DIAGNOSTIC PROCEDURES AND REAGENTS

SECOND EDITION

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DIAGNOSTIC PROCEDURES AND REAGENTS

TECHNICS FOR THE
LABORATORY DIAGNOSIS AND CONTROL OF THE
COMMUNICABLE DISEASES

SECOND EDITION

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PREFACE FOR THE SECOND EDITION

THIS, the second edition of *Diagnostic Procedures and Reagents,* consists of a complete revision of the chapters in the first edition and the addition of nine new ones. Each chapter treats the subject in such a manner as to bring together clearly and succinctly the basic science and resultant technical methods.

The book is planned to offer to the student a broad concept of the biology of the diseases discussed, to bring to the public health laboratory worker the recent technical practices which are significant in the recognition of these diseases, and to provide for the epidemiologist basic information which coördinates the laboratory and field investigator.

As the one whose business it has been to assemble the manuscripts I want to thank the referees for their prompt response and their coöperation in every way in spite of the additional burdens which the war emergency has placed upon them. After this job was done it was through the energy and interest of Dr. A. Parker Hitchens, Chairman of the Coördinating Committee of the Laboratory Section, and Dr. Reginald M. Atwater and his staff, that the editing and publishing were accomplished.

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CONTENTS

Culture Media
Stains, Reagents and Solutions

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I. CULTURE MEDIA

BACTERIAL nutrition is the subject of active investigation from numerous points of view, and important additions to our knowledge are constantly resulting. Notwithstanding, we probably do not yet know the optimum requirements for the artificial cultivation of any of the bacteria; we are still working somewhat empirically. It is understandable, therefore, that universal agreement upon a single method for the preparation of each culture medium in common use has not been attained. Hence, this section can only serve to direct attention to practices and formulae which have been found dependable and are in current use. A few directions can be mandatory; the majority are recommended in the interest of uniformity and comparability of routine technical procedure.

A. WATER

Distilled water shall be used in the preparation of all culture media.

B. CHEMICALS

Sodium chloride and other chemicals shall be of C.P. grade unless otherwise specified. American Chemical Society specifications should be used whenever the highest purity is an important consideration.

C. PEPTONE

Unless otherwise specified, Bacto-peptone, or any other peptone which comparative tests have shown to give equivalent results, shall be used.

D. MEAT EXTRACT

Bacto-beef extract, or any other brand giving equivalent results, shall be used.

E. MEAT INFUSION

All meat for making culture media shall be fresh and free from preservative of any kind. It shall be carefully freed from all fat, tendons, and fascia, and then ground finely in a meat chopper.

Nutrient media for special purposes may require the use of lean

beef, beef heart, liver, brain, veal, mutton, pork, horse meat, or other flesh. Potatoes and other vegetables are in common use as special sources of nutriment.

For general purposes, beef heart will be preferred because of its superior nutrient value and its lower cost.

F. "HORMONES"

Huntoon [1] suggested that substances—"hormones"—which possess growth enhancing properties for, at least, certain bacteria are removed from infusions and broths by straining fabrics, cotton, filter paper, etc. Glass, porcelain, and metals have no such adsorbent qualities for these "hormones." Until this point has been made the subject of conclusive research, there is probably enough evidence at hand to justify preparing infusion media without repeated straining through cotton or gauze and filtration through paper. Incidentally the avoidance of straining and filtering greatly simplifies the work to be done in the kitchen. The chief difficulty lies in overcoming the prejudices of the *Dieners*—and others—against innovations of this variety.

G. "HORMONE" BROTH

1. Infuse the ground meat in distilled water overnight at 4° C. to 6° C., the proportion being 500 gm. of meat to 1 liter of water. For double strength infusion, the proportion is 500 gm. to 500 ml. The container is a stock pot, enameled, glass lined, or of stainless steel.

2. After infusion skim off the fat with a metal or enameled spoon or skimmer.

3. Heat to 45° C., and hold between 45° C. and 50° C. for 1 hour.

4. Boil for ½ hour without stirring. Lift out the firm coagulum by means of a metal or enameled strainer; the infusion may be put through a metal sieve—this must not be of copper. Glass wool is permissible.

5. Add distilled water to make the volume up to the original amount.

6. Add peptone and salt and possibly other ingredients, and apply heat until solution is accomplished.

7. Adjust the reaction, bring the broth again to the boiling point, and check the reaction.

8. Instead of filtering the broth, pour it into glass cylinders or acid pitchers and allow it to stand until suspended particles have settled; the clear fluid is decanted or siphoned off.

H. AGAR *

The functions of agar in bacteriological studies are not fully explained when it is stated that this curious substance is merely a solidifying agent for fluid culture substrates (Hitchens [2]). Agar is not soluble in water at ordinary temperatures, but is miscible in all proportions at a temperature near 96° C. Then it remains fluid and miscible until its temperature is reduced to about 45° C.; here it separates and takes on many of the properties of a sponge—more accurately, those of a gum. There is a limit to its " swell," as can be seen when it is present in nutrient broth, to the extent of about 0.1 per cent. Above 0.2 per cent the agar cannot reach the limit of its swell, and then its relation to bacterial growth undergoes a remarkable change. At 2.0 per cent the agar " sponge " is under great pressure, as evidenced by the water of syneresis (often erroneously called water of condensation) which is squeezed out upon cooling and solidifying. At 2.0 per cent its function is probably only that of a solidifying agent. Agar in such proportion that it is permitted to reach the limit of its " swell " is in a physical state which does not permit diffusion, e.g., of air, and the lower levels of a tube of it will support the growth of anaerobic organisms (the color does not return to methylene blue after its reduction, in a tube of 0.1 per cent agar, through driving off its dissolved oxygen by heat). The upper " fringes " of the agar sponge have characteristics which suggest the physical constitution of animal tissues for they support the luxuriant growth of fragile *Neisseria* without the presence of proteins ordinarily considered necessary. Certain bacteria (e.g., *Clostridium perfringens*) " coagulate " the thin agar in this state.

" Semi-solid " and " semi-fluid " are convenient but erroneous names for media containing approximately 0.1 per cent of agar; they are actually slightly viscid. A semi-solid medium contains above 0.3 per cent.

The 0.1 per cent agar is made, obviously, by adding the proper amount of powder or jelly to nutrient broth of any formula or containing any special ingredients. The peculiar qualities of thin agar media are best demonstrated when in a tube of the medium there is a layer, possibly 3 mm. deep, of clear broth above the agar. This phe-

* Marion B. Coleman advises that the agar employed should be of a high degree of purity. Repurified agars or agar reclaimed from used culture media give more consistent results than most commercial products.[16]

nomenon is often observed when 0.1 per cent of agar is added to the broth; the amount actually required depends upon a quality of the lot of agar being used; the proper amount may be slightly more or less than 0.1 per cent.

I. AGAR JELLY

When purified and powdered agar is added to clear broth, filtration or sedimentation in the autoclave may not be necessary.

If shredded agar is used, it is convenient to make it into a heavy, clarified jelly, and add the required portions to double strength infusion or broth.

The shredded agar is made wet and cut into short lengths with scissors, tied up in cheese cloth, and soaked in running water overnight. The following day it is removed from the cloth and placed in a container of suitable size—with little or no added water—and autoclaved; it is allowed to remain in the autoclave until the following morning. Then the mass is turned out on a glass plate, the sediment is cut away, and the clear parts are placed in a series of small flasks; these are covered with wrapping paper and sterilized in the autoclave.

One of the flasks is used to determine the strength or concentration of the agar jelly. A convenient method is to place 10 gm. portions in tared evaporating dishes and by gentle heat to dry the agar to constant weight—without burning. The net weight of the dry agar multiplied by 10 gives the percentage of agar in the jelly. It is advisable to take the average of at least three 10 gm. portions.

To make 2 per cent agar if the jelly is found to be 8 per cent, the following formula would be used:

Agar jelly, 8%	500 gm.
Double strength infusion or broth	500 ml.
Distilled water, q. s. ad	1,000 ml.

In certain laboratories it is considered good practice to standardize the agar jelly at 4 per cent and then to mix equal quantities of this with double strength infusion or broth to make a 2 per cent agar medium.

J. "HORMONE" AGAR

The "hormones" are preserved and much inconvenience is avoided by allowing the agar medium to clarify itself by sedimentation.

The shredded or powdered agar may be put into the meat infusion with the peptone and other ingredients, or it may be added to the finished broth. The agar is brought into solution by heat, preferably in the autoclave. Great care must be taken to avoid burning if the free flame is used.

The mixture is titrated and its reaction adjusted and checked. It is poured into stoppered funnels or other containers which have flaring or straight sides (these facilitate dumping the agar out as a solid mass); it is placed in the autoclave (preferably in the afternoon when other work is finished), and the pressure is run up to 15 lbs. The heat is turned off, and the autoclave remains closed overnight. On the following morning the masses of agar are dumped out onto a glass plate; the sediment is cut away, and the clear portions are placed in flasks and sterilized. Handling should be avoided so far as possible.

K. MURRAY'S HORMONE AGAR [1a]

It has been ascertained as a result of many observations, that when the minced meat—heart muscle or liver—is extracted in distilled water at 75°–80°C., the accessory growth factors are liberated. Then they appear to be adsorbed on the agar during its setting and are retained by it when it is later melted for filtering, clearing, or having other ingredients added. Until the agar has set the phosphates must not be allowed to precipitate, otherwise the accessory growth factors will be removed with them. The accessory growth factors are not liberated at temperature below 75° C., and they are adsorbed on the coagulum at higher temperatures than 80° C.

1. Immediately the animal is killed, secure the meat to be used, trim it and mince it finely.
2. Extract it for 3 hours with distilled water (500 gm. to the liter—for single strength) at 75° to 80° C., on a water bath.
3. Strain the extract through an enameled strainer or glass wool as directed in " G " above.
4. Melt the required weight of washed fiber, or purified powdered agar in a small part of the extract and add it to the remainder, while still hot.
5. Heat the whole rapidly at 95° to 100° C., pour it into funnels and allow it to set, as in " J " above.

The agar, in this state, may be placed in suitable containers, sterilized, and stored. For use, it is melted, the special nutrients

required are added, the reaction adjusted and again cleared by sedimentation, if necessary. It is then dispensed into tubes, bottles, etc., and sterilized.

L. FERMENTABLE SUBSTANCES—CARBOHYDRATES, ALCOHOLS, AND GLUCOSIDES

These substances shall be of the highest purity; for use in culture media they shall be dissolved in distilled water, and their solutions sterilized by filtration.*

Ordinarily the solution of the sugar is made up in 10 per cent (or 20 per cent) concentration and mixed with the previously sterilized base medium in the proportion of 0.5 per cent or 1.0 per cent, using " flame " technic. Incubation before use, to test sterility, is mandatory.

The basic medium will generally be meat extract broth. Sometimes a sugar-free meat infusion will be recommended. To prepare the latter, adjust the reaction of the infusion to pH 7.2 and plant into it an active strain of *Escherichia coli;* incubate at 30° C. to 36° C. overnight or for 8 hours if it is to be used for certain organisms (e.g., some strains of *Corynebacterium diphtheriae*) which do not grow well after excessive growth of *Escherichia coli.* Sterilize and add peptone and salt as usual. The use of *Clostridium perfringens* has been recommended as a more certain " destroyer " of the muscle sugar. Absence of sugar must be confirmed by test. A portion of the infusion is filtered and placed in a Smith fermentation tube. It is sterilized and then planted with *Escherichia coli.* After 24 hours' incubation there must be growth in the open arm but none in the closed arm.

Biologic tests of finished media are useful for purposes of control. Tubes of each lot of medium may be inoculated with microörganisms known to differ in their ability to ferment the test substance.

Certain microörganisms, e.g., gonococci and meningococci, will require a weak percentage of agar or other added ingredient to support their growth. It must be remembered that if the growth promoting substance has any buffering effect it may obscure evidence of fermentation with acid production.

M. DEHYDRATED MEDIA

Many of the media which have been found useful and have been

* If this is made the invariable rule of the laboratory, errors due to decomposition through overheating will be avoided.

adopted for general and specific purposes are now obtainable in the dehydrated state. This development has much in its favor. Media can be made from the powder without delay and the technic of preparation is simple. An important feature is that in their manufacture large amounts are made at one time by a uniform procedure. Before distribution each lot is carefully tested to insure its specific suitability and correspondence with other lots. For media intended to be used in standard or comparable tests these are matters of great importance. In laboratories where careful cost studies have been made, it has been found that the adoption of dehydrated media is a measure of economy.

II. STERILIZATION

A. DRY WALL (HOT AIR)

The sterilizers shall be packed in such a way that air may circulate freely among their contents.

The temperature shall be raised to not less than 160° C. nor more than 180° C., and shall be held between these temperatures for 2 hours. No portion of the sterilizer shall be cooler than 160° C.

B. FRACTIONAL (STREAMING STEAM—USUALLY IN THE ARNOLD STERILIZER)

The value of the Arnold sterilizer is limited. It is customary to place media in it and hold them in the streaming steam for ½ hour after the temperature has reached 100° C. This means ½ hour from the time all parts of the substances being sterilized have reached 100° C., which may require a considerable period of time and can be learned only by observing trial flasks or tubes containing thermometers.

The exposure to 100° C. for 30 minutes is repeated on 3 successive days, the media being held at room temperature during the intervals; in hot weather they should be held at about 20° C.

C. AUTOCLAVE

1. *Routine method*

Unless otherwise stated, sterilization in the autoclave is accomplished by steam at a temperature of 121° C.; this temperature is attained at a pressure of 15 lbs. (2 atmospheres) of saturated steam. Allow-

ance must be made for the length of time required to heat the media in larger flasks up to this temperature. The temperature is maintained ordinarily for 20 minutes in the case of test tubes, and 30 minutes for small flasks; large flasks will require a longer time.

Unless the autoclave has an automatic valve for releasing air from the bottom of the chamber, the pet cock must be allowed to remain open until steam is escaping freely; in the older, upright form of autoclave this may require a considerable period of time (5 to 10 minutes).

2. *Special method for blood serum and egg media in the autoclave as used in the Ontario Department of Health Laboratories*

a. Arrange the tubes of the medium in a pan or rack at the appropriate slant and cover them with paper to avoid too sudden contact with steam and protect them from dripping condensation water.
b. Place them in the autoclave; close both the door and the air outlet valve.
c. Raise the pressure gradually to 15 lbs. without letting any of the air escape.
d. Maintain this pressure for 10 minutes, or until the temperature reaches at least 100° C.
e. Open the air outlet valve so slightly that the pressure will not vary more than ½ lb.—thus allowing the condensed water and some of the confined air to escape.
f. Close the valve and continue the sterilization process for 20 minutes at 15 lbs. pressure, or 20 minutes after the temperature has reached 100° C.

III. CULTURE MEDIA FORMULAE

A. LIST

1. Solution, Peptone for Single Carbohydrates
1a. Solution, Tryptone
2. Broth, Peptone, Infusion-free
3. Broth, Peptone, Infusion-free, with Sucrose
4. Broth, Extract
5. Broth, Bacto-tryptose Difco
5a. Broth, Bacto-tryptose, with Sodium Citrate
6. Broth, Meat Infusion, Plain (For a discussion of the basic principles underlying the preparation of meat infusions see Sec. E, above.)
7. Broth, Meat Infusion, Double Strength
8. Broth, Meat Infusion, with Carbohydrate

9. Broth, Pork Infusion
10. Broth, Liver Infusion
11. Broth, Beef Infusion with Proteose for Hemolytic Streptococci
12. Broth, Brilliant Green Lactose Peptone Bile
12a. Broth, Fuchsin Lactose
13. Broth, Meat Infusion, with Dextrose and Indicator
14. Broth, Meat Infusion, with Saccharose
15. Broth, Meat Infusion, Buffered, with Dextrose
16. Broth, Avery
17. Broth, Tryptic Digest, Douglas'
18. Ground Meat Medium for Anaerobes
19. Blood Culture Medium, Kracke
20. Enrichment Media
    a. Bile-glycerol-peptone Solution
    b. Solution, Peptone Tetrathionate
21. Milk with Indicator
22. Milk with Calcium Lactate
23. Gelatin, Extract
24. Agar, Bacto-tryptose Difco
25. Agar, Extract (For a discussion of the basic principles underlying the preparation of agar media see Sec. H, above.)
25a. Agar, Tryptone-glucose-extract-milk
26. Agar, Malt Extract
27. Agar, Sabouraud's Maltose
28. Agar, Peptone Tartrate, Jordan and Harmon
29. Agar, Staphylococcus Medium of Dolman, Wilson, and Cockcroft
30. Agar, Beef Infusion
30a. Agar, Hormone Veal Peptone
31. Agar, Beef Heart Infusion
32. Agar, Liver Infusion
33. Agar, Pork Infusion
34. Agar, Double Sugar, Russell's
35. Agar, Eosin-Methylene-Blue, Holt-Harris and Teague
36. Agar, Endo's, Robinson and Rettger
37. Agar, Bismuth Sulfite, Wilson and Blair
38. Agar, Bismuth Sulfite, Hajna's Modification
39a. Agar, Sodium Desoxycholate
39b. Agar, Sodium Desoxycholate Citrate
39c. Agar, Bile Salts Citrate (Difco "SS")
40. Agar, Bile Salt, MacConkey
41. Agar, Blood
42. Agar, Bordet-Gengou Potato Blood, Modified
43. Agar, Douglas'
44. Agar, Chocolate Blood, Douglas'
44a. Agar, Chocolate Blood Difco Proteose No. 3
45. Agar, Chocolate Blood
46. Agar, Potato Glycerin Hormone
47. Agar, Serum-dextrose

48. Agar, Ascitic Fluid, with Carbohydrate
48a. Agar, Blood-Glucose-Cystine, Francis
49. Agar, McLeod's Chocolate Tellurite, Modified
50. Agar, Cystine-Tellurite-Blood, for plating
51. Agar, Semi-fluid, Hitchens
51a. Agar, Semi-fluid, as used in the Laboratories of the New York State Department of Health
52. Agar, Semi-fluid, for fermentation tests
53. Agar, Semi-fluid, for *Neisseria meningitidis*
54. Loeffler's Blood Serum Mixture
55. Dorset's Egg Medium, Modified
55a. McCoy and Chapin's Egg Yolk Medium (Francis)
55b. Egg Medium (for Vi cultures)
56. Pai's Medium (Modification of McGuigan and Frobisher)
57. Petragnani's Medium, Modified (MacNabb)
58. Woolley's Potato Egg Medium, Modified (MacNabb)
59. Löwenstein's Medium, Modified (MacNabb)
60. Löwenstein's Medium with Silica
61. Schwabacher's Egg Yolk Saline Medium
62. Mueller's Medium for Gonococci and Meningococci
63. Broth, Thioglycollate
64. Agar, Veal Infusion Glycerin

B. PREPARATION

1. *Solution, Peptone, for Single Carbohydrates*[3]

Water	1 kg.
Peptone	10 gm.
Sodium chloride	5 gm.
Chlorphenol red (0.04 per cent aqueous solution) or other suitable indicator *	10 ml.

* Andrade's indicator is more satisfactory when studying bacillary incitants of enteric disease.

a. Dissolve the peptone and salt in the water with heat if necessary.

b. Filter through paper.

c. Add the indicator and adjust to pH 7.4–7.5.

d. Dispense in convenient amounts in bottles and autoclave for 20 minutes.

e. Per 100 ml. of base, add aseptically 1 gm. of carbohydrate in sterile aqueous solution and dispense in at least 2 ml. amounts in small tubes.

f. Dispense aseptically and incubate at 37° C. for approximately 48 hours before using. The tubes may be sealed with paraffin to prevent evaporation.