubes, give a colour range from yellow to orange.

At the end of 18–24 hours' incubation the inositol tubes are read: the tubes which are yellow (acid) are positives or I_1, while the tubes which are still green are negatives or I_2. These tubes are also incubated further, and finally all become yellow.

By these tests Kristensen and Bojlèn were able to divide paratyphoid B strains into six groups:

R_1I_1: rhamnose positive, Bitter positive, inositol positive,
R_1I_2: rhamnose positive, Bitter positive, inositol negative,
R_2I_1: rhamnose positive, Bitter negative, inositol positive,
R_2I_2: rhamnose positive, Bitter negative, inositol negative,
R_3I_1: rhamnose negative, — inositol positive,
R_3I_2: rhamnose negative, — inositol negative,

and among the cultures which they examined, they encountered all these except R_1I_2.

Organic salt fermentation tests

Kristensen and Bojlèn also used the organic salt fermentation test of Brown, Duncan and Henry, and they claim that brom-thymol-blue is suitable for indicating the acid change which occurs, and that it is not necessary to add the lead acetate solution; but if desired the lead acetate can be added in addition and then gives the usual reading.

The composition of the medium for these tests was as originally described and the first three salts were mostly used, viz. citrate, dextro-tartrate and laevo-tartrate, but also meso-tartrate and fumarate at times.

Distribution of fermentative types

(1) The anaerogenic strains. All the twenty-six strains were of the R_2I_1 type and were, therefore, epidemiologically a unit. Dr M. Kristensen informs us that his anaerogenic strains were also of this type, so it is possible, in the absence of further examples, to regard this merely as characteristic of the anaerogenic variety of *B. paratyphosus* B.

(2) The three aerogenic strains isolated from the same cases as the anaerogenics were of type R_2I_2. This observation might be taken to invalidate the epidemiological significance of the classification of Kristensen and Bojlèn, but it is just possible that the presence of these three strains may be explained by casual infections either of the patients or of utensils occurring in the wards and superimposed on the original "anaerogenic" infection. Actually the three specimens of faeces from which these were isolated were obtained on the same day. We have no proof, however, that any R_2I_2 case was in the ward at the same time.

(3) Among the twenty-eight aerogenic strains received from Scott the following distribution was found:

R_1I_2	R_2I_1	R_2I_2	R_3I_1
1	5	10	12

(4) All the thirty-eight aerogenic strains isolated in our laboratory during a period of four months were of the R_2I_1 type. As regards epidemiology there were seven strains from seven cases of paratyphoid fever in an outbreak in the Ryton district and two strains from Ryton sewage; an outbreak followed this in an institution to which one of the Ryton cases was sent and twenty strains were tested from fifteen persons so infected: another small outbreak at Sedgefield about the same time yielded seven strains from the seven cases examined: at Pegswood, a village 18 miles from Newcastle, two cases occurred at the same time and one secondary case in connection with one of these (three strains typed); a case from Stockton (57 miles away) gave five strains, isolated each from a different specimen; two cases in Darlington (36 miles away) gave two strains; and finally from the sewage in Consett where an outbreak of paratyphoid fever had occurred about a year before we isolated and typed a strain of *B. paratyphosus* B which we assume represents that epidemic.

The results of "fermentative typing," though satisfactory in the sense that they were sharp and easy to read, were disappointing from the epidemiological side. We expected to find the two outbreaks associated with Ryton to give, as they did, the same fermentative type, but the other outbreaks are unlikely to have had any epidemiological connection between them, yet all gave the same type. This type evidently predominated in the Northumberland-Durham area at that period but we can say no more.

(5) In order, therefore, to get some more information about the distribution of types in this country, we obtained freshly isolated cultures of *B. paratyphosus* B from various places which we typed with the results given in this table:

	R_1I_1	R_2I_1	R_2I_2	R_3I_1
Bradford	—	3	—	4
Glasgow	—	2	1	2
Wakefield	—	8	—	1
Copenhagen	—	1	—	1
National Collection of type cultures	1	2	1	1
Newcastle (4 new strains)	—	2	—	2

All the strains have been tested serologically and all agglutinated specifically with paratyphoid B serum.

In all the batches of cultures put up for typing we have inserted in the series varying numbers and strains of other Salmonella group organisms to act as controls against the typing, the agglutination reactions and the organic salt fermentation tests.

As regards fermentation of organic salts, all our strains behaved alike, fermentating citrate and laevo-tartrate but not attacking dextro-tartrate. With meso-tartrate they gave negatives with a few doubtful exceptions probably due to error in technique; they fermented fumarate. We preferred to the brom-thymol-blue used by Kristensen and Bojlèn the original indicator of Brown, Duncan and Henry, namely lead acetate. We have no doubt that this behaviour towards these organic salts is characteristic of *B. paratyphosus* B, though we have found one strain of the *gaertner* group which gives the same reactions.

Persistence of the fermentation types

We have examined in all 193 strains of *B. paratyphosus* B and have re-examined fifty-eight after varying periods. In only one instance have we observed a change in the second examination; a culture from a urinary "carrier" was recorded as R_2I_2 on the first examination, gave $?R_1I_2$ on the second and later was R_1I_2.

Occurrence of types

Kristensen and Bojlèn give the following table as to the numbers of Type strains they found in their examinations:

	R_1I_1	R_2I_1	R_3I_1	R_2I_2	R_3I_2	Total
No. of patients	8	247	139	19	1	414
No. of specimens	23	447	350	52	1	873
No. of strains	27	485	449	57	1	1019

R_2I_1 and R_3I_1 being by far the commonest types, R_3I_2 was only found once and R_1I_2 not at all.

As the number of strains we examined was so small and came from a rather limited area, it is not of much use to give figures for comparison; but some facts seem to show a very definite similarity to Kristensen's findings. In this area R_2I_1 is by far the commonest type found at present. All the

anaerogenic strains were of that type, and in the small outbreaks in the adjacent districts, aerogenic R_2I_1 was again the type present.

In Dr Scott's series, which came from a wider distribution, together with some strains from the National Collection, we found a good variety of types, R_1I_1, R_2I_1, R_3I_1 and R_2I_2 being present. We have also classified two strains as R_1I_2; one was only examined once and the other three times. Possibly they are both R_2I_2, a type although not very common, yet one which we have found a number of times. R_1I_1 we found only once, being one of the strains from the National Collection; it was examined three times and each time gave the same reading, and each time in parallel with quite different types; it is of French origin. R_3I_2 was not found at all as a paratyphoid B, but Kristensen and Bojlèn only found it once, so that it may possibly be quite a rare type. With the strains from other places in this country, even though the numbers were very small, yet different types were found. From Bradford with only seven strains, two types were found, R_2I_1 and R_3I_1. From Glasgow with only five strains, three types were found, R_2I_1, R_3I_1 and R_2I_2, and here also cases giving the same type of organism are definitely associated. From Wakefield with nine strains, two types were present, R_2I_1 and R_3I_1, although the latter only once. So that it appears that the two commonest types in Denmark are also apparently the prevalent types here.

In the types isolated from sewage we have:

Sewage	Consett (Scott)	anaerogenic	R_2I_1
,,	Consett	aerogenic	R_3I_1
,,	Stoke (Scott)	,,	R_2I_1
,,	Isle of Wight (Scott)	,,	R_2I_2
,,	Stella gut, Ryton	,,	R_2I_1
,,	Clara Vale, Ryton	,,	R_2I_1

Thus sewage strains show practically all our different varieties. It is unfortunate that we have only single cultures from each of these samples of sewage, since this is probably the only kind of specimen in which there is a possibility of obtaining more than one fermentative variety in the same specimen without invalidating Kristensen's idea of specificity.

We have paid little attention to the fermentative varieties shown by the various Salmonella group organisms other than *B. paratyphosus* B. It would be necessary to examine good numbers of definite serological types before coming to any conclusions. All we have done was to show with the other Salmonellas that the fermentative reactions were the same on repeated tests of the same strain. This was so, except with one *gaertner* strain (No. 1658) which changed from R_3I_2 to R_2I_2. A number of *aertrycke* or ?*aertrycke* strains which we tested gave quite different fermentative typing, but actually many of these strains had not been definitely typed serologically.

The next point is as to whether these fermentative types represent definite races or not. As far as we are able to judge from this small collection, we

are inclined to believe that actually they do so, in that we have found, in the same patient, the same type occurring in urine and faeces, blood and faeces; in faeces on repeated examinations up to as many as six positives and also in definitely associated cases. In fact we have never obtained a different fermentative type from one patient or associated case from that originally isolated, with the single exception of the aerogenic strains found among the anaerogenic cases. Here we should have been inclined to imagine that the aerogenic strains were just evidence of the anaerogenic types reverting to their normal activity, if it were not for the fact that the anaerogenic strains are R_2I_1, and the aerogenic strains R_2I_2. This latter fact makes us rather think that these aerogenic strains are possibly accidental contaminations as we have already suggested.

III. SUMMARY

A series of nine cases of paratyphoid fever due to infection with anaerogenic strains of *B. paratyphosus* B are recorded.

Biological examination of these strains shows no difference from the ordinary type of *B. paratyphosus* B except the inability to produce gas from any "sugar."

Serologically these strains are identical with the aerogenic type of the organism.

These anaerogenic strains retain their inability to produce gas from "sugar" after subculture on artificial media for a considerable period, and cultivation on a number of different fluid media failed to restore the gas producing power.

In the organic salt fermentation test *B. paratyphosus* B gives a characteristic reaction which is also given by the anaerogenic strains.

The fermentative varieties of *B. paratyphosus* B defined by Kristensen and Bojlèn are definite and can be identified as a matter of practical routine, but the test is delicate and requires care in the preparation of the media and especially in the Bitter test.

The existence of these apparently stable fermentative varieties of *B. paratyphosus* B is likely to be of use in epidemiological investigations.

We wish to thank a number of people whose assistance has made it possible for us to carry out this work. Dr J. A. Charles, Medical Officer of Health, Newcastle-on-Tyne, for permission to publish these cases; Dr E. F. Dawson-Walker, Assistant M.O.H., for histories and specimens from the cases. Dr W. M. Scott for many cultures and other assistance.

Dr M. Kristensen, Copenhagen, Dr M. A. C. Buckell, Bradford, Dr P. L. Sutherland, Wakefield, for paratyphoid cultures and Dr J. W. H. Morrison, M.O.H., Blaydon-on-Tyne, for a number of specimens.

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(*MS. received for publication* 5. II. 1934.—Ed.)