ANAEROGENIC FERMENTATION WITH PARATYPHOID BACILLI

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In the preceding paper we have described the conditions under which we found a set of twenty-six anaerogenic strains of *B. paratyphosus* B and their cultural and serological behaviour in comparison with normal strains of the same species. We propose now to describe experiments which throw some light on the reason for their abnormal behaviour towards fermentable sugars.

Penfold (1911) has shown that by selective growth on agar containing sodium monochloracetate, it is possible to get strains of *B. coli communis* which no longer produce gas from dextrose though such strains retain the power of gas production from alcohols (mannitol, dulcitol, etc.) and from formates. Arkwright (1931) states that anaerogenic variants may be obtained from aerogenic bacteria by means of mass culture in broth containing phenol, brilliant green, etc.

Since the occurrence of formic acid as a product of the fermentation of "sugars" by organisms of the coli-typhoid group was first noted by Harden (1901), many workers have reported on the marked increase of "gas" production from the addition of formic acid to a fermentable "sugar." Buchanan and Fulmer (1930) record the observations of a number of workers confirming this point. Grey (1914, 1920, 1924), especially, considers that it is necessary to use a mixture of glucose and sodium formate before deciding that a recently isolated strain of a coli-typhoid organism is definitely anaerogenic. He also points out that calcium formate is the most favourable salt to use, and that the alkali produced from the breaking down of the formate neutralises the acid produced from the breaking down of the glucose; thus leading to a much greater utilisation of both substances than when either is used alone. Further the addition of chalk to the medium greatly accelerates the fermentation, even when formates are present.

Technique

In our experiments we have used both the usual $5 \times \frac{1}{2}$ in. test tubes with Durham tubes and the Smith fermentation tubes in which a much more complete collection of gas formed during growth is possible. Our Smith tubes were about 9 cm. long and held about 5 c.c. of medium. All the media used contained 1 per cent. of bacto-peptone (Difco) along with 1 per cent. of the various organic salts tested. The indicator used was brom-thymol-blue and the *p*H of the medium was adjusted, if necessary, to 7·4, giving with this

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indicator a blue or bluish green colour. The tubes of medium were usually sterilised by steaming for 20 min. on 3 successive days but, as will be seen later, the autoclave and filtration sterilisation gave some interesting variations. The tubes were inoculated in the usual way from agar slope cultures varying in age from 20 hours to 10 days, the older cultures having been kept at room temperature after the preliminary 37° C. incubation. All the tests on fermentation were done at 37° C.: the cultures were kept for 7 days with readings at 1–2 day intervals.

Experiments

In our first series of experiments we grew both aerogenic and anaerogenic strains of *B. paratyphosus* B in peptone water with 1 per cent. of the sodium salts of the following organic acids in Smith tubes. Lactic, acetic and succinic salts yielded no gas with either aerogenics or anaerogenics, the only change in the medium being a slight increase in its alkalinity. With formic and pyruvic salts both types produced slightly increased alkalinity but the aerogenic strains produced gas, while the anaerogenics produced none. The gas produced by the former from sodium formate was at its maximum in 3 days and measured 3·0 cm. of the Smith tube; this is about the same amount as with 1 per cent. dextrose. With sodium pyruvate (0·25 per cent. pyruvic acid added to the peptone water and neutralised with $N/1$ soda to pH 7·4) the aerogenics produced some alkalinity and slightly less gas (2·0 cm.) than with dextrose or formate.

We next tried a combination of dextrose (0·5 per cent.) with each of these organic salts (1 per cent.). With such combinations, the aerogenics produced gas, roughly corresponding to the amount from dextrose alone, with lactate, acetate and pyruvate; with succinate an increase in gas (5·0 cm.), amounting to almost double that with dextrose alone, was observed; the medium became acid at first, as did the other combinations, but, unlike them, became alkaline in about 6 days. The anaerogenic strains produced no gas from any of these combinations but a change in the reaction of the medium of the same order as the aerogenics. With sodium formate, however, the combination with dextrose produced quite definite differences. The aerogenic strains all produced temporary acidity and greatly increased gas formation, the maximum possible (9·0 cm.) being reached in 48 hours and further gas escaping from the open limb of the Smith tube; later the reaction became distinctly alkaline and there was a tendency for the volume of gas to diminish, probably by absorption of CO_2 by the alkaline fluid. With the anaerogenic strains in this combination of dextrose and sodium formate, we got irregular results; most strains produced gas (from 1 to 6 cm.), reaching the maximum in about 4 days, with one batch of medium, while with another batch, which showed no difference with the aerogenic strains, only one or two of the anaerogenics showed gas production (among about twelve strains tested). In addition not all of the anaerogenics produced a final alkaline reaction, some remaining acid.

Very similar results were obtained with a combination of mannitol and sodium formate, the anaerogenic strains producing irregular amounts of gas more slowly than the aerogenics and varying with different batches of medium.

The reason for this variability in different batches of medium was next investigated and it was found that in peptone water containing "sugar" and sodium formate, sterilised, without heat, by filtration through a Chamberland candle or through an E.K. Seitz disc, none of the anaerogenic strains produced gas. In the same peptone water containing formate, autoclaved at 120° C. for 20 min., a certain proportion of the anaerogenic strains produced gas in small amount. In the same peptone medium, steamed at 100° C. for 20 min. on three successive days, a larger proportion of the anaerogenics produced gas and that in larger amount. With calcium formate we found, in agreement with Grey (1924), that gas production was more abundant and regular with the anaerogenic strains than with sodium formate. Less difference, however, between filtered and heated medium was observed, the former permitting slight gas or a bubble of gas or none with the anaerogenic strains, while the heated medium yielded volumes of gas measuring 2–4 cm. of the Smith tube and only slightly less than the aerogenic strains.

It seems clear from these experiments that the reason for the failure of the anaerogenic strains to produce gas from "sugars" is that they have lost the power of reducing the formate produced from their first attack on the sugar. They suggest also that heating formates to 100° C. or over may induce some slight change in it, sufficient to enable an organism weak in reducing power to complete the process of reduction. In ordinary "sugar" fermentation, the formate produced by the attack of the bacterium on the "sugar" has, of course, not been heated beyond 37° C.

SELECTION OF AEROGENIC RACES FROM ANAEROGENIC STRAINS

Since the anaerogenic strains, as shown above, could produce gas from a mixture of dextrose and formate, it seemed likely that, by continued cultivation in such a mixture, they might develop the power of completing the fermentation of dextrose alone to the stage of gas formation. Accordingly, an anaerogenic strain was subcultured daily for 10 days in tubes of peptone water containing 0·25 per cent. of dextrose and 1 per cent. of sodium formate (under aerobic conditions). It was then plated out on MacConkey agar and produced two types of colony, "large flat granular" and "small smooth." On picking off these into dextrose peptone water the former produced gas on 24 hours' incubation, while the latter, the smooth, produced none, even on prolonged growth at 37° C.

The "large flat granular" colonies were not typically "rough," since they had sharply cut edges, not crenated, but they were of tough consistency and moved *en masse* on the surface of the agar when touched, though on agar slope subculture the growth was apt to be adherent to the medium. In broth

there was pellicle formation as well as general turbidity. The bacteria were actively motile and gave cultural reactions characteristic of *B. paratyphosus* B, producing acid and gas in mannitol, dulcitol, arabinose, xylose, rhamnose, maltose and inositol, but no change in lactose or sucrose. Broth cultures agglutinated well with specific paratyphoid B serum. On subculture thrice in succession in dextrose peptone water, gas production remained a constant character.

The smooth colonies produced acid in the same set of "sugars" but in every case without gas: they agglutinated well with specific paratyphoid B serum: on subculture thrice in succession in dextrose peptone water, gas production remained in abeyance.

When these two type colonies were subcultured in dextrose peptone water containing 1 per cent. formate, the "large flat granular" produced as much gas as an aerogenic strain put up for comparison, whereas the "smooth" colony gave very little gas and turned the medium alkaline very slowly.

With another anerogenic strain grown in dextrose formate peptone water but plated from a 7-day old culture in that medium, very few "large flat granular" colonies appeared; the great majority of the colonies were "smooth," though varying somewhat in size, and the eight colonies tested were all anaerogenic. But on two successive passages at 48 hours' interval in the same medium, followed by five successive 48 hour cultures in dextrose peptone water, a small bubble of gas was produced each time and, on plating out, all the colonies were of the "large flat granular" type; seven of these picked off were now aerogenic and one still anaerogenic. Three of these were tested for cultural and serological behaviour and were all typical *B. paratyphosus* B except that they produced some scum and deposit as well as normal general turbidity in broth.

Incubated anaerobically in the same dextrose formate peptone water, sterilised by filtration without heat, another anaerogenic strain gave acid without gas. When plated out, this tube produced large flat granular colonies (as well as smooth) which however, on subculture, were still anaerogenic. Similarly incubated, but in *autoclaved* medium (acid and gas produced), the same strain gave four aerogenic colonies out of six tested.

Experiments substituting mannitol for dextrose gave similar results; continued passage in the mixture produced, on plating, increasing numbers of aerogenic colonies though anaerogenic colonies never disappeared entirely. When subcultured in mannitol peptone water anaerogenic strains produced only anaerogenic colonies and aerogenic strains produced only aerogenic colonies. It should be noted that there is not an absolute correlation between the "flat granular" appearance of a colony and aerogenic capacity, as is shown in the experiments described above.

STABILITY OF GAS PRODUCING POWERS

All the anaerogenic strains kept for 18 months on ordinary nutrient agar, with occasional subculture, have retained their character of inability to produce gas on growth in dextrose containing medium. Numerous colonies (80 in all) were picked from plates made with such old cultures and all failed to produce gas in dextrose peptone water.

On the other hand, one of the "large flat granular" colonies which appeared as an aerogenic variant in one of the experiments described, became "smooth" and soft after several subcultures on dextrose formate agar plates and was then found to have become anaerogenic though otherwise typical *B. paratyphosus* B. This dextrose formate agar is quite good for eliciting slime wall formation and if plating is done after its appearance, the colonies after a few subcultures finally change from the "large flat granular" type to "smooth" and soft ones. But subculture on ordinary agar slopes of four of the aerogenic variants from four different anaerogenic strains failed, after five passages each, to produce any but aerogenic cultures.

SUMMARY

1. Anaerogenic races of *B. paratyphosus* B are stable in ordinary artificial culture.

2. Aerogenic variants of anaerogenic strains can be produced (though the anaerogenic strain persists) by growth in dextrose or mannitol with a relatively large amount of sodium formate. Such variants can then produce gas from all the other "sugars" fermentable by *B. paratyphosus* B.

3. Failure to produce gas from sugars by anaerogenic races is due to deficiency in the power of breaking down the formate produced in the first stage of fermentation.

4. A mixture of dextrose and formate in peptone water reveals the latent capacity for gas production in anaerogenic races of *B. paratyphosus* B. This confirms the observations of Grey (1914, 1920, 1924).

Since this work was done, we have seen the paper of Pot and Tasman (1932) in which they succeed in isolating aerogenic variants from anaerogenic cultures of *B. paratyphosus* B by continued cultivation in 2 per cent. calcium formate, in a somewhat similar manner as we have done with the mixture of dextrose and sodium formate.

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