SEMISOLID MEDIA FOR CULTIVATION AND IDENTIFICATION OF THE SPORULATING ANAEROBES

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This paper amplifies the studies presented serially during the past four years at the annual meetings of our Society, on the use of semisolid media as applied to the routine isolation and identification of the sporulating anaerobic bacilli. These media have been applied tentatively to a variety of aerobes as well, with indications of practical utility here also. This development, however, is a subject for later presentation.

The sporulating anaerobes are commonly cultivated in deep solid agar. Formerly such cultures were usually protected by a vaseline or other oily cap, which appears to be an unessential complication, providing the depth of medium is sufficient.

Fluid media are also routinely used for certain procedures. For these a vaseline or mechanical seal is requisite, unless incubated in an oxygen-free environment.

However, it appears that the two principles may be combined, as was probably first shown by Pringsheim (1910), by adding only sufficient agar to the fluid base to check convection currents, thus inhibiting aeration. This observation was substantiated and amplified by Jackson and Muer (1911), Lignières (1919), Hitchens (1921, 1922), Murray and Headlee (1931), among others.

When this principle is applied to a liver-peptone broth all of the common anaerobes grow abundantly in the open and un-

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protected medium. No seal or anaerobic container is required, and anaerobic cultivation under these conditions is no more complicated than that of the common aerobes. During the past five years these simple methods have been successfully applied to some 450 strains of 30 or more species of anerobes.

In general the anaerobic group is not fastidious in its nutritional requirements. However, it is observed that the butyric group is not favored by the liver-base, but grows better in a semi-solid whey-base.

Having established the practicability of this simple method of cultivation, it appears that a basic semisolid medium might be adapted to the demonstration of certain differential physiologic reactions. Thus, the production of indol, of hydrogen sulfide, reduction of nitrates to nitrites, fermentation, gelatin liquefaction, and other reactions may be determined in a medium to which the necessary ingredients and reagents are added. Suitable mediums have been devised, which are described below. The formulas given represent the optimum present development. In certain cases it is obvious that improvement is possible, and it is hoped that this report will stimulate similar studies by others.

Practical applications. We shall not discuss anaerobic isolation further than to emphasize the utility of the anaerobic culture dish described by the writer (1930). Primary cultures are inoculated into deep brain medium, or into the semisolid stock culture medium described below. After incubation these cultures are examined for spores, after which they are heated for ten minutes at 80°C., plated, and representative colonies fished to deep brain. Replating for purity is desirable.

The Hall "marble-seal" tube is most useful for anaerobic enrichment of primary cultures. Also, the addition of a tint of crystal violet to the surface of the semisolid agar, or to the supernatant fluid in the Hall tube, as advised by Hall, serves to suppress the aerobic spore-formers.

By these, and other devices, a series of pure cultures of sporulating anaerobes may be obtained. The problem of identification, however, still remains. The novice in the anaerobic field will encounter difficulty here, due chiefly to the lack of any standard

procedure of identification. Many different media and reactions have been utilized, with the result that no two descriptions of the same organisms can be made to conform. The early history and terminology are replete with descriptions of what are now recognized as mixed cultures. Multiple names for identical organisms lead to confusion, complicated further by the recent general adoption of the generic name *Clostridium* for the group.

It is one of the primary purposes, then, of this study to advocate the uniform application of a selected series of differential reactions which are of demonstrated value. These tests should be uniformly applied to all species studied. The resulting descriptions will be at least comparable, and the differentiation will be in proportion to the number and suitability of the tests employed.

Such a series of tests has been applied here to a group of 328 cultures of 20 common anaerobic species. In addition a number of other species are under study, but the present data do not warrant their inclusion in this report.

The tests, as now applied, include: (1) Motility; (2) morphology; (3) spore form and position; (4) production of hydrogen sulfide; (5) production of indol; (6, 7, 8, 9) fermentation of glucose, lactose, sucrose and salicin compared with a sugar-free control; (10) nitrate reduction to nitrites; (11) iron-gelatin liquefaction; (12) iron-milk coagulation and digestion.

It is probable that, as more species are studied, further tests may be required. Thus the butyric and Welch groups may be split into types, or sub-species, by the use of other carbohydrates, as shown by Simmonds and others.

In the "Tentative Key" below, we have attempted to arrange these tests in such order as to effect first a physiologic grouping of the unknown organism, reserving morphology, motility and sporulation as secondary—not in importance, but in availability for early recognition. Thus the reaction of *Clostridium Welchii* in iron-milk almost identifies this organism, while spore form and position may be determined at times only with the greatest difficulty. In certain species the spore position is relatively fixed, while in others it may vary from equatorial to almost terminal.

We present here the formulas as now used. The technic of

procedures is outlined, and finally the results of the study are incorporated in a "Tentative Key," which is susceptible to expansion as further species are studied.

FORMULAS

1. Anaerobe plating (or deep-tube) medium (or use Difco Liver-Veal Agar):

Make liver infusion; boil 50 grams Bacto Liver Powder in 1 l. of distilled water. Filter through cotton and make to 1 l. by pouring hot water through the filter. Adjust the reaction to pH 7.3 to 7.4.

To make 1 l. of agar, take

1 l. liver infusion (above)
77 grams Difco North Gelatin Agar
37 grams Difco Brain-Heart Infusion Powder
2 grams sodium nitrate

Boil to dissolve and add water to make 1 l. Retitrate, and adjust to pH 7.3 to 7.4. Autoclave in flask, and cool to solidify and to fix precipitates. When solidified, remelt and add water to make 1 l. Tube about 8 cm. deep, preferably in 200 x 15 mm. tubes. Autoclave for 15 to 20 minutes at 15 pounds pressure.

2. Semisolid stock culture medium (or use Difco Brain-Liver-Heart Semisolid Agar):

To make 1 l. of agar, take

1 l. liver infusion (above)
37 grams Difco Brain-Heart Infusion Powder
10 grams Bacto Tryptone
5 grams NaCl
2.5 grams agar flakes

Boil to dissolve and add water to make 1 l. Retitrate and adjust to pH 7.3 to 7.4. Filter twice through cotton with slight suction, and tube about 6 cm. deep, preferably in 150 x 13 mm. tubes. Autoclave as usual.

3. Sugar-free fermentation base.

To make 1 l., take

10 grams Difco Neopeptone

10 grams Difco Tryptone
2.5 grams agar flakes
1 l. distilled water

Boil to dissolve and add water to make 1 l. Adjust the reaction to pH 7.3 to 7.4. Andrade or other indicators may be added or omitted. These are usually reduced, although many organisms show typical fermentative reactions.

Divide into lots, and add 1 per cent of the desired sugars. Reserve one lot to be tubed without sugar as a control. Tube about 8 cm. deep in 200 x 15 mm., or better 200 x 13 mm. tubes. Autoclave as usual.

4. Nitrate semisolid agar.

To make 1 l., take

5 grams Difco Tryptone
5 grams Difco Neopeptone
2.5 grams agar flakes
1 l. distilled water

Boil to dissolve and add water to make to 1 l. Adjust the reaction to pH 7.3 to 7.4, then add

1 gram potassium nitrate
0.5 gram glucose

Dissolve and mix well. Tube about 8 cm. deep in 200 x 15 mm., or 200 x 13 mm. tubes. Autoclave as usual.

5. Iron-milk.

Mix fresh whole milk well and tube about 8 cm. deep in 200 x 15 mm., or in 200 x 13 mm. tubes. Add to each tube one strip of No. 26 gauge black stove-pipe iron, cut about 50 x 7 mm. Autoclave as usual, but reduce the pressure slowly when completed to avoid wetting or blowing of plugs.

6. Iron-gelatin.

To make 1 l., take

128 grams Difco Nutrient Gelatin
1 gram dextrose
1 l. distilled water

Dissolve in a double boiler to avoid scorching. Adjust the

reaction to pH 7.3 to 7.4, and add water to make 1 l. Tube about 8 cm. deep in 200 x 15 mm., or in 200 x 13 mm. tubes. Add to each tube one strip of iron (as in Iron-Milk), and autoclave as usual.

7. Lead acetate semisolid agar.

To make 1 l., take 36 grams Difco Lead Acetate Agar (2 per cent agar). Infuse this for 30 minutes, shaking frequently, in 1 l. warm (37°C.) distilled water to dissolve all ingredients except the agar. Filter through cotton, with slight suction, to remove the agar. Make the volume to 1 l. by pouring warm water through the cotton filter. Add to this 2.5 grams agar flakes, and boil to dissolve the agar. Make the volume to 1 l., and adjust the reaction to pH 7.3 to 7.4. Tube about 8 cm. deep, and autoclave as usual.

Note: Prepared by this method the medium displays quantitatively distinctive reactions with the various anaerobes. We have been unable as yet to reproduce these distinctions by other formulas or other modes of preparation.

GENERAL PROCEDURE OF IDENTIFICATION

In this plan all intricate anaerobic apparatus, common only to research laboratories, is eliminated. Isolation, cultivation and identification are accomplished with apparatus and media readily available. Thus, anaerobic studies can be conducted by these methods in even the most meagerly equipped hospital laboratory.

The basic requirements for such studies are satisfied by:

1. The customary anaerobic jars, if available, for plate cultivation, although we favor the use of the anaerobic culture dish of the writer.[2] Deep tubes of agar may be used by those preferring this method.
2. A suitable solid plating, or deep-tube, medium (Bacto Liver-Veal Agar).
3. A semisolid stock culture medium (Bacto Brain-Liver-Heart Semisolid Agar).

[2] Obtained from Fisher Scientific Company, Pittsburgh, Pa.

4. A supply of Pasteur pipettes drawn from 3 to 4 mm. tubing.
5. Marble-seal Hall tubes for anaerobic enrichment. These are very useful, but are not required.
6. Brain medium tubes for storage of pure cultures (von Hibler's formula).
   6a. Corn-Liver medium of McClung and McCoy (1934) for storage of *Clostridium butyricum* types.
7. Special diagnostic media, chiefly semisolid, as listed above.

When assured, by replating, that pure cultures have been isolated, we establish stock cultures in deep brain, or in corn-liver. These cultures are sealed with paraffin, and are reserved in the dark at room temperature. They are opened again only as a last resort, as in case of loss or contamination of the current subcultures.

At the same time a working culture is established in the semi-solid stock culture medium (S. S. C.). Our cultures, in this medium, have all survived 54 days continuous incubation at 37°C., and all tested have been found viable after 8 months storage at room temperature. This allows ample time for all reasonable requirements. However, duplicate storage in deep brain or in corn-liver serves as a precaution against possible loss.

In preparing for the identification, a fresh subculture in S. S. C. medium is incubated not over 24 hours, in order to have active vegetative growth. A complete set of the diagnostic media is placed in a rack. If these are not used on the day of their preparation all tubes are boiled for 10 minutes and cooled just before inoculation.

The stock culture is held at a convenient angle in a burette clamp, with the mouth near a flame. A Pasteur pipette is well flamed, the sealed end snipped with flamed forceps, and the pipette is inserted and rested within the culture tube. Each diagnostic medium is then inoculated from the bottom upward with about 0.1 cc. of culture, cautiously blowing to expel the contents without bubbling air. The entire set of cultures is then incubated at 37°C.

DISCUSSION AND INTERPRETATION

1. Motility tests. It should be strongly emphasized that motility tests must be made early; in fact, preferably, as soon as growth is evidenced by perceptible turbidity. Many incorrect descriptions are based upon delay even to only 24 hours. Thus, *Clostridium bifermentans* and *Clostridium luciliae*, commonly described as non-motile, are found uniformly motile if examined at from 4 to 6 hours. Both of these species are commonly non-motile after 18 hours incubation.

No special precautions appear necessary. We remove a sample, preferably from the S. S. C. medium, by Pasteur pipette to an open slide, dropping on a cover-glass and examining as soon as convection currents subside. We have not found it necessary to seal the cover-glass with vaseline, or to examine in flattened capillary pipettes, as advocated by some workers.

The S. S. C. medium is recommended for the study of motility, although others may be used, excepting those containing fermentable carbohydrates. Negative results require, of course, repeated confirmation from a variety of media, all tests being made as early as growth is perceptible.

2. Morphology. Gram stains are commonly made at 18 to 24 hours. Because of the common morphologic similarity of the group, microscopic examination may yield little information. Even in pure cultures the limits of size and shape are such that one often cannot certify the purity. Obvious contaminants may be recognized, and certain species may be tentatively grouped. Thus, *Clostridium Welchii* may be differentiated readily from *Clostridium tertium*, or *Clostridium tetani* from *Clostridium bifermentans.*

Deep brain is, in certain respects, preferable to the S. S. C. medium for microscopic examination. In the former the morphology may be more uniform, and the Gram reaction more stable. In the latter, however, the field is entirely free from confusing debris. Unusual care is required to avoid over-decolorizing the Gram stain. Acetone alcohol must not be used as the decolorizer. The method of Claudius, as cited by Henry (1916–1917) is one of the most useful modifications.

When stained from young cultures the sporulating anaerobes appear inherently Gram-positive. Thus, this method yields little information of value, except that certain species, such as *Clostridium Welchii* and *Clostridium Sordelli*, among others, are strongly positive even in rather old cultures. This feature may then assist in the tentative grouping of unknown species.

3. Spores. We do not lay primary emphasis upon sporulation, except where it is readily observable, or where the spore has a definite and obvious shape or position. Thus, *Clostridium tertium*, with its abundant terminal spores may be readily differentiated from *Cl. Welchii* or *Cl. Sordelli*, which have central to excentric spores. *Cl. tetani* may also be distinguished from *Cl. Sordelli*, but not so readily from *Clostridium tetanomorphum*, which also has terminal spores with only a slight ovoid tendency.

Depending upon the species encountered, sporulation may or may not be particularly useful. We include it only as a secondary feature, as contrasted with its common primary position in other current systems, appreciating the fact that many will disagree. It should be noted, however, that we utilize physiology, rather than morphology, as a primary basis of classification.

4. Hydrogen sulfide. We regard this test as most useful. However, the method used at present is rather empirical. Our differentiations are based solely upon the above formula. We have been unable to obtain these quantitative differentiations when the medium is prepared by the methods used for the other semisolid mediums. The concentration of the agar appears to exert an action comparable to that recently reported by Vaughan and Levine (1936).

With our formula three distinctive reactions may be observed. Thus, *Cl. sporogenes*, *Cl. botulinum*, *Cl. Welchii* and others, show early and intense blackening at 12 to 24 hours. *Cl. tetani*, *Cl. lentoputrescens* and *Cl. tertium* show but a slight diffuse smoky browning, while *Cl. multifermentans* and *Cl. tetanomorphum*, among others, give no reaction at all.

We have experimented with the Difco Peptone Iron Agar of Levine (1932–1934), and feel that this may supplant later the lead acetate agar. However, we have not yet developed a

formula permitting the differentiation afforded by the lead acetate.

It is probable, moreover, that all of the sporulating anaerobes produce more or less hydrogen sulfide. We have developed formulas of both peptone iron and lead acetate in which all species tested blacken strongly.

5. Indol. This test is performed in the sugar-free fermentation control, after reading the pH reaction. No discrepancies have been observed between the Ehrlich and the vanillin tests. The vanillin test, however, is advocated because of a reaction described below as the "Vanillin Violet" test, which is shown by only 3 species in our series.

The minimum safe time of testing is about 72 hours. Tests may be made up to 7 days or more, with little change in reaction. Comparative tests of 12 peptones showed an obvious superiority of a combination of Difco Neopeptone and Tryptone. In this medium our strains of *Cl. tetani* and *Cl. lentoputrescens* gave a positive reaction of some degree, while negative reactions were observed in practically all other peptones tested.

When traces of glucose were added to the medium, to increase growth of the fermentative species, it appeared to exert a "protein-sparing" action on the peptones, inhibiting, or suppressing entirely, the formation of indol. In some cases it seemed that this might be due to the resulting acidity. However, *Clostridium sphenoides* gave a strong reaction in 1 per cent sucrose and lactose and a negative test in 1 per cent glucose and salicin, although all carbohydrates were fermented. Thus, it appears that the specific sugar, rather than the acidity, was the responsible factor.

"Vanillin Violet" test. When 10 drops of 5 per cent vanillin in 95 per cent alcohol are added to the culture, followed by 10 drops of concentrated HCl, the orange indol reaction appears in degree according to the species tested. Certain cultures, however, including *Cl. sporogenes, Cl. tyrosinogenes* and *Cl. botulinum* (*A* and *B*), showed no orange, but rather a faint violet. On further addition of the reagents this color is intensified, diffusing as a deep violet color. The nature of this reaction is not known, but

Hall suggests that it is probably due to skatol or some closely related body.

This reaction indicates, in our series, one of these three species, and it is interesting to note that it occurs in mixed as well as in pure cultures. Thus, it was observed in a culture of *Cl. Welchii* containing *Cl. sporogenes* as a contaminant. This test may, then, be of value in checking cultures for freedom from *Cl. sporogenes*, probably the most common anaerobic contaminant.

6, 7, 8, 9. Fermentation tests. We have used, thus far, only four carbohydrates. Others may be required later as other species are studied. For example, the *Cl. butyricum* group seems to include a variety of fermentative types, whose reactions may later prove correlated with other cultural distinctions.

We utilize only the end reaction as evidence of fermentation. Presence and amount of gas cannot be relied upon, as emphasized by Hall, Heller and others. We do not use vaseline caps, or attempt any measurement of gas. Of course, great excess of gas and growth, as contrasted with the sugar-free control, is indicative. It may often serve as a fairly reliable index, but nothing more.

In general, 36 hours incubation appears to be the optimum time for testing acidity. However, certain species, which grow rather poorly, continue slowly to increase in acidity up to at least 7 days. This may explain some of the discrepancies in past studies of *Clostridium Chauvoei, Clostridium fallax* and other rather feebly growing anaerobes.

We have tried adding various indicators to the medium. However, as observed by others, these are commonly reduced to an inactive form. By reducing the peptone content some indicators remain reasonably stable and active, but as the peptones are reduced in amount many species, such as *Cl. Chauvoei* and *Cl. Novyi*, may fail to grow. Hence, we have discontinued their use in the medium, and follow Hall's procedure using the spot plate, or remove aseptically by pipette some 5 drops of culture and test this in tubes of properly diluted indicator. If carefully done, the culture may be reincubated for subsequent retest.

There is no purpose in reading the exact pH in routine iden-

tification. A definite acid reaction, as contrasted with the sugar-free control, is sufficient indication of fermentation. With few exceptions the reaction is sharp and obvious. In such exceptions a repetition of the test at 24 to 48 hours later usually confirms the reaction.

10. Nitrate reduction to nitrites. This test, usually considered a simple one, has given considerable difficulty. This has been largely overcome by the present formula of the medium. The difficulty is apparently due to several factors which require a delicate balance, as recently summarized by Conn (1936).

Thus, in order to obtain the growth requisite for reducing the nitrate, it is almost necessary to add a trace or more of glucose. Yet, when this is added to facilitate growth of the fermentative types, nitrate reduction may be distinctly, or completely, suppressed.

To compensate for this inhibition we have added larger amounts of peptone. Here, again, a second inhibition is observed. An excess of peptone, as well as traces of glucose, seriously interferes with the accuracy of the test. Moreover, of several test organisms, each responds differently to the varying concentrations of each factor. For example, *Cl. Welchii*, *Cl. multifermentans*, *Cl. tertium* and *Cl. septicum* all behave differently from each other.

In the formula above, however, the peptone, glucose and nitrate have been so adjusted that uniformly positive reactions are obtained. We believe that this formula may yet be improved, and are working toward this end.

The test is usually performed after 72 hours incubation, although the reaction does not vary thereafter up to 7 days. We prefer the use of 0.6 per cent dimethyl-alpha-naphthylamine (Eastman), with sulfanilic acid, both in dilute acetic acid.

11. Iron-gelatin liquefaction. This test is performed in Difco Nutrient Gelatin to which is added 0.1 per cent of glucose and a strip of iron.[3] The Standard Methods gelatin formula is to be avoided. In this simple gelatin solution many anaerobes fail to

[3] Powdered iron reduced by hydrogen may be used, as advocated for milk by Hastings and McCoy (1932), although we prefer the iron strip, as suggested by H. G. Dunham of Difco Laboratories.

liquefy in any reasonable time, while in our formula the same organisms commonly liquefy completely in 24 to 48 hours. This is particularly striking in the case of *Cl. septicum* and *Cl. luciliae.*

All cultures are incubated at 37°C., and are tested daily in ice water until liquefied, or, if negative, at least for 30 days. No time limit can be set for the negative cultures, but we have not observed any species showing liquefaction delayed beyond 10 days. If incubated beyond 10 days, the plugs should be paraffined.

The iron-gelatin shows a distinctive orange to wine-red color when inoculated with *Clostridium histolyticum.* The orange color appears at about 24 hours, deepens to red at about 48 hours, then slowly and variably fades in 3 to 5 days or more. The reaction appears specific for *Cl. histolyticum,* and establishes a valuable presumptive test for this organism, having the same significance as the reaction of *Cl. Welchii* in iron-milk. The reaction is displayed in mixed, as well as in pure cultures, but its particular value lies in its application to pure cultures.

12. Iron-milk. The reactions in this medium are utilized for the primary group differentiation. As noted above, Hastings and McCoy used powdered reduced iron for the detection of *Cl. sporogenes* in milk. For a time we used this with satisfaction, until the idea of the iron strip was suggested by Dunham. The results are so distinctly superior that this principle has been adapted to our purpose.

This medium affords 5 distinctive and useful reactions, which permit ready grouping of the unknown strains. With some species the reaction is striking and early; in others, especially the non-reactors, delay is necessarily encountered. However, failure to react has the usual value of other negative tests.

These reactions have been so often repeated that one may conclude that they are uniformly characteristic, and practically unvarying in reliability. Even such selective organisms as *Cl. Chauvoei, Cl. luciliae* and *Cl. Novyi* present no difficulty of cultivation in this simple medium. No vaseline seal, or other anaerobic conditions are required for the cultivation of any of our species.

The group reactions in iron-milk and their interpretation

I. Active gaseous fermentation, with early coagulation (12 to 48 hours). The clot is violently disrupted, with no subsequent digestion or blackening. This reaction suggests commonly *Cl. Welchii*, although other anaerobes, including *Cl. aerofoetidum*, *Cl. multifermentans* and members of the *Cl. butyricum* group, approach or even equal it in intensity.

As was repeatedly observed by Hall, this "stormy" reaction in milk inoculated with material from gangrenous wounds, or other positive sources, suggests the presence of *Cl. Welchii*. However, it is indicative only, in iron-milk, since several other anaerobes may cause a similar reaction.

II. Inactive gaseous fermentation, with coagulation quite uniformly delayed (4 to 6 days). No digestion or blackening of the clot is observed, although a dirty gray-brown discoloration may occur if evaporation exposes the end of the iron strip. Such changes are of no significance. Furthermore, cultures requiring prolonged incubation should have the plugs paraffined after the first 5 to 6 days.

In this group we find *Cl. sphenoides*, *Cl. fallax*, *Cl. tertium* and *Cl. septicum* showing a fine stream of small gas bubbles rising in 12 to 24 hours. This gas evolution continues for several days, never becoming more than barely perceptible. Upon shaking the tube gently a "shower" of bubbles is released to rise in a fine foam.

Coagulation occurs suddenly and almost uniformly in 5 to 6 days, but, since most of the gas has already escaped, the clot shows only a slight splitting. It is never torn as in the preceding group. If the plugs are paraffined no further change, other than a slight graying, is observed for many days or weeks.

III. Inactive gaseous fermentation, long continued, with late (if any) coagulation; no digestion or blackening. This group differs from group II only in that coagulation is uncommon, and rarely appears before 12 days, if at all. Fermentation is very slight, being evidenced often by only single bubbles which arise only on shaking. The evolution of gas, however, is indefinitely prolonged, gas bubbles being observed after as long as 60 days incubation.

In this group we place *Cl. Novyi, Cl. Chauvoei* and *Cl. luciliae.*

IV. Inactive gaseous fermentation, with digestion and blackening. This reaction is almost as striking as the "stormy" reaction of *Cl. Welchii.* In 12 to 24 hours the milk becomes watery and more or less translucent. A soft semi-coagulation may then occur (*Cl. botulinum A* and *B*), or it does not (*Cl. sporogenes, Cl. histolyticum* and *Cl. bifermentans*). Little or no gas is observed, at most only an occasional bubble arising on shaking.

After the initial translucence, and up to 5 to 7 days, according to the species, a fine, then coarse flocculence, or a soft coagulation develops. This darkens with variable rapidity, often becoming coal-black within 24 hours (*Cl. sporogenes*). Digestion proceeds, and fine or coarse flocculi settle as a black sediment, leaving a yellow to greenish supernatant fluid. Paraffined tubes ultimately become almost clear, with only a small amount of black precipitate.

In general, these changes are rapid and complete within 5 to 6 days, except for the prolonged settling. This excepts *Cl. botulinum A* and *B*, which usually clot the milk softly in 2 to 5 days, with blackening delayed until 5 to 7 days, after which further changes follow those of the other members of the group. This coagulation and delayed digestion, at least among our 13 strains, seems to be a reliable index of the species. Together with fermentation of salicin and the "Vanillin Violet" reaction, it suffices to identify the species.

V. No gaseous fermentation, with no digestion or blackening. Coagulation is rather constant, but long delayed, by *Cl. tetani* and *Cl. lentoputrescens.* This is not an acid coagulation, but is due rather to a weak coagulase. It appears, at the earliest, in from 15 to 20 days. A few strains have coagulated at 30 days or later, while others have failed to coagulate even after some 5 months incubation.

The fact that these cultures are growing actively is easily demonstrable by microscopic examination, or by subculture. The negative early milk reactions are readily supported by the other reactions, such as indol formation, gelatin liquefaction and failure to ferment carbohydrates.

Cl. tetanomorphum follows the same negative course in iron-

milk. However, it ferments glucose and gives a very striking indol reaction. Our strains have shown no tendency to coagulate iron-milk, even after 60 days incubation. This adds a belated confirmation of its identity. Its ovoid spores offer further differentiation from *Cl. tetani.*

The results of all these reactions and observations are presented in the form of a "Tentative Key."

"TENTATIVE KEY" TO THE SPORULATING ANAEROBES

I. Iron-Milk, Active gaseous fermentation; Early coagulation (12–48 hours); No digestion of clot; No blackening;
  A. Lead Acetate, Strongly blackened;
    1. Sucrose, Fermented; (Salicin,—Not fermented);
      Nitrite +; Indol −; Violet −; Gelatin +; Motility −;
        Glucose +; Lactose +;
          Spores ovoid, central-excentric; Not swelling rod;
            (Spores infrequently observed, − not in fermentable sugars);
              .....1. *Clostridium Welchii*
                (44 cultures)
    2. Sucrose, Not fermented; (Salicin, − Fermented);
      Nitrite +; Indol −; Violet −; Gelatin +; Motility +;
        Glucose +; Lactose +;
          Spores ovoid, excentric-subterminal; Distinctly swelling rod;
            (Spores infrequently observed, apparently not in fermentable sugars);
              .....2. *Clostridium aerofoetidum*
                (1 culture)
  B. Lead Acetate, Not blackened; No browning;
      Nitrite +; Indol −; Violet −; Gelatin −; Motility +;
        Glucose +; Lactose +; Sucrose +; Salicin +;
          Spores ovoid, central-excentric; Not swelling rod;
            (Spores abundant, even in fermentable sugars);
            a. Glycerol, − Fermented;
              .....3. *Clostridium multifermentans*
                (13 cultures)
            b. Glycerol, − Not fermented[4]
              .....4. *Clostridium butyricum* (Group)
                (11 cultures of various species)

II. Iron-Milk, Inactive gaseous fermentation; Late coagulation at 4–6 days; No digestion of clot; No blackening;
  A. Lead Acetate, − Strongly blackened;

[4] Our strains are in accord with the common impression that members of the group do not ferment glycerol. We are advised, however, by McCoy, that this is not entirely and uniformly true.

1. Indol, Positive;
Nitrite +; Violet −; Gelatin −; Motility +;
Glucose +; Lactose +; Sucrose +;[4] Salicin +;
([4] pH in Sucrose reduced from 7.2 to 6.4 only);
Spores almost spherical, abundant, subterminal, becoming terminal; Swelling rod;
Vegetative cells distinctly navicular, pointed;
.....5. *Clostridium sphenoides*
(1 culture)

2. Indol, Negative;
Nitrite +; Violet −; Gelatin −; Motility +;
Glucose +; Lactose +; Sucrose +; Salicin +;
Spores ovoid, excentric-subterminal; Swelling rod;
(Spores infrequently observed, not abundant);
Vegetative cells slender, round to pointed ends;
.....6. *Clostridium fallax*
(1 culture)

B. Lead Acetate, Slight smoky browning; Not blackened;
Nitrite +; Indol −; Violet −; Gelatin −; Motility +;
Glucose +; Lactose +; Sucrose +; Salicin +;
Spores ovoid, terminal; Swelling rod;
(Spores abundant, even in fermentable sugars);
Grows aerobically, (Microaerophile);
.....7. *Clostridium tertium*
(9 cultures)

C. Lead Acetate, No blackening; No browning;
Nitrite +; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Lactose +; Sucrose −; Salicin +;
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;
.....8. *Clostridium septicum*
(Vibrion septique)
(*Clostridium oedematis-maligni*)
(92 cultures)

III. Iron-Milk, Inactive gaseous fermentation (long continued);
Late, if any, coagulation (10–20–30 days, or not even at 60 days); No digestion; No blackening;

A. Lead Acetate, Strongly blackened;
1. Lactose, Not fermented;
Nitrite −; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Sucrose −; Salicin −;
Spores ovoid, not abundant, excentric-subterminal;
Swelling rod;
(Some few strains coagulate Iron-Milk (5–12 days), others not 30 days, although gas bubbles are constantly evolved);
.....9. *Clostridium Novyi*
(*Clostridium oedematiens*)
(28 cultures)

1. Lactose, Fermented;
Nitrite +; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Sucrose +; Salicin −;
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;
(Some strains coagulate Iron-Milk (12 days); others not at 56 days);
.....10. *Clostridium Chauvoei*
(7 cultures)

B. Lead Acetate, No blackening; No browning;
Nitrite −; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −; Salicin −;
Spores ovoid, not abundant, terminal; Slightly swelling rod;
(No coagulation of Iron-Milk (30 days), although gas bubbles are constantly evolved (15–20 days));
.....11. *Clostridium luciliae*
(*Clostridium botulinum* C)
(9 cultures)

IV. Iron-Milk, Inactive gaseous fermentation; More or less rapid digestion (with or without previous clotting); Strongly blackened, early (48 hours) or late (8–9 days);

A. Lead Acetate, Strongly and rapidly blackened (24–48 hours);

1. Salicin, − Fermented;
Nitrite −; Indol −; Violet +; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −;
Spores ovoid, not usually abundant, excentric-subterminal;
Swelling rod;
(Iron-Milk softly coagulated (2–5 days); First blackened (5–7 days); Clot slowly digested (10–20 days));
(Differentiated by toxin-antitoxin);
.....12. *Clostridium botulinum* A and B
(13 cultures)

2. Salicin, Not fermented; (See *Clostridium centrosporogenes*);
a. Indol, − Negative; (Vanillin Violet +);
Nitrite −; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −;
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;
(Iron-Milk not coagulated; Translucent, then flocculent precipitate; Rapidly blackened (24–48 hours); Rapidly digested (8–10 days));
1. Tyrosine crystals not observed;
.....13. *Clostridium sporogenes*
(11 cultures)
2. Tyrosine crystals in old cultures;
.....14. *Clostridium tyrosinogenes*
(4 cultures)

b. Indol, Positive; (Vanillin Violet −);
Nitrite −; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −; Salicin −;[5]
Spores ovoid, abundant, central-excentric;
Not markedly, if at all, swelling rod;
1. Pathogenic;

.....15. *Clostridium Sordelli*
(10 cultures)

2. Non-pathogenic;

.....16. *Clostridium bifermentans*
(*Clostridium centrosporogenes*)[5]
(23 cultures)

B. Lead Acetate, Not blackened; No browning;
Nitrite −; Indol −; Violet −; Gelatin +; Motility +;
(Wine-red color in Iron-Gelatin (24–48 hours))
Glucose −; Lactose −; Sucrose −; Salicin −;
(No sugars fermented)
Grows aerobically, (Microaerophile);
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;

.....17. *Clostridium histolyticum*
(21 cultures)

V. Iron-Milk, No gaseous fermentation; No digestion; No blackening; Coagulation late, if any, (15–20 days or more);
A. Lead Acetate, Not blackened, but showing smoky browning at 24–48 hours, not measurably increased on incubation;
Nitrite −; Indol +;[6] Violet −; Gelatin +; Motility +;
Glucose −; Lactose −; Sucrose −; Salicin −; (No sugars fermented);
Spores spherical, not abundant, terminal;
Swelling rod;
a. Toxic;

.....18. *Clostridium tetani*
(15 cultures)

b. Non-toxic;

.....19. *Clostridium lentoputrescens*
(*Clostridium putrificum*)
(10 cultures)

B. Lead Acetate, Not blackened; No trace of browning;
Nitrite −; Indol +; Violet −; Gelatin −; Motility +;
Glucose +; Lactose −; Sucrose −; Salicin −[7]
Spores spherical to perceptibly ovoid, terminal;
Swelling rod;

.....20. *Clostridium tetanomorphum*
(5 cultures)

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[5] (5 of 13 cultures, labeled *Cl. centrosporogenes*, frankly fermented *salicin.*)
[6] Occasional strains are Indol-weak, or apparently Indol-negative.
[7] Some strains lower pH slightly (from 7.2 to 6.8).

SUMMARY

This report, based upon some 5 years study of 328 strains of 20 species of the sporulating anaerobes, proposes the use of a series of selected physiologic tests, supported by morphologic characters, which tests should be applied uniformly to all unknown species of the anaerobe group.

These tests are applied chiefly in semisolid media which permit abundant growth under conditions eliminating all of the tedious routine of the customary anaerobic technic.

Formulas for these media, methods of performing tests, and interpretations of the reactions are outlined.

Certain newly observed reactions are recorded, which are valuable in the tentative recognition of a few species.

These reactions and observations are arranged in a definite plan, and a "Tentative Key" to 20 common species is presented. In most instances these suffice to identify the species. In a few cases they offer only "group" identification, as of the butyric or Welch group.

It is obvious that this "Tenative Key" is open to modification and addition of other reactions as more species are studied.

Some five years of study have amply demonstrated the utility of these methods. Their simplicity makes possible the easy and certain cultivation and identification of the sporulating anaerobes even in the small laboratory, and by relatively inexperienced technicians. Such, at least, is the opinion offered by other schools and laboratories which have adopted these methods, even in their present state of development.

It should be emphasized that this report is presented, not as a completed study, but rather as one indicating a new form of approach to the problem of cultivating a group of bacteria commonly regarded as especially difficult to deal with. Many species remain to be studied, particularly the thermophiles, as well as the confusing butyric group.

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