ADVANTAGES OF CULTURE MEDIUMS CONTAINING SMALL PERCENTAGES OF AGAR

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The peculiar characteristics of agar have made it an important factor in the development of the science of bacteriology. Too frequently it is considered merely an inert constituent of culture mediums useful because of the changes in its physical state at various temperatures, while the effects on bacterial metabolism of different percentages of it are ignored. The maximum concentration in common use is fixed more by convenience in filtration than by economy or influence on growth.

It is true that semisolid mediums have been suggested from time to time. Rosenthal[1] and Klie[2] experimented with concentrations of gelatin as low as 2.5%. The luxuriant growth obtained probably as a result of diffusion of the inhibiting metabolic products and the extension through the soft jelly of the colonies of motile bacteria caused gelatin to be preferred by many workers and resulted in its being given up with reluctance in favor of the more convenient agar. Hiss[3] and later Hesse[4] found that 0.5% agar permitted flagellated bacteria to travel through it and suggested this medium as a means for differentiating between the motile typhoid bacillus and the nonmotile colon bacillus. Jackson and Melia[5] working with the Hesse medium found that about 0.4% of dried agar was the equivalent of the 0.5% recommended by those who had not taken into consideration the moisture content of thread agar. North[6] found that semisolid mediums were more suitable for preserving stock cultures, doubtless for the reason that unlike solid mediums they did not hold the concentrated products of growth in direct contact with the bacteria. The gelatin-agar medium, attributed by North to Guarnari, contains but 0.3% of agar. Lignières[7] has more recently made the claim that a semisolid medium containing but 0.25% of agar is superior to broth or solid mediums for the cultivation of anaerobic bacteria. That there are advantages in still lower percentages than have heretofore been employed has been revealed in the work reported in the following. The extreme simplicity of such a procedure has caused me to feel that it could not be new to any one but myself, and I have sought diligently to find references to previous work along the same line, but so far unsuccessfully.

Received for publication June 6, 1921.

1 Deutsch. Arch. f. klin. Med., 1895, 55, p. 513.

2 Centralbl. f. Bakteriol., I, O., 1896, 20, p. 49.

3 Jour. Exper. Med., 1897, 2, p. 677.

4 Centralbl. f. Bakteriol., I, O., 1908, 46, p. 89; Ztschr. f. Hyg. u. Infektionskr., 1908, 58, p. 441.

5 Jour. Infect. Dis., 1909, 6, p. 194.

6 Jour. Med. Research, 1909, 20, p. 359.

7 Compt. rend. Soc. de biol., 1919, 82, p. 1091.

Our interest in this subject was stimulated by seeing colonies of Clostridium histolyticum in deep agar which were considered by J. F. Donner, late lieutenant, Sanitary Corps, U. S. A., entirely atypical, in that they are unusually large and filamentous. In seeking an explanation it was decided to study some of the factors which might influence colony formation. The first of these taken up for investigation, because it seemed and has proved to be one of the chief causes of variation, was concentration of the agar.

In the study of the pathogenic anaerobic bacteria, the type of colony formed in the solid agar medium used is considered a valuable differential characteristic. Usually, however, no statement is made in published reports concerning the percentage of agar used in the medium, and we seldom find attention paid to the fact that the thread agar of commerce contains impurities, and, according to Whitaker [8] from 18 to 20% of moisture. Ayers, Mudge and Rupp [9] have recently called attention to the influence on bacterial growth of some of the impurities in commercial sugar which may be largely removed through washing, as noted by the Committee on Standard Methods of the Laboratory Section of the American Public Health Association.[10]

PREPARATION OF CULTURE MEDIUM

The culture medium selected for the study of colony formation was similar to the putrid meat medium of Veillon.[11] In order to have the various lots, containing the different percentages of agar, uniform in every other respect, double strength broth was made, and to this was added an equal volume of agar in distilled water. One kilo of ground lean beef stirred into 1 liter of water was incubated at 37 C. for 48 hours. This was then strained and heated in a water bath to boiling and strained again. Then were added peptone, 40 gm. (2% in the final medium) and KNO_3, 4 gm. (0.2% in the final medium). When the peptone had dissolved, the broth was titrated and the reaction adjusted to P_H 7.5. It was then filtered and autoclaved at 15 lbs. of pressure for 30 minutes. Just before mixing with the agar, enough glucose was added to make 0.2% in the final medium.

To make the agar jelly, the thread agar was dried thoroughly, weighed, washed in running water over night and made up with distilled water to 6%. The agar was dissolved in the autoclave, reaction adjusted to P_H 7.5 and cleared by straining through cotton and gauze. In making the mixtures the amount of hot, fluid, 6% agar necessary to obtain the various percentages was diluted with hot distilled water to a volume equal to that of the double strength broth—also hot. For example, to make 500 c c, 1% nutrient agar and to make 83 c c, 6% agar was mixed with 167 c c distilled water and 250 c c double

[8] Jour. Am. Pub. Health Assn., 1911, 1, p. 632.
[9] Jour. Bacteriol., 1920, 5, p. 589.
[10] Standard Methods for Examination of Water and Sewage, 1920, p. 93.
[11] Arch de med. et pharm. mil., 1918, 69, p. 15.

strength broth. The reaction was checked, the medium was tubed without further filtration and the tubes were sterilized in the autoclave at 15 lbs. of pressure for 20 minutes. Before inoculation the reaction was again checked.

TECHNIC OF INOCULATION

For inoculation, the tubes of medium were heated in a water bath for one-half hour and chilled rapidly to about 45 C. A 24-hour meat broth culture served as the seed. A sterile glass capillary sealed at the end was dipped into the broth culture and carried successively through 9 tubes of the agar without reinoculating. The glass inoculating rod was inserted into the warm fluid and moved about carefully to avoid whipping air into the medium. By this dilution method of inoculating, the first 3 tubes generally showed innumerable colonies, while succeeding tubes showed a rapidly diminishing number. Nearly always, one or two of the tubes contained only from 1 to 5 colonies.

PERCENTAGE OF AGAR AND COLONY MORPHOLOGY

The strain of C. histolyticum in question, from a single organism, isolated by Lieut. Donner with the Barbour apparatus, usually produces colonies like the illustration shown in Weinberg and Seguin, plate 5, figure 5.[12] The naked eye appearance of these colonies is much like a crumb of very dry bread. That is, they are irregularly star-shaped and have a fairly sharp contour.

In this series, the colonies in agar of from 1.25% to 3% developed the typical bread crumb morphology. In the 1% agar the colonies became much larger and spherical in shape, growing radially from a central nucleus. They finally became fairly dense and regular in contour, and had somewhat the puff-ball appearance certain of the molds assume when growing suspended in broth. These colonies are shown in Fig.1. The largest of the colonies in deep agar measured about 10 mm. in diameter. This was in the lower half of the tube, the one other colony in the culture being in the upper half with a distance of nearly 15 mm. between. Both colonies were identical in appearance and the upper was used to confirm the identity of the culture; this was done by microscopic examination, sugar reactions, colonies of subcultures in higher percentages of agar and finally by animal inoculation.

The colonies in the 0.75% agar were similar—possibly less dense. One of the 0.75% tubes contained nearly 100 colonies. These exhibited no tendency to coalesce; in fact, colonies approaching one another were flattened somewhat, with a narrow layer of clear agar between, as though the products of their growth inhibited further extension.

The 0.5% and the 0.1% agar showed a most luxuriant diffuse growth; the former was nearly solid while the latter seemed quite fluid.

[12] Le Gangrene Gazeuse, 1918, p. 169.

In none of the cultures was there any growth within 5 mm. of the surface; this superficial layer, probably invaded by oxygen, was not clouded to the slightest degree.

As noted in the foregoing, various amounts of agar, less than enough to make a firm, solid medium, have been recommended from time to time. The medium of Guarnari, containing 0.3% and that of Lignières [7] containing 0.25%, apparently reach the lowest proportions used and investigated heretofore. It is clear from our work, however, that these workers in stopping at 0.3% and 0.25% did not exhaust the advantages of dilution, nor did they reach the optimum point.

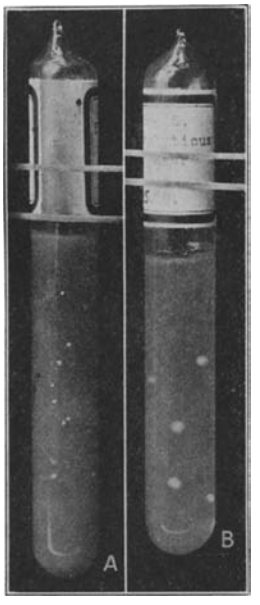

Fig. 1.—Colonies of Clostridium histolyticum in Veillon agar; incubated 48 hours; A, 2% agar; B, 1% agar.

The growth of C. histolyticum in the 0.1% agar was so luxuriant, it was decided to study the suitability of the lower percentages for the growth of both aerobes and anaerobes and to observe the relation of agar in various concentrations to the penetration of oxygen, using methylene blue as an indicator.

THE GROWTH OF ANAEROBIC BACTERIA IN 0.1% AGAR

Primary Cultivation.—A point of interest in this connection was the possible value of nutrient thin agar as an enriching medium or for the

primary culture directly from the infected wound or for other infectious material. If it might be found useful for this purpose, its simplicity and ease of preparation would be strong points in its favor.

Six samples of earth were collected from various places and planted into freshly heated, plain beef-infusion-broth-0.1%-agar, reaction PH 7.6, without glucose. In 18 hours the growth was heavy; microscopically, few spores were found; these were oval and somewhat rectangular. After 5 days every flask contained round spores and many bacilli morphologically like tetanus. Small quantities of the lower layers of the culture were removed and heated in sealed capillary tubes to 70 C. for 30 minutes. The heated material was planted into freshly heated 0.1% agar. At the end of 24 hours' incubation, the growth was luxuriant. Small amounts were passed through Mandler filters and 0.5 c c of each was injected subcantaneously into guinea-pigs. Two of the 6 animals died with symptoms of tetanus in about 48 hours.

Stock Cultures.—Some of the war wound anaerobes in the collection at the Army Medical School have been planted in 0.1% agar and all have grown well; these include Clostridium tetani, C. welchii, C. histolyticum, C. septicum (Vibrion septique), C. edematicum, C. purtificum, C. sporogenes, and also types A and B of C botulinum. The ease with which cultures of anaerobic bacteria in this medium may be successfully inoculated, makes it possible to study their physiologic characteristics as easily as those of aerobic bacteria. Noteworthy is the simplicity with which the carbohydrate relations of the various anaerobes may be observed.

Toxin Production.—Since the anaerobic bacilli grow well in this medium, apparently more luxuriantly than in broth, interesting possibilities are suggested with regard to toxin production. In preparation for work along this line methods for eliminating the agar, in case its thorough removal should be found necessary, have been investigated. If only small amounts are being prepared, the agar may be packed by centrifugation and the supernatant broth decanted. This will remove most of the agar but not all and if the toxin should be intended for intravenous injection, it might not be adequate unless centrifugation is repeated two or three times. For larger amounts such as may be used for the immunization of horses to produce antitoxins and for more thorough elimination of the agar, the culture may be precipitated by the addition of an excess of ammonium sulphate. The agar will be thrown out with the toxin. The precipitate obtained may then be

kneaded to eliminate as much salt as possible and dried over sulphuric acid in vacuo. On dissolving the desiccated mass the toxin will go into solution and the agar, before it has swelled to any appreciable extent, may be removed by filtration.

THE GROWTH OF AEROBIC BACTERIA IN 0.1% AGAR

In order to ascertain the range of suitablitiy of the medium for the more common aerobic bacteria, flasks and tubes were prepared and

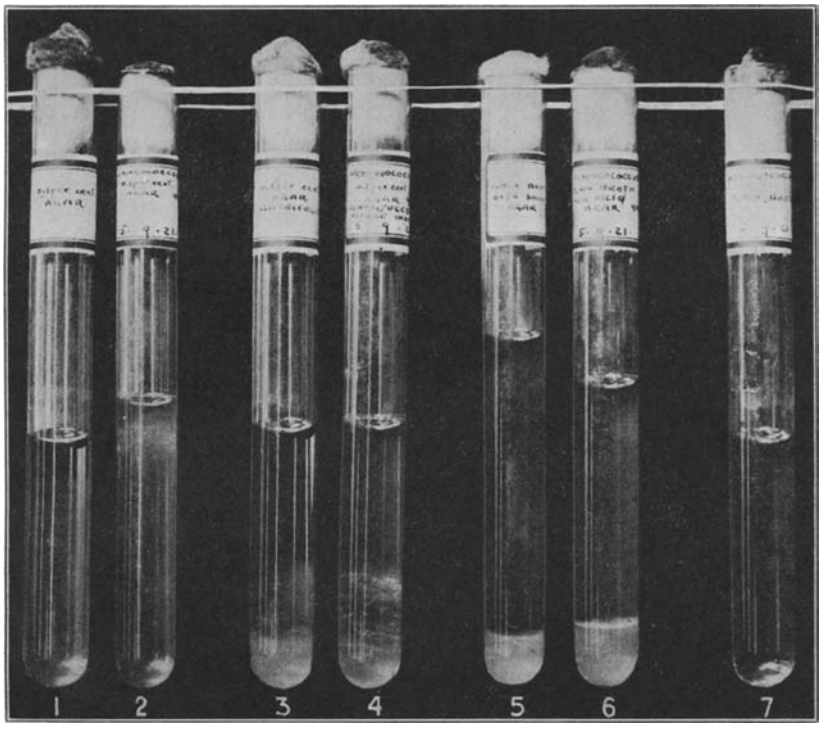

Fig. 2.—(a) Control broth. Tube 7 shows no growth, although planted at the same time. (b) Undisturbed 0.1% agar. Tube 1, uninoculated, shows clear broth above the slightly cloudy agar. Tube 2, inoculated, shows heavy granular cloud at upper level of agar with clouding of broth above. (c) 0.1% agar packed by centrifugation. Tube 3, uninoculated, shows deeper layer of clear broth with agar in the bottom of the tube and masses of agar floating above. Tube 4, inoculated, shows particles of agar accentuated by the growth surrounding them and slight clouding of the broth above. (d) Not illustrated. The appearance of the tubes was similar to "C". (e) Solid agar with control broth above. Tube 5, usinoculated; tube 6 shows growth on the surface of the agar and clouding of the broth above.

planted with various stock cultures. Naturally, any of the organisms which grow well in ordinary broth grew well in the 0.1% agar. In many instances growth in the latter was more luxuriant than in broth

or on solid agar. Several of the common contaminants from air and water, members of the colon-typhoid-dysentery group and the gram-positive cocci all grew well; then some of the more delicate bacteria were studied.

The meningococcus and the gonococcus grow most luxuriantly and in a manner which promises to add something of value to the cultivation and study of these gram-negative cocci. The luxuriance of growth suggested contamination so strongly that it was necessary to spend much time in the repeated examination of the cultures to rule out this factor. It should be added that the growth of these cocci is possibly no more luxuriant in the weak nutrient agar than in 1% starch in broth, but in the latter medium both organisms grow without differential characteristics and apparently never form pellicles. The starch jelly, however, settles out just as the agar does, and the growth seems to occur chiefly on its surface. Vedder [13] called attention to starch as favoring the growth of the gonococcus. The similarity of relations between the growth of these gram-negative cocci on starch and on agar is striking in view of the chemical and physical similarity of the two carbohydrates.

The meningococcus settles on and develops about the particles of agar, but it also grows as a difuse cloud in the fluid above the agar; after 24 or 48 hours' incubation, pellicle formation commences.[14] The pellicle increases in thickness until it falls of its own weight or when the tube is disturbed. The growth is exclusively aerobic. Some recently isolated strains grow as well as do the old laboratory strains.

Some studies have been made with a view to learning more about the function of the agar and its relation to the growth. The same lot of broth and the same lot of agar jelly were used throughout. The 0.1% agar and the control broth, treated in several ways and then inoculated, gave the following results noted and illustrated in Fig. 2. The purity of these cultures was checked by plating and by microscopic examination after staining by Gram.

(a) Control broth; no growth except of some of the more hardy strains

(b) Undisturbed 0.1% agar; heavy growth at the surface of the agar and in the supernatant broth

[13] Jour. Infect. Dis., 1915, 16, p. 385.

[14] For the collection of recently isolated strains, I am indebted to Miss Alice C. Evans, of the Hygienic Laboratory, U. S. P. H. S. One of these strains, "HL-412" grows, not in the supernatant broth, but, like the gonococcus, exclusively about the particles of agar. It ferments glucose and maltose, but not sucrose, lactose or mannit.

(c) 0.1% agar packed by centrifugation, leaving a deeper layer of broth above; gradual accentuation in outline of the particles of agar; finally good-sized grayish white granules resulting from growth on these particles; gradual clouding of the broth above.

(d) The supernatant broth from tubes centrifuged as in (c); the first attempts to remove the supernatant broth without carrying over some of the agar, were not successful. In the tubes containing a few small particles at the bottom of the tube, the results were identical with those noted under (c). A second attempt was apparently more successful, but at the bottom of even these tubes there were a few masses, almost transparent, about which there seemed to be some growth, and the broth above was slightly cloudy. In these tubes there was definite growth, but it is not possible to rule out absolutely the presence of agar.

(e) The agar remaining in the tubes after removal of the supernatant broth (d) was heated and chilled, making a homogenous solid mass in the bottom of the tube; above this was carefully placed 10 c c of the control broth; gradual accentuation of the surface of the agar with definite colony formation there and slight clouding of the supernatant broth.

The luxuriance of growth in the tube containing undisturbed 0.1% agar, the relation of growth to the agar in the tubes which had been centrifuged and the very sparse growth in the removed supernatant broth can leave no doubt on the point at issue. The agar itself forms a kind of trellis for the coccus and thereby furnishes conditions favorable to its development. Whether or not the meningococcus actually utilizes the agar in its metabolism, we do not know.

Tubes of 0.05% agar, to which had been added several of the carbohydrates and the Andrade indicator, were inoculated with various strains of the meningococcus. The change in color was somewhat slower in developing in glucose than in maltose, requiring 72 hours for some strains. Acid formation was apparent with most of the strains tested within 48 hours. Tubes containing sucrose, lactose, galactose and mannit showed no change in color.

The viability of the meningococcus in mediums containing sugars is interesting. Apparently, in contradistinction to certain other organisms, some of the sugars, especially those which are fermented with the formation of acid, act in some way to prolong the life of the organisms. Possibly they exert a protein-sparing action. After 14

days' incubation of the 14 strains tested subcultures to blood agar from sucrose showed none living; from lactose, 1 living; from mannit, 1 living, from galactose, 9 living; from maltose, 11 living; from glucose, 13 living. The average reaction of the cultures after 16 days' incubation was: sucrose, lactose, mannit and galactose, PH 8.2; glucose, PH 6.8; maltose, PH 5.8. The reaction before inoculation was about PH 7.5.

In the supernatant broth of the meningococcus cultures, there are substances poisonous to white mice.

The gonococcus does not regularly cloud the supernatant broth, but grows either on or within the upper layers of the agar. Particles of culture pushed down toward the bottom of the tube apparently do not develop; in other words, the gonococcus, except in highly nutrient mediums, prefers to grow neither aerobically nor anaerobically. The growth is from the first granular; it seems as though the gonococci, unlike the meningococci, grow exclusively in contact with the particles of agar. As in the case of the meningococcus, pellicle formation is seen, however, after 24 or 48 hours.

The virulence or toxicity of these extremely rich cultures deserves investigation.

The two explanations for pellicle formation which immediately present themselves are: first, that the organisms have exhausted the oxygen supply and find at or just below the surface of the broth the exact conditions they need; the other is that the location of growth is related in some way to surface tension and, as suggested by Larsen,[15] with increasing surface tension, resulting from growth, pellicle formation is favored.

The Army Medical School gonococcus strain 3 at the end of 20 days in a 250 c c flask containing about 200 c c 0.1% agar showed heavy granular growth and pellicle formation. Subcultures demonstrated the purity of the growth. The fluid between the pellicle and the agar below was clear. Tested by Captain Williams it gave a positive complement-fixation test with antigonococcus rabbit serum and (commercial therapeutic) antimeningococcus serum, and with the rabbit serum it gave a positive precipitin test, using the technic of Robinson and Meader.[16]

[15] Abs. Bact., 1921, 5, p. 2.
[16] Jour. Urol., 1920, 4, p. 551.

The tubercle bacillus did not grow on 0.1% agar without glcerol but it has grown luxuriantly on the medium containing 4% glycerol. The pellicle did not start to sink until it was approximately three times as thick as the maximum growth commonly seen on glycerol broth. When it did begin to fall, at the end of about 6 weeks, it settled only to the surface of the agar. The center was sufficiently supported to keep it above the fluid and at 2 months it seems the bacilli are still multiplying. It is impossible to avoid speculation with regard to the possiblities of developing antigens in such cultures.

The acne bacillus has been grown recently by Captain Davis in 0.1% agar. He tells me the growth is best if he adds glucose and glycerol to the medium, and that the development of the culture is far more luxuriant than he has been able to obtain on any of the other mediums suggested for this organism. The appearance of the growth is interesting. Those bacilli which on inoculation settled to the surface of the agar, grew there, and becoming heavier gradually sank straight downward through the agar. In settling they left paths of growth resulting in a series of perpendicular lines. The part of the growth above the agar is slightly pigmented so that the entire culture looks like a sheaf of much elongated comedones!

TESTING VACCINES AND SERUMS FOR STERILITY

It is clear from the foregoing that in 0.1% agar we have a medium peculiarly suited to the detection of bacterial contamination, whether it be aerobic or anaerobic. No special apparatus, technic or culture medium is required, and the growth obtained is likely to be more luxuriant and therefore less easily overlooked, than with the use of current methods. The method is specially applicable to the examination of those substances which, like the so-called biologic products, contain a percentage of antiseptic.

In the preparation of the medium for this purpose, it is sufficient to add the proper amount of a previously prepared and assayed agar jelly to stock broth and sterilize. With careful technic the sterile agar may be added to hot sterile broth (as we frequently add some of the less staple sugars) and used without further heating. If the medium is prepared ahead, it should, of course, be placed in a water bath or Arnold just before use to drive off suspended oxygen, and then chilled rapidly. It is our custom to control the efficiency of the heating by

placing a few drops of 1% aqueous methylene blue in an extra flask or tube; heating is continued until the methylene blue is decolorized, except for the surface of the medium.

The amount of vaccine or serum to be planted in a tube or flask of 0.1% agar depends on its content in phenol or trikresol. The preservative must be diluted to a concentration of not more than 0.01%. The Army Medical School vaccines contain 0.25% of trikresol; the maximum amount that may be added to the culture medium is therefore 1 in 25.

In applying this method in the course of our routine work it has been used up to the present as an addition to the regular tests and not as a substitute for them. Every indication so far points to its being entirely dependable.

THE PHYSICAL PROPERTIES OF LOW PERCENTAGES OF AGAR IN NUTRIENT BROTH

In seeking an explanation for these results, chemical differences between solid and semifluid agar mediums do not seem to come under consideration. This is emphasized by the luxuriant growths obtained in the water of condensation of an agar slant where the constituents are qualitatively identical. The factors of importance must therefore concern differences in the physical condition of mediums containing high and low percentages of agar. Consequently, some attention has been given to these differences with the results recorded in the following.

We have made no attempt to prepare ash-free or electrolyte-free agar or to remove the nitrogen, of which crude agar contains 6.3% [17] according to Bordet and Zunz.[18] It is to be expected that different lots of crude threads even after washing will vary in jellying power and consequently in the size of the gel mass formed at the same percentage concentration. This is true of a lot of purified and powered agar, of foreign manufacture, that has recently come into our possession. Its jellying power is about twice that of the lot of thread agar used in this work. In the earlier observations with 0.1% agar in broth we had noted that agar is not miscible with broth in all proportions but below a certain point separates as a gel leaving the clear broth above. Attention seems not to have been called to this phenomenon in culture

[17] This figure is surisingly high. If it is correct, it seems likely that some of the N must be present in some other form than as protein N.

[18] Ztschr. f. Immunitäts., I, O., 1915, 23, p. 49.

mediums heretofore. Bordet and Zuns,[19] in their work with purified agar—pararabin—prepared by the method given by Grafe,[20] noted a separation but apparently this was more in the nature of a flocculation. They say that at 0.5% the upper part of the fluid is clear, while abundant whitish floccules are deposited and that ordinary agar is more coherent and more transparent. C. R. Smith[21] makes practically the same statement with regard to gelatin.

Ash-free gelatin swells in water to about 7 or 8 volumes. If such a gelatin is melted and cooled a clear, stable jelly is produced. If, however, a weaker jelly is prepared, synaeresis takes place, with the production of a cloudy jelly. One-half per cent. jelly will floculate into jelly particles (probably 7 times swollen) and can be filtered off completely from the extruded water which shows no traces of gelatin.

In the case of low percentages of agar in broth the process is distinctly that of the formation of a gel leaving the clear broth above. The relative viscosity of plain and glucose broth and of the extruded broth from 0.05% and 0.06% plain and glucose agar was determined by permitting equal volumes to fall through a capillary tube, the time being measured in seconds. The supernatant broth has the same viscosity as that of the control broth, of the same lot, to which no agar has been added. This is an indication that, like gelatin, agar is not soluble in water at temperatures below the point at which it gels. Sometimes floccules are seen just above the jelly mass, and the gel may be broken up into flakes by shaking the tube. The gel is at first only little less clear than the broth; later, after standing around for a few days and especially if shaken to break up the gel, it may become slightly cloudy. With clear mediums, the separation may be almost invisible. If the jelly is broken up by whipping it with a capillary glass rod, the flakes settle more compactly and occupy less volume than did the undisturbed gel. Once broken up in this way there is, of course, no change in the physical state of the agar until it is heated to near the boiling point.

In the solid mediums, containing at least 1% of agar, the colloidal particles are relatively closely packed together; in fact, this is true down to a concentration of about 0.2%. The squeezing out of fluid is noted in all agar mediums; this fluid constitutes most of what we call water of condensation. It seems from our work that, at 0.2% and less, the collodial particles having reached the limit of their dissociation do not naturally remain further apart but that equal amounts of agar

[19] Loc. cit., p. 42.
[20] Abderhalden, E.: Biochemisches Handlexikon, 1911, 2, pp. 27 and 73.
[21] Jour. Am. Chem. Soc., 1921, 43 (in press).

form gel masses of equal volume regardless of any increase in the volume of broth. On standing, the gel continues to contract and may not reach its equilibrium for several days. It is rather easily packed by centrifugation, and the weight of a luxuriant growth may force it downward. It is understood that this applies only to the conditions of temperature and composition and reaction of the broth with which we have been working. We believe it will apply proportionally to all ordinary lots of agar, but the relative amounts necessary to obtain the desired synaeresis may need to be ascertained for each lot. At 0.06% of our present lot the gel finally occupies about one-half the volume of the medium. This holds for test tubes of about three-fourths inch diameter. The separation in flasks is apparently greater since the greater bulk in shrinking leaves a relatively deeper layer of broth above. At present, we prefer 0.07% of agar for test tubes and 0.1% for 250 c c Erlenmeyer flasks.

The impression that the gel is in a state of equilibrium and therefore constant in density was formed by studying the appearance of tubes in series containing graduated amounts of agar and methylene blue. The use of this dye as an indicator of anaerobiosis has received much attention recently, notably by Hall[22] and Gates and Olitzky.[23] The following percentages of agar were made up (with 1% peptone, beef infusion broth, 0.5% sodium chlorid) according to the method given in the foregoing: 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.15, 0.20, 0.25 and broth controls; in repeating these observations we started with 0.01 and went up to 2.0%; the results were the same, the tubes outside this range added nothing of value or interest. These were filled into three-fourths inch test tubes, 15 c c to each, and before sterilization, 0.1 c c 1% aqueous solution of methylene blue was added to 3 tubes of each percentage. One series received 1% of glucose, the other received none. After sterilization in the autoclave for 15 minutes the reaction of the glucose series was a little higher than PH 7.4; that of the plain series a little less than PH 7.6.

The tubes were chilled on removal from the autoclave and placed in the incubator. The depth of the blue color was measured at intervals and the tubes have been retained up to the present. The glucose exhibited clearly its reducing effect; the broth without agar required 48 hours to lose its greenish color and take on the blue of the dye. The glucose-free broth became quite blue in 18 hours.

[22] Jour. Bacteriol., 1921, 6, p. 1.
[23] Jour. Exp. Med., 1921, 33, p. 51.

The appearance of the tubes containing agar was increasingly interesting from day to day. Except for the fact that the glucose tubes always showed more reduction and less penetration, there was no noteworthy difference. It was formerly my belief that the agar in uniform suspension throughout the broth acted merely as a hindrance and that the rate of diffusion of the oxygen depended on the relative solidity or fluidity of the semisolid medium as determined by its agar content. This impression is permitted by Gates and Olitzky,[23] who, in commenting on their results, make the following statement: "Even so small an amount as 0.02% of agar may inhibit the diffusion of oxygen to the depths of the culture tube, or at least so retard it that dextrose broth is able to maintain anaerobic conditions below a certain level. This level occurred at 1 cm. from the surface when 0.5% of agar was employed." It seems worth while to quote their table since the results obtained so nearly coincide with ours:

TABLE 1

FORMATION OF OXYGEN INTO SEMISOLID MEDIUM

Tubes	Amount of Agar, %	Penetration of Oxygen from Surface of Medium		
		After 5 Hours, Cm.	After 24 Hours, Cm.	After 48 Hours, Cm.
1	0	1.0	4.0	Complete
2	0.02	1.0	1.5	3.3
3	0.04	0.9	1.2	2.2
4	0.06	0.9	1.1	1.3
5	0.1	0.8	1.0	1.1
6	0.2	0.8	1.0	1.1
7	0.5	0.7	0.9	1.0

While inspecting the first set of our tubes, 42 hours after removal from the autoclave, an important point was noted which these authors had failed to record. This refers to the synaeresis of the agar and its relation to the diffusion of oxygen into the medium. The penetration of oxygen is not only retarded, it is stopped or controlled by the agar in glucose broth. When the culture medium is still hot from the autoclave or water bath, the particles of agar are diffused through it as in a solution, but as it cools gel formation occurs in very low concentration, agar being insoluble at room and incubator temperatures; the gel mass contracts leaving the clear extruded broth above. The percentage of agar in the broth determines the relative volume of the gel mass; at 0.2% and above the entire volume of the medium is occupied. Below this point, the colloidal particles of agar tend to draw together to an equilibrium, and regardless of the relative amounts of agar and

broth the density of the gel is finally the same. The gel in this state permits the penetration of oxygen into the medium. As the gel contracts, the clear broth above becomes colored and the limit of contraction is reached only after several days. These points are illustrated in table 2.

The readings after 40 days in the incubator show that the agar, for practical purposes, permanently excludes oxygen. In the higher

TABLE 2

PENETRATION OF OXYGEN INTO CULTURE MEDIUMS CONTAINING SMALL PERCENTAGES OF AGAR AND 0.1 C C OF A 1% AQUEOUS SOLUTION OF METHYLENE BLUE—AUTOCLAVED, CHILLED, INCUBATED

Per-centages of Agar	1% Glucose					No Glucose				
	18 Hours	42 Hours	72 Hours	5 Days	40 Days	18 Hours	42 Hours	72 Hours	5 Days	40 Days
Control broth	Green	Blue green	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
0.05	0.9	2.0*	3.3	4.2†	Blue†	1.3	3.2*	4.4	5.5†	Blue†
		0.7	0.7	1.5			1.8	2.0	2.5	
0.06	0.9	1.5	2.5	3.0	‡	1.3	2.2	3.3	3.7	Blue†
		0.7	0.7	0.8			1.5	1.5	1.6	
0.07	0.8	1.3	2.3	2.8	‡	1.3	2.0	2.8	3.6	‡
		0.8	0.7	1.0			1.5	1.3	1.8	
0.08	0.8	1.1	1.6	2.0	1.5	1.3	1.8	2.4	3.0	3.0
		0.8	0.7	1.0	‡		1.4	1.3	2.0	‡
0.09	0.8	1.0	1.3	1.6	1.5	1.3	1.7	2.1	2.6	2.8
		0.7	0.8	1.0	0.5		1.5	1.3	1.6	0.8
0.1	0.8	1.0	1.3	1.5	1.6	1.3	1.8	2.1	2.3	2.5
		0.6	0.8	0.9	0.5		1.7	1.3	1.6	1.2
0.15	0.8	1.0	1.2	1.4	1.4	1.3	1.7	1.9	2.1	2.7
		0.8	0.7	1.0	0.7		1.7	1.7	1.8	1.7
0.2	0.8	1.0	1.1	1.3	1.4‡	1.3	1.7	1.9	2.2	2.3‡
		1.0	1.1	1.3	1.4		1.7	1.9	2.2	2.3
0.25	1.2	1.6	1.9	2.2	2.5	1.3	1.6	1.8	2.1	2.4
		1.6	1.9	2.2	2.3		1.6	1.8	2.1	2.4

* The upper figures represent the total depth of penetration of the oxygen, the lower, the depth of penetration into the agar, as shown by the return of color to the methylene blue. The measurements are expressed in centimeters and are taken from the bottom of the meniscus downward.

† Exact measurements are difficult on account of the separation of the agar from the sides of the tubes.

‡ Unsatisfactory for exact measurement chiefly on account of evaporation.

concentration the Liesegang rings, noted by Hall,[22] are present. These may easily be due to changes in temperature especially at times of removal of the tubes from the incubator. For comparison with the earlier readings the figures are of little value for there has been about 40% evaporation, the tubes have been brought out for examination repeatedly and the relations between agar and broth considerably disturbed. The agar, in the tubes containing 0.05%, is so small in amount it is difficult to tell how far the agar has been penetrated since it is almost surrounded by the colored broth. In the tubes containing

0.25%, the agar is penetrated farther and the color seems to be slowly advancing, in the 0.06 to the 0.2% tubes a point, practically stationary, was reached within five days. Agar itself in distilled water or in physiologic salt solution exhibits no reducing effect on methylene blue.

DISCUSSION

There are two outstanding points which make 0.1% agar of interest as a practical culture medium for the growth of bacteria. The first of these concerns the superior anaerobic qualities of the thin agar jelly, the second concerns not only the greater luxuriance of growth of many of the bacteria but also the successful cultivation of certain species which grow only with difficulty or not at all on the ordinary mediums.

With regard to the first point, our conception of the physical state of the agar seems to offer an explanation. When meat infusion broth containing agar is heated to near the boiling point, the agar goes into a state of solution and remains in this state until the temperature falls to slightly below 40 C. On cooling, the agar gels and the phenomenon of synaeresis is exhibited by the extrusion of a certain amount of the broth. The agar, insoluble in the broth, separates to form a gel in which the colloidal particles are in a state of equilibrium and apparently in the dispersing phase with the broth in the dispersed phase. This gel resists the penetration of oxygen since the undisturbed films of agar supported by the reducing action of the peptone and glucose offer a barrier to its advance. It is conceivable that in the case of solid mediums, the relations between the particles of agar and broth are modified by forces which act to destroy or fracture the continuity of the agar films. Thus, the broth, in a less confined state, is permitted to aid the penetration of oxygen but is likely to do so irregularly, depending on the degree of disturbance that has occurred in the process of cooling. The results obtained by Hall,[22] as noted in his table 4, support such an hypothesis. He found that 3% agar was penetrated more rapidly than 2 or 1%, but that 2% was penetrated slightly less rapidly than 1%.

The favorable influence of the absence of oxygen is supplemented by the thin consistency of the agar permitting ready extension of the bacteria through it by development or motility. They thus find it possible to utilize all the nutriment present in a way that is impossible in solid mediums.

On the second point, we find in the literature of the cultivation of spirochetes some strikingly suggestive statements. Especially illuminating is the observation of Noguchi:[24] "It is very important to employ samples of ascitic fluids which contain no bile, but which form a loose fibrin in the culture tube, for many specimens are unsuitable just because they contain too much bile or do not cause the formation of fibrin when mixed with the fresh tissue in the culture tube."

Zinsser, Hopkins and Ruth Gilbert,[25] in attempting to find a more simple technic for the cultivation of Treponema pallidum, found that clear fluid serum mediums are unsuitable, and it was necessary to coagulate the serum partially as Schereschewsky [26] had done originally. They say

> It is interesting to note that we were not able to grow the culture on horse serum without tissue unless the serum had been heated and gelatinized. When the serum had been heated in this way, our strain grew both with and without the addition of tissue, and not only on the horse serum but also on similarly prepared sheep and beef serum.

These observations, together with those of our own noted in the foregoing, suggest that we are dealing with a fundamental principle in the artificial cultivation of disease-producing organisms. Until the work of Noguchi, efforts of bacteriologists had been directed almost exclusively to the production of artificial culture mediums identical chemically with the particular tissues and tissue fluids most favorable to the bacterium in question, as exhibited by the part of the body in which it most frequently found parasitic residence. Bacteria are classified into facultative and strict parasites according to their ability to grow in liquid mediums or on solid mediums which may bear no physical resemblance to their tissue habitat. We consider the colon bacillus practically a saprophyte because we can cultivate it with so little difficulty—in the body, it grows in the contents of the intestine. The spirochetes and some of the gram-negative cocci, and doubtless other bacteria, seem to require formed substances resembling tissues or cells to which they may cling or on which they may settle while taking their nourishment from surrounding fluids. We may find that for certain bacteria, when placed in mediums physically similar to the tissues, chemical composition of the fluids will be of as little consequence as physical conditions are to those bacteria of which the colon bacillus is the type.

[24] Jour. Exp. Med., 1912, 16, p. 199.
[25] Ibid., 1915, 21, p. 213.
[26] Deutsch. med. Wchnschr., 1909, 35, p. 835.

SUMMARY

In agar culture mediums containing 0.1% agar, the gel, composed of colloidal particles in a state of equilibrium, resists the penetration of oxygen and consequently offers excellent conditions for the development of anaerobic bacteria. It is therefore suggested as a medium for the primary cultivation of specimens suspected of containing anaerobic bacteria, and for the more convenient study of pure cultures especially with regard to their physiologic relations. The broth above the agar is naturally an excellent culture medium for the aerobes which develop well in ordinary mediums; in addition, the presence of the agar underlying or suspended through the broth makes it, like water of condensation, a good medium for certain others, the gonococcus and meningococcus, for instance, which grow with difficulty or not at all in ordinary mediums.

Culture mediums containing low percentages of agar, since they offer at the same time suitable conditions for the development of both aerobic and anaerobic bacteria, should be valuable for bacteria requiring partial oxygen tension and for the detection of contamination in such substances as the so-called biologic products. The medium should be especially valuable for this purpose since the disinfectant generally used for the preservation of biologic products is diluted and removed from direct contact with any bacteria that may be present just as the metabolic products of the gonococcus, for instance, are diluted and removed.

It is suggested that this medium, composed of a definite stroma of agar containing droplets of broth, with a supernatant reservoir of broth, bears a certain important physical resemblance to the tissues and their fluids. With this basic medium, chemical "differentiation" may be accomplished, if necessary, through the addition of special substances. It therefore presents advantages over ordinary mediums and offers possibilities for the development of our knowledge of groups of bacteria whose study has heretofore been surrounded by great difficulties.

Not least among the points in favor of the medium is its great simplicity in that it contains only the ingredients of our most commonly used culture substrate—ordinary nutrient agar.