HYDROGEN SULPHIDE PRODUCTION BY ANAEROBIC SPORE-BEARING BACTERIA

MORTON CHARLES KAHN

Department of Hygiene, Cornell University Medical College, New York City

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That commercial peptones vary in the amount of hydrogen sulphide which they will yield when incorporated into suitable culture media supporting growth of various species of bacteria capable of elaborating this substance, has been pointed out by Myers (1920), Thompson (1920–1921) and Tilley (1923a). This last named investigator also demonstrated that differences in hydrogen sulphide production may be shown for various strains of bacteria within the same species. In a later communication, Tilley (1923b) reported that while unoxidized sulphur in the form of cystin yielded an abundance of hydrogen sulphide, the unoxidized sulphur of commercial peptones may consist largely of some compound or compounds other than cystin, and not utilizable by bacteria for the production of H_2S.

To obviate this difficulty and to avoid the necessity of testing each lot of pepton individually when hydrogen sulphide tests are to be made, Tilley advocated the addition of known quantities of an unoxidized sulphur in the form of sodium thiosulphate to media containing the usual lead acetate indicator. In this way Tilley succeeded in producing a uniform black coloration of the media when the bacteria planted were able to elaborate hydrogen sulphide, regardless of the initial cystin content of the pepton used.

As no report has heretofore appeared in regard to the hydrogen sulphide producing properties of the anaerobic spore-bearing bacterial group, a study of this subject has been undertaken, and for this purpose I have used the sodium thiosulphate media as advocated by Tilley, but modified somewhat to suit the requirements

of this group of organisms. Instead of using 3 per cent of pepton, 1 per cent was employed and 0.5 per cent of agar instead of 3 per cent, as anaerobes find some difficulty in growing in a very stiff substance. Casein digest fluid (Kahn, 1922) was also added as an enriching agent as some of the anaerobes will not multiply actively in a plain pepton-meat infusion mixture. The formula, then, for the substance used to support growth and test for H_2S in these experiments was as follows: Beef heart infusion, 1000 cc., pepton (Difco) 10 grams, casein digest fluid 30 cc., agar 5 grams, sodium thiosulphate crystals, c.p. (Baker) 2.5 grams. The above ingredients were heated to 100°C. for thirty minutes to dissolve, the reaction adjusted to pH 7.2, filtered through cotton and flannel, tubed in 10 cc. amounts and sterilized in the autoclave at 15 pounds for twenty minutes.

Just prior to inoculating, the tubes were boiled for fifteen minutes to expel as much of the dissolved oxygen as possible, rapidly cooled to 46°C. and 0.1 cc. of a sterilized 10 per cent solution of lead acetate added to each tube. Inoculations were made from 0.5 per cent casein digest agar cultures with the aid of a Pasteur pipette, employing about 0.25 cc. of inoculum. In all experiments with anaerobic spore-bearing organisms definite results are more likely to be obtained within a minimum of time if relatively large amounts of culture are used for transplanting. Often a mere loopful of material when seeded from one tube to another will not be sufficient to bring about a desired growth or reaction until considerable time has elapsed. Anaerobiosis was induced in all cultures by applying a cap of sterile vaseline about $\frac{3}{4}$ inch in height. As has already been reported (Kahn, 1922) this method of inducing growth was found to be an effective and simple one, capable of satisfying the oxygen requirements of the most fastidious species. The tubes were incubated at 37°C. for twenty days and daily observations made during this period.

The following types of spore bearing anaerobes were employed in these experiments, the numbers in parenthesis indicating the number of strains tested: the *Vibrion septique* (3), *B. oedematiens* (*B. novyi*) (2), *B. welchii* (5), *B. fallax* (1), *B. tertius* (1), *B. chauvoei* (1), *B. sphenoides* (1), *B. putrificus* (3), *B. aerofoetidis* (2), *B.*

tetani (2), *B. bifermentans* (3), *B. sporogenes* (3), *B. centrosporogenes* (1), *B. botulinus* (2), *B. histolyticus* (4). These cultures were all offsprings of single bacterial cells isolated by the Barber technic and there would seem to be no question of their purity. Suitable tests for aerobic contamination were made by inoculations on casein digest agar slants. I was unable to detect any such contamination during the investigation.

The species of bacteria capable of elaborating hydrogen sulphide in this medium invariably produced an intense black coloration of the entire substance. This reaction was quite obvious with the positive strains after twenty-four hours and very marked after two days incubation. The organisms seemingly incapable of elaborating H_2S did not produce any alteration in the color of the medium which exhibited a turbid grey appearance due to the addition of the lead acetate.

On the basis of the results shown in table 1, it seems feasible to divide the anaerobic spore-bearers into two groups, i.e., producers and non-producers of hydrogen sulphide. Those anaerobic spore bearers capable of elaborating H_2S were found to be *B. aerofoetidis, B. putrificus, B. tetani, B. bifermentans, B. sporogenes, B. centrosporogenes, B. botulinus, B. sphenoides, B. welchii* and *B. tertius,* while the following were found to be incapable of producing this substance: *B. fallax, B. oedematiens, B. chauvoei* and the *Vibrion septique. B. histolyticus* was the only organism encountered giving what might be termed a borderline reaction. Although 3 strains of this species were investigated and the tests duplicated on several occasions, the reaction about to be described was invariably obtained. After four days incubation the tubes seeded with this organism still resembled the controls in color, leading one to believe that although a strongly proteolytic species, *B. histolyticus* was incapable of elaborating H_2S, whereas with the frankly positive group the jet black coloration was produced after forty-eight hours. On the fifth day, however, the cultures of *B. histolyticus* were found to have taken on a distinct tan hue and after ten days had elapsed this color deepened somewhat, but was hardly comparable to the black coloration produced by the definitely positive types. On the basis of this test it seems

TABLE 1

Hydrogen sulphide production by anaerobic spore-bearing bacteria in lead acetate sodium thiosulphate medium

SPECIES OF ANAEROBE	NUMBER OF STRAINS	24 HOURS	48 HOURS	72 HOURS	4 DAYS	5 DAYS	6 DAYS	10 DAYS	12 DAYS	15 DAYS	20 DAYS
Vibrion septique	3	−	−	−	−	−	−	−	−	−	−
B. oedematiens	2	−	−	−	−	−	−	−	−	−	−
B. chauvoei	1	−	−	−	−	−	−	−	−	−	−
B. fallax	1	−	−	−	−	−	−	−	−	−	−
B. tetani	2	+	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. putrificus	3	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. aerofoetidis	3	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. bifermentans	3	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. sporogenes	4	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. centrosporogenes	1	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. botulinus	3	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. histolyticus	3	−	−	−	−	+	++	++	++	++	++
B. welchii	5	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. tertius	1	++	++++	+++	++++	++++	++++	++++	++++	++++	++++
B. sphenoides	1	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B. bellonensis	1	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++

+ indicates presence of blackening.

TABLE 2

Hydrogen sulphide production by anaerobic spore bearing bacteria in lead acetate peptone agar. No sodium thiosulphate added

SPECIES OF ANAEROBE	NUMBER OF STRAINS	24 HOURS	48 HOURS	72 HOURS	4 DAYS	5 DAYS	6 DAYS	10 DAYS	12 DAYS	15 DAYS	20 DAYS
Vibrion septique	3	−	−	−	−	−	−	−	−	−	−
B. oedematiens	2	−	−	−	−	−	−	±	±	±	±
B. chauvoei	1	−	−	−	−	−	−	−	−	−	−
B. fallax	1	−	−	−	−	−	−	−	−	−	−
B. tetani	2	++	++	++	++	++	++	+++	+++	+++	+++
B. putrificus	3	++	++	++	++	++	++	++	+++	+++	+++
B. aerofoetidis	3	++	++	++	++	++	++	++	++	++	++
B. bifermentans	3	−	+	+	++	++	++	+++	+++	+++	+++
B. sporogenes	4	++	+++	+++	+++	++++	++++	++++	++++	++++	++++
B. centrosporogenes	1	++	+++	+++	+++	+++	+++	+++	++++	++++	+++++
B. botulinus	3	+	++	+++	+++	+++	+++	+++	+++	++++	++++
B. histolyticus	3	−	−	−	−	−	−	−	−	−	−
B. welchii	5	−	+	+	+	+	++	++	++	++	+++
B. tertius	1	−	+	+	+	+	+	+	++	++	++
B. sphenoides	1	+	+	+	++	++	++	++	++	++	++
B. bellonensis	1	+	++	++	++	++	++	++	++	++	++

+ indicates presence of blackening.

that *B. histolyticus* is extremely slow and weak in its H_2S elaborating properties but that there is some activity in this regard cannot well be disputed.

A second series of experiments was undertaken to test the hydrogen sulphide activities of this group of bacteria in a medium containing lead acetate but where no unoxidized sulphur was added in the form of sodium thiosulphate. The conditions of this experiment were otherwise identical with the first. The results are reported in table 2. It will be noticed that the result, where one depends on the inherent cystin content of the pepton as a mother substance of H_2S, is less distinct and takes a considerably longer time to become manifest than where sodium thiosulphate is added. The degree of blackening is not as intense and also is produced in such varying degree that it would obviously tend to make more difficult the interpretation of the test. The reactions, also, lagged somewhat as compared with those taking place in the medium to which sodium thiosulphate had been added. *B. histolyticus* failed to darken this type of substance at all, suggesting that unoxydized sulphur in the form of sodium thiosulphate is more available than cystin for this species as a source of hydrogen sulphide. These several findings establish the value of adding sodium thiosulphate to a medium when tests for hydrogen sulphide are made with members of this group.

Uninoculated controls were carried with each of the above experiments and exhibited no change in coloration during the observation period.

DISCUSSION

For years observers have considered the ability or lack of ability of certain anaerobic spore bearers to blacken animal tissue when incorporated into suitable culture media, as a cultural characteristic of differential importance. This phenomenon has been variously explained. Rettger (1906) considered the blackening due to hydrogen sulphide production. Von Hibler (1908), according to Hall and Petterson (1924), correctly attributed the blackening to the formation of iron sulphide in meat or brain

mediums by sulphuretted hydrogen set free from the protein molecule. Henry (1917) thought that this discoloration may have been due to a tyrosine derivative brought about by the interaction of tyrosine and tyrosinase, or a humin-like substance due to the condensing of sugar with an amino body. Hall and Peterson (1924) have recently published a painstaking investigation on the possible mechanism of this phenomenon and have arrived at the conclusion that it is due to the iron content of brain medium, when such is used alone, or to the iron content of pepton, when this substance is added. They were able to demonstrate tha iron, as it is found in various brands of commercial pepton, varies widely in the amount present, Difco pepton being most constant in this regard. When iron was added to their media, containing various brands of pepton, the following variations with the same species were found as regards the time taken to produce blackening: *B. welchii*, two to five days; *B. novyi* (*B. oedematiens*) two to five days; *B. tetani*, one to five days. When no iron was added to the basic substance containing various brands of pepton, even wider variation was encountered with some of the organisms, for example: *B. sporogenes*, two to four days, *B. histolyticus*, two to twenty-two days, *B. tyrosinogenes*, two to seven days, *B. welchii*, seven days, several of the brands of pepton not giving rise to any blackening with *B. welchii* after forty-five days and with *B. tetani* after one to thirty days. Some of the brands showed no blackening with *B. tetani* after forty-five days.

Thus we see that wide variations are liable to occur when recording the blackening phenomenon produced by various members of the anaerobic spore bearing group. Such discrepancies would seriously handicap accurate observation of this important cultural characteristic, depending on the brand of pepton used, when readings are taken solely from meat or brain mash with or without the addition of iron. The modified medium of Tilley, as described here, was found to yield uniform results as regards the detection of the elaboration of hydrogen sulphide by these bacteria. Rapid and constant blackening took place where a positive reaction occurred, making the test clear-cut and readily

interpreted; while on the other hand where no H_2S was liberated the medium remained uncolored as did the uninoculated controls.

In view of the variations liable to be encountered in the cystin content of various peptones used in the preparation of media, as reported by Tilley, or of the iron content, as demonstrated by Hall and Peterson, and in the possible variation of the iron content of natural brain and muscle tissue, where no pepton is added, it would seem advisable to substitute a medium more accurate in its composition for the recording of this blackening phenomenon and for this purpose we would like to recommend the modified medium of Tilley, as described here.

It is interesting to note that hydrogen sulphide production may not necessarily be an indicator of proteolysis from the bacteriological point of view, as evidenced by the reduction in volume of native protein, for *B. welchii*, *B. tertius* and *B. sphenoides*, non-proteolytic species of anaerobes, are able to elaborate H_2S quickly and strongly as judged by this test, while *B. histolyticus*, an active digestor of native protein, produces H_2S very slowly and in small amount.

CONCLUSION

The medium of Tilley, containing sodium thiosulphate and lead acetate, modified for culturing anaerobic spore bearing bacteria was found to be satisfactory for testing the hydrogen sulphide elaborating ability of this group. Sixteen different species (36 strains) were used in these tests.

There was found to be no essential correlation between the native protein digesting ability and the H_2S producing properties of these organisms.

It is suggested that Tilley's medium be employed in testing the so-called blackening ability of the anaerobic spore bearers instead of the less delicate and indefinite meat mash and brain preparations.

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