which should be taken to avoid cholera. The first looks to the exclusion of living cholera spirilla from the gastro-intestinal tract; the second refers to preserving any natural resistance, particularly on the part of the intestine, to injury due to the presence of that organism in the intestinal contents. It is believed that the toxins produced by the cholera spirillum are not absorbed through intact intestinal epithelium, but gain entrace to the circulation only after the epithelium has become damaged, necrosed, or desquamated. Therefore all dietary indiscretions which might cause intestinal irritation are to be avoided. Over-fatigue and exposure to cold are to be carefully guarded against. But it appears wise not suddenly to change the ordinary mode of life, but simply to follow the usual habits in a temperate manner. The free use of alcohol appears to be extremely dangerous. Those addicted to its use are particularly susceptible, and when attacked by the disease the prognosis is bad in people of intemperate habits. Care in the character of the food is important. All articles which might carry infection should be avoided entirely, or partaken of only after being cooked. This is especially the case with water and milk, which should be heated to the boiling point. Prolonged boiling is not necessary, but to insure the death of the spirillum it is safest actually to boil liquid articles of food. It has been recommended to take acid drinks, such as very weak hydrochloric acid, as a prophylactic measure, but it seems doubtful whether this is a wise measure, in view of the danger to the digestion if sufficient acid be taken to act as an effective agent in killing the spirillum. Particular care should be exercised against infection by those attending cases of cholera. They should disinfect the hands immediately after they have come in contact with the dejecta or vomitus from the patient. A two- or three-per-cent. solution of carbolic acid, 1 in 1,000 solution of mercuric chloride, or a solution of "chloride of lime" may be used for this purpose. It is possible that peroxide of hydrogen might find useful application for this purpose and in cleansing the patient, particularly for rinsing the mouth; its freedom from toxic properties and odor and its acid reaction particularly commending it.

The public measures for preventing the spread of cholera must be directed toward the destruction of the spirillum, and, when that is not possible, the checking of its dissemination. Each case that occurs must be regarded as a possible centre from which other cases may arise. The patient must, therefore, be isolated and the utmost care taken to destroy all the spirilla in the dejecta and vomitus, on the bedding and clothing, and on the hands or persons of those attending him. As the patient must be kept as quiet as possible, the evacuations must be received in a bed-pan and disinfected before being disposed of. For this purpose milk of lime has been found very efficacious. This may be prepared as follows: One volume of quicklime, which must not be air-slaked, is placed in a large vessel containing an equal volume of water. This is gradually absorbed by the lime, which then falls to powder. Three volumes of water are then added and the whole is stirred to a thin creamy fluid. Of this equal parts should be added to the dejecta and the mixture allowed to stand for an hour, when disinfection may be considered complete. Fresh "chloride of lime" may also be used, a large heaping tablespoonful of the powder being added to each quart of the evacuations and thoroughly mixed with them. In half an hour the mixture may be regarded as harmless. The hands of the attendants should be disinfected immediately after coming in contact with the patient. The supernatant fluid obtained by mixing 2 parts of fresh "chloride of lime" and 100 parts of water, shaking, and allowing the sediment to settle, may be used, or a two- to three-per-cent. solution of carbolic acid, or 1 in 2,000 corrosive sublimate. These solutions may also be used to cleanse the patient. Sheets and linen may be soaked in 1 in 1,000 solution of corrosive sublimate, the above solution of carbolic acid, or in a three-per-cent. solution of green or soft (potash) soap, with or without the addition of five per cent. of crude carbolic acid. The fluid must completely cover the articles. The simple soap solution must act for twenty-four hours; the carbolized soap solution will disinfect within twelve hours. Weak formalin, two per cent., may also be used where its odor and irritating properties are unobjectionable. In emergencies, where facilities for carrying on such disinfection cannot be obtained, boiling or burning may be employed to destroy the spirilla.

The bodies of the dead should be disinfected or wrapped in a sheet wet with 1 in 1,000 corrosive sublimate solution, and placed in a tight coffin upon a layer of some absorbent material. The sick-room must be disinfected with sulphurous acid or formaldehyde, or thoroughly washed with 1 in 1,000 corrosive sublimate, and be well aired and dried out before being again occupied.

Harold C. Ernst.

Revised by *Edward K. Dunham.*

BACTERIOLOGICAL TECHNIQUE.—The methods for the artificial cultivation of bacteria are of fundamental importance in bacteriology, and for that reason deserve very careful consideration. Nutrient media of various kinds are used, but the three most commonly employed are bouillon, gelatin, and agar. These in turn may be variously modified as the needs of the work may require. In addition, other media are used, such as blood, serum, exudates, eggs, urine, milk, potatoes, and the like. These will be severally considered.

Bouillon.—To prepare beef tea, or bouillon as it is called, 500 gm. of lean, chopped beef (Hamburger steak) are placed in a suitable enamelled vessel or in a one-and-a-half-litre flask and 1,000 c.c. of ordinary tap water are added, and the whole is thoroughly mixed. This may now be set aside in an ice-box for twenty-four hours so as to bring the soluble constituents into solution; or, what is preferable, it may be placed in a water-bath and warmed at a temperature not exceeding 60° C. for an hour. In this way the nutrient substances are dissolved out and much time is saved. It is not desirable at this point to heat the fluid above the temperature given, inasmuch as that would lead to the coagulation of the albuminous constituents, which, if they are allowed to remain in solution, will facilitate the subsequent clarification of the medium. When the digestion is completed, whether carried out at a low temperature or in the water-bath, the liquid is strained through well-washed, starch-free muslin, or the juice may be expressed by means of a meat press. The liquid thus obtained is of a dark red appearance, due to the presence of hæmoglobin.

One thousand cubic centimetres of the meat extract are then placed in a clean flask or vessel, and 10 gm. of dry, powdered peptone (Witte's) and 5 gm. of common salt are added and the whole is then warmed at about 55° to 60° C. till the peptone has dissolved. The next step is to render the medium suitably alkaline, since bacteria as a rule require a slightly alkaline soil. This manipulation requires special care, for, if improperly done, the finished product may be cloudy, or may even have a deposit, or may even be unfit for the growth of bacteria. The clouding and the formation of a precipitate can be avoided by boiling the meat extract after adding just enough alkali to neutralize the fluid. For this purpose 5 c.c. of *normal* sodium hydrate (four-per-cent. solution) are added to the litre of meat extract. This amount is usually sufficient to make the extract neutral to litmus. The liquid is then heated in a boiling water-bath or over a free flame for about fifteen minutes, after which it is filtered through a moist plaited filter and allowed to cool to about 50° C. As stated, bacteria thrive best when the medium is slightly alkaline. Hence 10 c.c. of the normal sodium hydrate are now added to impart the desired alkalinity, after which the liquid is again boiled for twenty to thirty minutes, and finally filtered through moist paper.

Inasmuch as considerable water is usually lost by vaporization during the preparation of the medium, it is advisable either to indicate the volume at the beginning

of the operation by a suitable mark on the vessel, or, better, to take the weight of the fluid before and after heating. The difference in the volume or weight is finally made up by the addition of the corresponding amount of distilled water. The finished bouillon should make up to the original volume of meat extract, that is, 1,000 c.c.

The beef tea thus prepared is now filled into tubes or into flasks, as the case may be, and sterilized by steam. This process will be described later. It is hardly necessary to add that the bouillon after being tubed and sterilized should be perfectly clear, without a deposit, and should have a slight alkaline reaction.

For cultivating the gonococcus Thalmann recommends using the ordinary bouillon, to which has been added two-thirds to three-fourths of the amount of alkali necessary to make it neutral to phenolphthalein.

Sugar-free Bouillon.—The bouillon as just prepared always contains some sugar derived from the muscle tissue employed. For many purposes this sugar content is undesirable, and must be removed in some way. One procedure is to allow the meat extract to ferment at a low temperature, 10° to 15° C., for two days. Another is to place the meat extract at 37° C. for twenty-four hours. Neither one of these methods will give results which can be relied upon. The best procedure is to add to the meat extract a rich fluid culture of some acid-producing organism, such as Bacillus coli (Smith), or B. lactis aërogenes (Durham), and then set it aside to ferment at 37° C. for twenty-four hours or longer. The frothy liquid is then carefully neutralized by the addition of normal sodium hydrate, peptone and salt added, then boiled, cooled, and rendered alkaline according to the directions given under the preparation of bouillon. The sugar-free bouillon thus prepared does not contain indol, as might at first be supposed. It is preferable to the Dunham peptone solution mentioned below for testing for the presence of indol, since a good reaction is given in sixteen hours, whereas the cultures in Dunham's solution often require several days before giving a positive test.

Martin's Bouillon.—The thoroughly mixed meat suspension (500 gm. of chopped beef and 1,000 c.c. of water) is set aside at about 37° C. for twenty hours so as to destroy the sugar normally present. The liquid is then strained through well-washed muslin, and to 1,000 c.c. of the filtrate 5 gm. of common salt are added, after which the liquid is neutralized and finally rendered alkaline by the addition of 7 c.c. of normal alkali per litre of bouillon. Ordinary peptone is not added, inasmuch as it is likely to contain sugar. Instead, Martin adds to this bouillon an equal volume of a rich peptone solution made by digesting the stomach of a pig. This latter solution is prepared as follows: A pig's stomach is cleaned and cut up into small pieces, and to 200 gm. of this finely divided tissue 1,000 c.c. of water and 10 c.c. of concentrated hydrochloric acid are added and the mixture is set aside at 50° C. for about twelve hours. The digested fluid is then decanted through a filter of absorbent cotton and the strongly acid reaction is reduced by the addition of 25 c.c. of a sixteen-per-cent. solution of sodium hydrate. The liquid is then carefully neutralized, after which it is rendered alkaline by the addition of 7 c.c. of normal sodium hydrate per litre. The mixture of equal volumes of the sugar-free bouillon and the peptone solution is heated, filtered, and tubed or placed in flasks.

Peckham's Bouillon.—This is made by taking finely chopped beef, which must be as old as it can be obtained in order that it may be free from muscle sugar, and adding 225 gm. of it to 500 c.c. of water. The mixture is rendered slightly alkaline with sodium carbonate, after which it is placed in a water-bath at 40° C., and 4 gm. of trypsin are added. After digesting for an hour the fluid is again rendered alkaline with sodium carbonate. In from one to one and a half hours the digestion should be arrested, otherwise traces of indol may be detected. At the end of this period the mixture is boiled and strained through gauze and filtered cold through wet filter paper to remove the fat. Five grams of salt and enough water to make up to one litre are then added. The acidity of the clear straw-colored filtrate is then reduced to the desired point. The most suitable reaction for the development of colon and like bacilli is when the medium contains such an amount of free acid as to require from 20 to 30 c.c. per litre of a decinormal sodium-hydrate solution to bring it to a point neutral to phenolphthalein.

Artificial digestion of muscle tissue by means of pepsin and trypsin is resorted to in the preparation of Deycke's agar.

Dunham's Peptone Solution.—This is prepared by dissolving 10 gm. of Witte's peptone and 5 gm. of common salt in 1,000 c.c. of ordinary tap water. The solution is then tubed and sterilized by steam. This medium is used to detect the formation of indol by bacteria, but inasmuch as many organisms fail to grow in it and others require several days before giving a reaction, it has not been found to be as suitable as the sugar-free bouillon given above.

Glucose Bouillon.—This is used to test for acid and gas production. It is made by adding to the ordinary bouillon, or better to that which is sugar-free, one or two per cent. of glucose. The two-per-cent. solution is most commonly employed. The sterilization of sugar-containing media by steam requires special care to prevent oxidation of the carbohydrate present. As a rule the steaming should not exceed ten or fifteen minutes each day on three successive days.

Instead of glucose other carbohydrates may be added to the bouillon in one- or two-per-cent. concentration. Thus galactose, lactose, saccharose, maltose, dextrin, and the alcohol mannite are frequently used to differentiate between organisms which otherwise closely resemble each other.

Glycerin Bouillon.—This is especially used for cultivating the tubercle bacillus. It is made by adding five per cent. of glycerin to the ordinary bouillon. The mixture is then tubed and sterilized in the usual way.

Carbolic Bouillon.—This is made so as to contain 0.1 per cent. of carbolic acid. One gram of acid may be added to one litre of bouillon. The better procedure is to add 1 c.c. of a one-per-cent. carbolic acid to 9 c.c. of bouillon. It is advisable to incubate the tubes for several days so as to eliminate any possible contamination. The medium is useful for examining water for the colon bacillus, especially when the bacterial contents are very high. The presence of the antiseptic serves to check or prevent the growth of many organisms which would otherwise develop. It should be borne in mind that weak colon and typhoid bacilli are likewise restrained.

The tubes after inoculation with the water are incubated for twenty-four hours at 38° C., after which lactose litmus agar plates are made, which are then examined for red colonies. Of course all red colonies are not to be regarded without further study as the colon bacillus.

Parietti's Bouillon.—A mixture of carbolic acid and hydrochloric acid is first prepared by adding 4 c.c. of the latter to 100 c.c. of a five-per-cent. carbolic solution. This solution after standing a few days is added in portions of 0.1, 0.2, 0.3 c.c. to portions of 10 c.c. each of sterile bouillon.

Colored Bouillon.—Various coloring agents are added to the nutritive media in order to bring out the acid-producing or the reducing properties of bacteria. The substances which are most commonly used for this purpose are litmus, neutral red, saffranin, and sodium indigo sulphate. The first two are particularly useful, and are prepared the same as the corresponding agar or gelatin media, which see.

Gelatin.—The ordinary nutrient gelatin is really nothing more than bouillon to which ten per cent. of gelatin has been added so as to impart solidity with the additional advantage that the medium is transparent. The method of preparation is as follows: To 1,000 c.c. of the meat extract, prepared according to the directions given under bouillon, 100 gm. of the best sheet gelatin are added; likewise 10 gm. of Witte's peptone and 5 gm. of common salt. The whole is then warmed in a water-

bath at 60° C. until the gelatin has passed into solution. The liquid is then neutralized and enough alkali added in excess so as to impart a suitable alkalinity. As ordinarily prepared the nutrient gelatin requires from 30 to 35 c.c. of normal alkali to effect neutralization. An additional 10 c.c. will give the desired alkalinity. Hence 40 c.c. of the normal alkali may be added at once and the liquid tested with litmus paper. If the liquid is not distinctly alkaline more of the reagent may be added until the desired alkalinity is obtained. The method of standardizing media by means of phenolphthalein will be given later.

When the proper amount of alkali has been added to the gelatin solution the latter is then placed in a water-bath, the water of which is then raised to the boiling temperature. The gelatin is kept immersed in the actively boiling water for three-quarters to one hour. The albuminous constituents of the meat extract coagulate in flakes, and at the same time clarify the liquid so that on subsequent filtration the gelatin will be perfectly clear. The gelatin is then filtered through a plaited filter, which should, however, be first warmed by passing through it several hundred cubic centimetres of boiling water. If the paper and funnel are sufficiently warmed in this way there is no likelihood of the gelatin solidifying on the filter. The filtered gelatin should be perfectly clear, should possess a slight alkaline reaction, and should solidify when cooled under running tap water. If it meets these requirements it is then filled into sterile tubes to a depth of one and a half to two inches, and the entire lot of tubes are then sterilized by steaming for a quarter of an hour on each of three consecutive days.

Whenever nutrient gelatin is mentioned in bacteriological work it is understood to be a ten-per-cent. solution. This medium melts at about 23° C. That is warm summer temperature, and for that reason it is sometimes advisable to add more gelatin to the preparation in order to make it more solid. A twelve- or even a fifteen-per-cent. solution of gelatin is used under these conditions. Again, at other times it is desirable to employ a gelatin which is relatively quite soft, and in that case a five- or eight-per-cent. solution may be made use of. Obviously the amount of alkali necessary to neutralize such media will vary from that required for the ordinary gelatin. The great value of the gelatin medium lies in the fact that it can be readily melted and again resolidified, and in its transparency. Moreover, many bacteria give rise to soluble ferments or enzymes which peptonize or liquefy the gelatin, whereas others are not able to do this. It becomes possible therefore to divide bacteria into two large groups, according as to whether they liquefy or do not liquefy gelatin.

Glucose Gelatin.—This is made by adding to the clear filtered gelatin, prepared as above, two per cent. of glucose. The material is then tubed and sterilized the same as ordinary gelatin. This medium is particularly useful for the cultivation of anaërobic bacteria.

Glucose Litmus Gelatin.—To the glucose gelatin a concentrated solution of litmus is added so as to impart to the medium a deep blue color. This is then tubed and sterilized. During the steaming of this medium the litmus is usually decolored, but on subsequent cooling the blue color returns. If such a medium is overheated in the process of sterilization the sugar will be altered, and as a result the color of the litmus will change to more or less of a red.

For special purposes other sugars may be added to the gelatin, as in the case of bouillon. A lactose litmus gelatin is very useful in differentiating various organisms. The amount added is usually one or two per cent.

Elsner's Medium.—The addition of gelatin to a potato extract, instead of to a meat infusion, was first resorted to by Holz. Elsner's medium is essentially Holz's potato gelatin, to which one per cent. of potassium iodide is added. It can be used to good advantage in differentiating between the typhoid and colon bacilli, but at the same time it should be remembered that it does not afford an absolute means of detecting the former organism. The method of preparation is as follows: 1,000 gm. of well-cleaned potatoes are cut up into lumps which are then mashed as fine as possible, which can be done best by passing the material through a fruit press. The finely mashed potatoes are then placed in a meat press and pressure is applied. In this way about 400 c.c. of a dark liquid is obtained from the kilogram of potatoes. The potato juice is then set aside in an ice chest overnight, after which it is filtered through cotton. Ten per cent. of gelatin and one per cent. of potassium iodide are then added to the dark liquid, and the mixture is warmed at about 40° C. until the gelatin melts. Inasmuch as the reaction of this material varies considerably it is necessary now to determine the exact degree of acidity present, and then to reduce this by the addition of the proper amount of alkali, so that the resulting medium has an acidity such that it would require the addition of 20 c.c. of normal alkali per litre to make the solution neutral. The acidity of the gelatin is determined by titrating a portion, say 10 c.c., with decinormal sodium hydrate, using litmus paper as an indicator. If, for example, 10 c.c. require 3.2 c.c. of the decinormal alkali, it will be necessary to reduce the acidity by adding 1.2 c.c. of decinormal alkali, or better 0.12 c.c. of normal alkali for every 10 c.c. of gelatin made. When the proper degree of acidity has been imparted to the medium, the gelatin is then placed in a boiling water-bath for three-quarters of an hour until all the proteids have coagulated, after which it is filtered through paper, filled into sterile tubes, and sterilized by steaming for fifteen minutes on each of three consecutive days.

Fish Gelatin.—Five hundred grams of chopped fish are added to 1,000 c.c. of water, and the material is digested the same as given above for ordinary gelatin. To the strained liquid 100 gm. of gelatin, 40 gm. of salt, 5 gm. of glycerin, and 5 gm. of asparagin are added, and the mixture when perfectly fluid is rendered slightly alkaline. It is then heated, tubed, and sterilized as above. This medium is particularly useful for the growth of phosphorescing bacteria.

Nutrient Agar.—One drawback to the ordinary gelatin is that it cannot be used as a solid medium at temperatures above 23° C. This has led to the introduction of agar-agar as a stiffening agent. This substance is a seaweed gathered off the coast of Asia. It has no nutritive qualities of its own, and is unacted upon by bacteria. The preparation of nutrient agar is very simple. Ordinary bouillon is first made according to the directions already given. The agar may be obtained as a powder or in threads; in the latter case the agar is cut up into very small pieces, and 20 gm. (two per cent.) is then added to the litre of bouillon, which should be in a large flask, or, better, in an enamelled jar. The vessel and contents should then be weighed, after which the liquid should be gently boiled until the agar has completely dissolved. The vessel is now again weighed, and the difference between the two weights is made up by the addition of the proper amount of distilled water.

It is advisable to place the agar now in a water-bath at about 50° C. for several hours in order to allow the sediment to settle as much as possible. The filtration of a two-per-cent. agar is a very slow and tedious process even when carried out in a steam sterilizer. It is sufficient for practically all purposes to filter through a layer of cotton. The filtrate thus obtained is almost, if not entirely, clear, and whatever little sediment may be present does not in the least interfere with the usefulness of the medium.

The filter is prepared by placing a piece of ordinary cotton, about two inches square, in the angle of a large funnel, and then while it is held down by means of a glass rod, a litre or so of very hot water is passed through, once or twice, so as thoroughly to warm the funnel. Eventually the sedimented agar is carefully and slowly decanted on to the cotton filter. If desirable the agar can be filtered a second time. A very convenient arrangement for the rapid filtration of agar through cotton is shown in Fig. 5052. This consists essentially of

a Witte's perforated porcelain plate, which is steadied in place in the funnel by means of a glass rod which passes through the centre. The plate is covered with a layer of cotton on which a similar porcelain plate is placed to prevent the cotton from floating. The funnel is inserted into a strong vacuum flask, which is connected with a Chapman air pump. Boiling water is first passed through the filter to warm it thoroughly, after which the agar is added and suction applied. As soon as the pump begins to act the top plate can be removed.

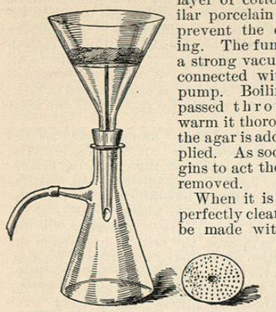

FIG. 5052.—Filtration Through Cotton Over a Porcelain Plate. (Novy.)

When it is desired to make a perfectly clear medium it should be made with only one or one and a half per cent. of agar instead of two per cent., as given above. Such agar is softer and can be passed through a previously moistened filter paper, especially if the funnel is placed in a steam sterilizer or in a funnel-shaped copper water-bath, such as is shown in Figs. 5053 and 5054.

The filtered agar is then tubed and sterilized by steaming one-half hour on each of three consecutive days, after which it is kept in an upright position. When it is desired to make inclined or slant agar tubes, as many of these as are needed are melted in a water-bath and then inclined so that the agar comes within an inch of the cotton plug.

Thalmann's Agar.—Five hundred grams of meat are boiled for one-quarter of an hour with 1,000 c.c. of distilled water, after which the mass is made up to the original weight and strained through muslin. One per cent. of peptone and 0.5 per cent. of salt are then added, and the liquid is boiled, after which it is again made up to the original weight, cooled, and filtered. One and one-half per cent. agar is then added and the weighed liquid is boiled in a concentrated salt-water bath for about three-quarters of an hour, after which it is again made up to the original weight. Thirty cubic centimetres are then titrated with normal sodium hydrate, using phenolphthalein as an indicator. The amount of alkali necessary to neutralize the entire amount of agar is ascertained and two-thirds of this quantity is then added, in portions and while shaking, to the agar. After heating fifteen minutes the material is filled into tubes.

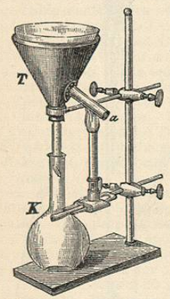

FIG. 5053.—Double-Walled Hot-Water Funnel.

According to Thalmann and others this medium is adapted for the cultivation of the gonococcus, especially for diagnostic purposes. A little of the pus is mixed with the water of condensation, and then by means of a wire, rod, or cotton swab the suspension is thoroughly spread over the surface of a series of inclined tubes or over Petri dishes. These when kept for twenty-four hours at 36°–37° show small, glistening colonies, which are single or confluent and appear like highly refractive drops.

The medium is not suitable for subcultures, and Thalmann recommends that the colonies be transplanted to serum bouillon. This is prepared by adding to some bouillon two-thirds to three-fourths of the amount of alkali needed to neutralize the liquid. After heating and filtering, an equal volume of hog serum is added and the mixture tubed. The tubes are inclined and heated for one to two hours at 70° on the first and also on the second day, and for one hour at 100° on the third day. According to Wassermann hog serum is just as good as human serum for cultivating the gonococcus.

Glucose Agar.—This is made by adding to the filtered agar, or to so much of it as may be wanted, two per cent. of glucose. The medium is then tubed and sterilized in the ordinary way. It is used especially for the growth of yeasts and anaërobic bacteria. If desired it may be colored with litmus as in the case of gelatin.

Rothberger's Neutral-red Agar.—This can be made by adding to a 0.3 per-cent. glucose agar one per cent. of a saturated aqueous neutral red solution. The typhoid bacillus does not change the color or produce gas, whereas the colon discharges the red and leaves a fluorescing color. The inoculation can be made either by planting a shake culture or by making a stab culture, which can then be covered with a layer of agar to exclude air changes. The addition of neutral red to bouillon is of service in water examinations (Irons, Jordan).

Lactose Litmus Agar.—This medium was introduced by Wurtz, and is very useful in differentiating between the typhoid and colon bacilli. Acid formation in the case of the latter is indicated by a change in the reaction of the litmus. If this medium is made by the addition of two per cent. of lactose and litmus to the ordinary agar it will be found that even typhoid bacilli will give a slight acid reaction. This, however, is not due to the fermentation of the lactose, but to the small amounts of muscle sugar derived from the meat. It is therefore desirable that the agar for this purpose should be made out of sugar-free bouillon, which can be prepared according to the directions already given. Prolonged boiling of the agar must be avoided, inasmuch as the agar itself, since it is a complex carbohydrate, may split off some sugar.

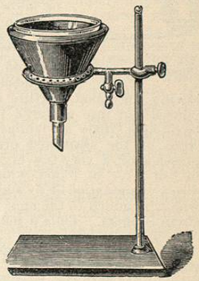

FIG. 5054.—Single-Wall Hot-Water Funnel with Ring Burner.

It is often preferable to make the plain lactose agar and to add to the tubed and sterilized medium, whenever needed, by means of a sterile pipette, a sterile litmus solution. Obviously other indicators, such as rosolic acid, neutral red, etc., may be added in the same way.

Glycerin Agar.—To the ordinary nutrient agar prepared as above, five per cent. of glycerin is added. The addition of glycerin serves to keep the surface of the medium moist, and at the same time imparts nutritive qualities to the agar. This medium is very valuable for the growth of diphtheria, glanders, pneumonia, and tubercle bacilli.

Mannite Agar.—Mannite, which like glycerin is a polyatomic alcohol, was first used by Norris and Hiss as a means of differentiating the typhoid from the dysentery bacillus. The latter organism does not give rise to acid production when grown on mannite media, whereas the typhoid bacillus does. The agar should be prepared from sugar-free bouillon, and to it one or two per cent. of mannite is then added. Litmus may be added to the bulk medium before it is tubed, or the sterile litmus solution may be added to the sterile tubed agar by means of a pipette whenever needed.

Pfeiffer's Blood Agar.—This is made by spreading over the surface of ordinary inclined agar a few drops of human blood. On the surface thus prepared he was able to cultivate the influenza bacillus. The blood from the

lower animals can be used in like manner to good advantage.

The human blood required for this and similar purposes can be drawn without difficulty by means of a sterile syringe from the large median veins just below the flexure of the elbow. The superficial circulation should first be diminished by means of a rubber tube tied about the middle of the arm. The surface of the skin over the vein to be punctured is thoroughly cleaned by means of a disinfecting solution, such as mercuric chloride or lysol. The needle of the sterile syringe is then introduced into the vein, and as the piston is slowly withdrawn the syringe fills with blood. Five or ten cubic centimetres of blood can thus be obtained in a few minutes. When the needle is withdrawn a compress of cotton, soaked in mercuric chloride, should be applied to the wound. The blood must be at once transferred to either the surface of inclined agar or to previously melted agar, cooled to 50° C. In the latter case it is mixed at once, and the tube is then set aside in an inclined position to solidify.

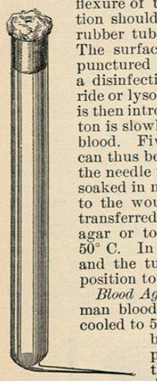

FIG. 5055.—Blood Pipette, Novy Form.

Blood Agar Mixture.—As just described human blood may be mixed with melted agar, cooled to 50° C., after which the mixture may be allowed to solidify in an inclined position. For diagnostic purposes this procedure has been utilized to detect the presence in the blood of typhoid bacilli, gonococci, and other organisms. Instead, however, of allowing the blood mixture to solidify in the tube it is poured out into a sterile Petri dish, and in this way a blood-agar plate is obtained, on which eventually colonies of the suspected organism may develop. The presence of a very few organisms can thus be detected in 1 or 2 c.c. of blood, which would not be possible by direct examination or by staining. The amount of blood which is added to the agar may be varied according to circumstances. Thus it may be one to four, one to two, or even one to one.

Blood from the lower animals can be drawn under strictly aseptic conditions into sterile Nuttall's blood pipettes, or into the modified form of Novy, shown in Fig. 5055. This can be easily made from test tubes of various sizes, according to the kind of animal to be bled. Thus a five-eighth by five-inch test tube may be used for bleeding a mouse or rat, while a one- by eight-inch tube would be used in the case of a rabbit. The bottom of the test tube and the end of a piece of glass tubing are softened in the flame of a blast lamp and then brought together. A narrow blast flame is then directed against the test tube about an inch from the bottom. On slow rotation in a horizontal position a thickened constriction results, and as soon as this is sufficiently thick the two ends are pushed apart slowly. A tapering capillary results, which is then sealed in the flame at a point about two inches from the tube proper. The tube is then plugged with cotton and sterilized by dry heat. When it is desired to prepare sterile defibrinated blood a drawn-out tube or a narrow glass rod is passed through the centre of the plug. By moving this about, after the blood has been received in the pipette, complete defibrination can be obtained, and that without any contamination from the outside.

In the case of the larger animals the blood is best drawn from the carotid artery. For this purpose the animal is anæsthetized and the artery exposed for about an inch. After the first incision it is advisable to avoid the use of cutting instruments, and instead to separate the tissues with the fingers. Pressure forceps are then applied at the distal end of the artery. Another pair are then applied about an inch below this point. A finger is then placed under the clamped portion of the artery and a very slight opening is made into the blood-vessel. The blades of a very narrow pointed pair of forceps are then introduced into the opening, and, when distended, the tip of the sterile blood pipette can readily be introduced. Before this is done, however, the tip should be scratched with a file, then broken off, and the open end should be flamed for a moment to insure sterility and to round off the sharp edge. As soon as the pipette is in position the lower clamp is removed, when the blood rapidly rises in the tube. If defibrinated blood is desired, the blood should be stirred by an assistant. When scrum is wanted this stirring is omitted. As soon as blood ceases to flow, the pipette is removed and the tip is sealed in the blast lamp.

Obviously in the case of small animals, such as the mouse or rat, this procedure is not applicable. The blood may be drawn up into a syringe from the artery. A much better way, however, which has been used for several years in the author's laboratory, is to take the blood directly from the heart into a small pipette of the same form as that used for the larger animals. For this purpose the thorax is opened, the heart is freed from the pericardium and raised by means of oval-tipped forceps. The end of the pipette is then introduced into the right ventricle. Suction may be applied to the end of the pipette in order to obtain the fullest possible yield.

Blood can be drawn from very large animals, such as the horse, by introducing a trocar into the jugular vein. This is the procedure which is followed in the preparation of antitoxins. The trocar is connected by means of a short rubber tube with a glass tube, which is inserted into the receiving cylinder. In this way several litres of blood can be drawn from the horse at each bleeding.

In ordinary laboratory work the blood which has been collected in the glass pipettes is then transferred to melted agar, which has been previously cooled in the water-bath to 50° C. The amount of blood which is added to each tube will vary with the purpose in view. It may be one part of defibrinated blood to ten of agar, or one to five, one to two, or one to one, as the case may be. Exceptionally two to one and three to one are used. The blood is then mixed with the agar and the tubes are set aside to solidify in an inclined position. The transfer of the blood to the tubes is best accomplished by means of a sterile drawn-out bulb pipette, such as is shown in Fig. 5084, *e*.

The blood agar thus prepared requires no further sterilization, for if the operation has been properly carried out no organisms will be present. The tubes can be used for culture purposes at once, or they may be kept for several days to allow any organisms which might be present to develop. This blood medium is invaluable for the cultivation of various pathogenic organisms. On such media it has been possible, for example, to grow for the first time pathogenic protozoa—the trypanosoma Lewisi of rats and the trypanosoma Brucei, the cause of nagana or the tsetse fly disease (Novy and McNeal).

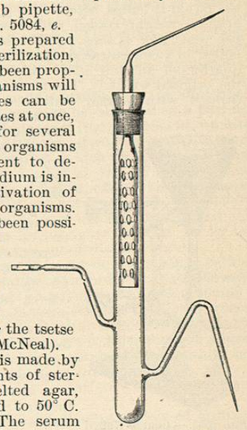

FIG. 5056.—Blood Pipette of Latapie.

Serum Agar.—This is made by adding variable amounts of sterile serum to the melted agar, which has been cooled to 50° C. in the water-bath. The serum can be obtained by collecting the blood, as given above, in sterile pipettes. The blood is allowed to clot, and eventually when the serum has separated it can be drawn up into sterile bulb pipettes and transferred to the melted agar. The largest yield of serum is obtained by using the Latapie pipette shown in Fig. 5056. This consists of an inner tube, which is

freely perforated and the narrow, outer end of which is drawn out into a capillary for insertion into the blood-vessel. This tube is held in position within the outer one by means of a rubber stopper. The outer receiving tube, which is about an inch in diameter, is provided with two side tubes, one of which is drawn out and sealed while the other is plugged with cotton. The entire pipette is first sterilized by steaming in an autoclave. The tip of the inner tube is then broken, flamed, and inserted into the carotid artery of a rabbit or other animal. The blood should not fill the pipette beyond the inner tube. The tip is then sealed and the pipette is allowed to remain in a vertical position until the blood has firmly clotted. It is then inverted and the serum, as it is squeezed out of the clot, falls to the bottom. The purpose of the perforated inner tube is to allow more complete shrinking of the clot. The serum drains away at once from the clot, and is therefore perfectly clear. When it is desired to remove the serum the tip of the side tube is scratched with a file, then broken off, and the end is flamed to insure absence of bacteria. The tube is then inserted into a sterile test tube or flask and by blowing into the other side tube the serum is forced out. It can then be distributed to the agar tubes by means of a sterile bulb pipette. These are then allowed to solidify in an inclined position. As in the case of blood agar the medium prepared in this way is perfectly sterile, if the manipulation is properly carried out. Inasmuch as sterilization by heat is avoided, the proteid constituents of the serum remain in as near to the native condition as possible. Such serum agar makes an excellent medium for the pneumococcus and for other organisms. Obviously, serum-agar plates can be prepared, if it is so desired, in which case the melted and cooled agar is inoculated with the organism to be cultivated, after which the serum is added and mixed with the agar, which is then poured out into sterile Petri dishes.

Serum agar, made by adding human blood serum to melted agar, has been used for the cultivation of the gonococcus (Wertheimer). Ascitic or pleuritic fluid may also be added to agar in the proportion of one part of the fluid to two parts of the agar. Such agar is used especially for the cultivation of the gonococcus. The ascitic, pleuritic, or hydrocele fluids may be sterilized by fractional sterilization or by filtration through a Berkefeld filter under pressure.

Wassermann's Serum-Nutrose Agar.—This also has been found useful for cultivating the gonococcus. Five cubic centimetres of hog serum are added to 30 to 35 c.c. of water, 2–3 c.c. of glycerin, and 0.8–0.9 gm. of nutrose. Nutrose is a sodium phosphate casein compound, and when added to serum prevents coagulation on boiling. The solution is boiled for twenty minutes, after which it is added in equal parts to two per cent. peptone agar in test tubes. This mixture is then poured into Petri dishes. Nutrose has been used also in the preparation of the Drigalski-Conradi agar. Hog serum, which is said to be as good as human serum for cultivating the gonococcus, has been employed also by Thalmann.

Drigalski and Conradi Agar.—This is a meat-peptone-nutrose agar containing lactose, litmus, and crystal violet. The preparation is as follows: 1. A mixture of three pounds of meat and two litres of water is allowed to stand for twenty-four hours; the expressed meat juice is then boiled for one hour and filtered. To the filtrate are added 20 gm. of Witte's peptone, 20 gm. of nutrose, 10 gm. of sodium chloride, and the whole is boiled for one hour and filtered. To this filtrate 60 gm. of agar are added and the liquid is boiled for three hours, or one hour in an autoclave. It is then rendered alkaline to litmus paper, boiled half an hour, and filtered. 2. A solution of litmus is prepared according to Kubel-Tiemann as follows: The powdered commercial litmus is repeatedly extracted with hot distilled water. The liquid is acidulated with dilute acetic acid and evaporated to syrupy consistence on a water-bath. The thick fluid is then diluted by the gradual addition of ninety-per-cent. alcohol, transferred to a flask, and an excess of ninety-per-cent. alcohol is added. This precipitates the blue pigment, while the red dye and the potassium acetate remain in solution. The precipitate is filtered and washed with alcohol, then dissolved in distilled water, after which the solution is warmed and filtered. The filtrate is then added gradually to very dilute sulphuric acid (one or two drops of acid to 200 c.c. of water) till the color changes to a wine red. The concentrated blue is then added till the blue color is restored; 260 c.c. of this litmus solution are boiled for ten minutes, then 30 gm. of pure lactose are added, and the boiling is continued for fifteen minutes. 3. The hot litmus is added to the hot agar, mixed, and the reaction is made slightly alkaline; 4 c.c. of a hot sterile solution of ten-per-cent. anhydrous soda and 20 c.c. of a freshly prepared solution of 0.1 gm. of crystal violet in 100 c.c. of warm sterile water are then added, after which the material is filled into tubes or flasks. Excessive heating should be avoided, inasmuch as it alters the lactose. The crystal violet is intended to restrict the development of the unimportant bacteria.

The Drigalski-Conradi medium has been recommended for the isolation of the typhoid bacillus. For this purpose the fæces should be diluted with ten to twenty volumes of salt solution. The authors employ large plates, 15–20 cm. in diameter. The agar is poured into the dishes to a depth of at least 2 mm., and the cover is then kept off till the moisture has dried off the surface of the agar. By means of a 5 mm. glass rod, bent at right angles and previously dipped in the suspension, a series of streaks are made over a number of the dishes. The inoculated plates are then kept at 37° C. for twenty-four hours. The colon colonies are large, opaque, and red, while the typhoid are small, glassy, and resemble dew drops. The further identification of the suspected colony is made by applying the agglutination test and by growing in Rothberger's neutral red agar.

Gelatin Agar.—Several formulas have been proposed for the preparation of this medium. Each finds its special application. That of Capaldi was recommended for the isolation of the typhoid bacillus from fæces. It is made by dissolving 20 gm. of Witte's peptone, 10 gm. of gelatin, 10 gm. of glucose or of mannite, 5 gm. of sodium chloride, and 5 gm. of potassium chloride in 1,000 c.c. of water. The solution is filtered and two per cent. of agar is added and dissolved by boiling, after which it is rendered alkaline by the addition of 10 c.c. of normal alkali. The filtered solution is then tubed and sterilized by steaming.

Stoddart's medium is a gelatin agar which contains five per cent. of gelatin, one per cent. of peptone, and a half per cent. each of agar and of salt. A litre of meat extract is prepared in the usual way. In this 10 gm. of peptone and 5 gm. of salt are dissolved, and the solution is then divided into two parts. To one portion ten per cent. of gelatin is added, and when this has dissolved, the solution is neutralized and an excess of 10 c.c. of normal alkali per litre is added. The other half of the meat extract is likewise neutralized, and then 10 c.c. of the normal alkali are added per litre to impart the requisite reaction. The liquid is then measured or weighed, boiled, and filtered. Five grams of cut agar are added to the bouillon, which is then boiled until the agar dissolves. Distilled water is added to make up to the original volume or weight, after which the two liquids are combined and allowed to sediment. The entire product is finally filtered through cotton or, better, through paper. The medium is filled into tubes which are then steamed for fifteen minutes on each of three consecutive days. To use this medium, it is poured out into sterile Petri dishes, and when solidified the centre is touched with the organism to be tested. The typhoid bacillus, on account of its motility, spreads rapidly over the surface as an almost transparent growth, whereas that of the colon bacillus spreads less and is easily visible.

Guarnieri's gelatin agar is made in a somewhat similar

manner. Three grams of powdered agar are emulsified with 50 c.c. of distilled water, and this is then added to a solution of 50 gm. of gelatin in 750 c.c. of meat extract. The whole is boiled till the agar has dissolved, when a solution of 25 gm. of Witte's peptone and 5 gm. of salt are added. The entire liquid, which now makes up to one litre, is then carefully neutralized with normal alkali, using litmus as an indicator. The medium is tubed and sterilized as usual. It has been used to advantage in the cultivation of the pneumococcus.

Weil's Meat-Potato Agar.—The potato juice is prepared as in the method of Holz or Elsner; 300 c.c. of this are added to 200 c.c. of slightly alkaline bouillon; 3.75 gm. of agar are then dissolved in the liquid, thus yielding a 0.75-per-cent. agar solution. The typhoid bacillus presents threaded colonies on this medium, the same as in Elsner, Hiss, and Piorkowski media.

Substitutes for the Meat Infusion.—In the preparation of the foregoing media a meat infusion served as the basis in each case. In special instances, but not as routine procedure, these media may be modified by using the commercial Liebig's beef extract in place of the meat infusion. The chief advantage lies in the fact that the beef extract can be kept always on hand. At the same time it must be remembered that media made up with such extract are by no means as nutritious as those made up with the meat infusion. The amount of Liebig's extract which is used varies with different workers. In general, from 1 to 3 gm. are added to one litre of water; 5 and even 10 gm. are also used. To this solution peptone and salt may be added in the usual amounts. The liquid when rendered alkaline and filtered constitutes a Liebig's extract bouillon. In the same way gelatin and agar media are prepared.

Peptone Substitutes.—Several compounds have been suggested as substitutes for Witte's peptone. In Martin's and Peckham's bouillon and in Deycke's agar this peptone is replaced by that which is formed by the digestion of the muscle tissue. In other media derivatives of albumen or casein are employed. Heyden's "Nährstoff" is a digested egg albuminate, while nutrose is a casein compound. The addition of lecithin, protogen, and hæmoglobin, etc., is made with the object of improving the nutritive qualities of the media.

Hiss' Tube Medium.—This is used as a means of testing for the typhoid bacillus. It is made by adding 5 gm. of Liebig's extract, 5 gm. of salt, and 5 gm. of agar to 1,000 c.c. of water. The mixture is then heated until the agar has dissolved, after which the water which is lost by evaporation is replaced and then eight per cent. gelatin is added. As soon as the gelatin has dissolved, the liquid is partially neutralized by the addition of normal alkali. The reaction is left acid, and to such an extent that it would require 15 c.c. of normal alkali per litre to make the solution neutral to phenolphthalein. The liquid is then cooled to 60° C. and cleared by the addition of the white of an egg stirred up in about 25 c.c. of water. The liquid is then boiled for a few minutes, after which 10 gm. of glucose are added. After sedimentation at 50° C. the medium can be filtered through paper or cotton and tubed. This medium is used only for stab cultures. Diffusion of the growth through the medium in the case of very motile organisms, such as the typhoid bacillus and the production or absence of gas, are the criteria sought for.

Hiss' Plate Medium.—Hiss utilized the tendency of the typhoid bacillus to form threaded colonies when grown on soft media, as a means of differentiation from the colon bacillus. The medium, as first proposed, contained 15 gm. of agar, 15 gm. of gelatin, 5 gm. each of Liebig's extract and of sodium chloride, 10 gm. of dextrose, and 1,000 c.c. of distilled water. This was cleared by the addition of the whites of two eggs and filtered through absorbent cotton. The reaction was left acid, and of such extent that it would require the addition of 2 c.c. of normal alkali to make it neutral to phenolphthalein. Subsequently Hiss made various modifications of this formula, eliminating the unnecessary constituents. The simplest combination, which was found to give excellent results, was made by adding 15 gm. of agar and 5 gm. of Liebig's extract to 1,000 c.c. of distilled water. No acid or alkali was added. The medium was cleared by the whites of two eggs and filtered through cotton. Plate cultures, made at 37° C., show excellent differentiation between the colonies of typhoid and colon bacilli in twenty-four hours. The former show threaded colonies, the latter do not.

Hesse's Nährstoff-Heyden Agar.—The "Nährstoff-Heyden" is an albumose made from egg albumen. It should first be stirred up in a beaker with a little water, and then added to the liquid. For cultivating the tubercle bacillus the medium consists of: 5 gm. Nährstoff-Heyden, 5 gm. salt, 30 gm. glycerin, 10 gm. agar, and 1,000 c.c. of distilled water; 5 c.c. of normal soda solution are added. The latter represents a 14.3 per cent. of the crystalline salt ($Na_2CO_3 + 10\ H_2O$) and not 28.6 per cent., as stated by Hesse. The Hesse-Niedner agar, which has been recommended for the study of water bacteria, is made by dissolving 7.5 gm. of Nährstoff-Heyden and 12.5 gm. of agar in 1,000 c.c. of distilled water. Gage and Phelps dissolved one per cent. each of agar and of the Nährstoff in 1,000 c.c. of distilled water, and made the solution neutral to phenolphthalein.

Blood Serum.—The preparation of serum from small animals has been described at length under serum agar. When it is desirable to use large quantities of serum it is advisable to collect ox blood at a slaughter-house. The more care taken in collecting the blood under aseptic conditions the less troublesome will be the subsequent sterilization. A convenient receptacle is a half-gallon battery jar covered with paper and previously sterilized. The spurting blood is received directly into the jar, after which the paper cap is replaced and the blood set aside until it firmly clots. It can then be transported to the laboratory and set aside in a cool place for the serum to separate. The serum can then be drawn up by means of an aspirator into a sterile globe receiver, such as is shown as a part of Fig. 5060. It can then be conveniently filled into test tubes or into flasks.

The earliest method of sterilizing blood serum is that of Koch by fractional heating. The tubes were placed in an inclined position in a serum coagulator shown in Fig. 5057. The Roux water bath, shown in Fig. 5063, is particularly useful for this purpose. The serum tubes are immersed in the water at 58° C. The serum tubes are heated for an hour at 58° C. on each of seven successive days. This low temperature is selected in order to accomplish the sterilization and yet keep the serum in a

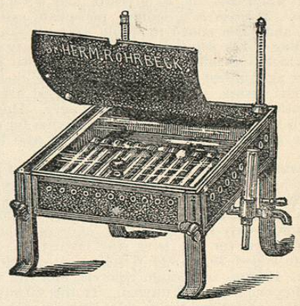

FIG. 5057.—Koch's Blood-Serum Coagulator.

fluid condition. Unfortunately bacteria may be present in the serum which will actually grow at the temperature employed, and in that case this method of sterilization is inapplicable. Some have endeavored to obviate this difficulty by filtering the serum through a Berkefeld

bougie. Martin suggested that one to two per cent. of chloroform be added to the serum, which is then set aside for several months, after which the chloroform can be driven off by heating at 65°. Fraenkel dispensed with the sterilization, relying entirely upon the aseptic collection of the serum. When the serum is collected with the care outlined above, it will be found that very few bacteria are present. Consequently after the tubes have been filled with the serum they may be incubated for several days, and at the end of that time the contaminated ones can be discarded. This procedure is preferable to those just given. The sterile serum is then coagulated in an inclined position by raising the temperature of the sterilizer to 65° C., and keeping it there until the serum has become solid. The medium thus prepared is transparent and solid. When a higher temperature is used the serum coagulates to an opaque white mass.

Inasmuch as the above methods require much time and skill and are in themselves very tedious, they have been largely supplanted by fractional sterilization in steam. For this purpose the tubes are first placed in an inclined position, either in a dry-heat oven, or, better, in the coagulator, and then heated to 85° to 95° C. until firm coagulation results. If this is not looked after the medium will be torn up by gas bubbles during the next step. The coagulated serum tubes are then placed in wire baskets and steamed, as in the case of agar, for half an hour on each of three consecutive days. The medium thus prepared is fully as useful as that which is transparent.

Löffler's Blood Serum.—This consists of one part of a one-per-cent. glucose bouillon and of three parts of blood serum. This mixture is filled into tubes and sterilized in the manner just given. It is used very extensively for the diagnosis of diphtheria.

Glycerin Serum.—Five per cent. or more of glycerin is added as in the case of glycerin agar. The sterilization is the same as that just given. It is used for the cultivation of the tubercle bacillus.

Serum Water Media.—When serum is diluted with five to ten parts of water it can be sterilized by steaming without coagulation taking place. Hiss employed such a medium in differentiating between the pneumococcus and streptococcus; also in distinguishing between the dysentery and allied organisms. He prepares the medium by adding one part of clear beef serum to two parts of distilled water. The mixture is first heated to 100° for a short time, so as to destroy the glycolytic enzyme which is present, after which one per cent. of the sugar desired is added. Dextrose, galactose, mannite, maltose, lactose, saccharose, inulin, and dextrin have been thus used. The medium is colored by the addition of one per cent. of a five-per-cent. aqueous litmus solution. The medium is then tubed and steamed for ten minutes on three consecutive days.

Marmorek's Media.—In order to maintain streptococci at their maximum virulence Marmorek used several media, preference being given to them in the following order:

1. Human serum 2 parts, bouillon 1 part.
2. Pleuritic or ascitic fluid 1 part, bouillon 2 parts.
3. Serum of mule or ass 2 parts, bouillon 1 part.
4. Horse serum 2 parts, bouillon 1 part.

These media can be sterilized by fractional heating at low temperature, or, better, by filtration through a Berkefeld bougie.

Thalmann's serum bouillon for cultivating the gonococcus has been mentioned in connection with his agar.

Milk.—This is an excellent medium for diagnostic purposes. It is advisable to use centrifugated milk if possible. Otherwise the whole milk is placed in a beaker or flask and steamed for about half an hour. When partially cooled it can be poured into a large separatory funnel, or into a bulb receiver shown as part of Fig. 5060, and allowed to stand thus overnight. The underlying layer of fat-free milk can then be filled directly into tubes. These are then sterilized by steaming half an hour on each of three consecutive days. When time is an object the whole milk may be filled directly into tubes. If desired the milk may be colored with litmus. Instead of milk, whey may be used to good advantage. This can be prepared by coagulating the milk with rennet. The liquid is first separated by means of cheesecloth and finally put through paper. It is then colored with litmus, filled into tubes, and sterilized. Care must be taken not to overheat the milk lest the lactose undergo more or less oxidation. Whey-gelatin and whey-agar are used for special purposes.

Urine.—By discarding the first portion of urine which is passed the remainder can be collected in sterile flasks and will be free from bacteria. Such urine may be used directly for studying the various fermentations which it may undergo. To prepare a urine gelatin the secretion should be diluted so as to have a specific gravity not to exceed 1.010. Ten per cent. of gelatin is then added, and when it has dissolved the reaction is made to correspond to that of the original urine. Heller's urine gelatin is prepared in the same way, but has one per cent. peptone and a half per cent. of salt. After solution the liquid is rendered faintly alkaline, then filtered and tubed.

Piorkowski Urine Gelatin.—Normal urine of 1.020 specific gravity is collected for two days, and is allowed to become slightly alkaline in reaction. Then 1.5 per cent. peptone and 3.3 per cent. gelatin are added, and the mixture is heated for one hour on the water-bath, after which it is filtered and filled into tubes. These are sterilized by heating for fifteen minutes at 100° on the first day, and for ten minutes on the second day. The medium is used to differentiate the typhoid from the colon bacillus. Petri plates are made and developed at 22° C. for twenty-four hours. While the colon colonies are roundish, finely granular, sharp-bordered, and yellowish, the typhoid colonies are small and show a more or less marked threaded border. This method has given good results in connection with the examination of typhoid fæces.

Urine Agar.—This can be prepared by adding to the freshly passed urine two per cent. of finely cut agar. The mixture is then boiled until solution results, when it is filtered through cotton or paper as in the case of ordinary agar. This agar is then filled into tubes and sterilized by steaming.

Another way of preparing a urine agar is to collect the urine, after discarding the first portion which is passed, in a sterile flask, and then to transfer it by means of a pipette, as in the case of blood or serum, to the melted and cooled agar. One part of urine to two parts of agar is ordinarily used. Normal or albuminous urine may be used for this purpose, and with very little care the urine can be collected entirely free from bacteria. Such urine agar has been used to advantage for growing the gonococcus.

Internal Organs.—For special use the several media, such as bouillon, agar, and gelatin, may be made up with the finely divided organs in place of the minced meat. At times the solid organs are sterilized and used as such. The steamed brain, for example, when cut up into slices and sterilized, can be used for cultivating the tubercle bacillus (Ficker); and also the gonococcus (Thalmann).

Egg Media.—Hueppe first suggested the use of fresh eggs as a culture medium. For this purpose the shell is thoroughly cleaned and disinfected with mercuric chloride. A small opening is then punched through the shell, and through this the organism to be tested is introduced into the inside. The opening is then sealed with a bit of sterile paper and collodion. Another procedure is to insert through the opening in the shell a rather wide, drawn-out tube pipette. On applying suction, especially with the aid of an aspirator, the contents of the egg can be drawn up into the bulb, and can then be distributed to tubes (Novy).

The egg may be used as a solid opaque medium according to Wesener. The egg is thoroughly agitated so as to mix the yolk with the albumen. It is then coagu-

lated at 75° to 80° C., after which the shell is removed and the contents are cut up into slices and placed in suitable dishes and sterilized by steam. In like manner the coagulated white of the egg may be cut up into slices and tubed. A transparent, coagulated egg albumen may be prepared by converting it into an alkali albuminate, as suggested by Tarchanow and by Karlinski. For this purpose the egg is placed in ten-per-cent. potash for fourteen days, after which the shell is removed and the solidified egg is cut up into slices, tubed, and sterilized.

Potatoes.—These may be prepared in several ways. The old method, introduced by Koch, is still used where mass cultures are desired. The potatoes are scrubbed clean under the tap, and any bad spots carefully removed by means of a knife. They are then placed in boiling water or steamed for three-quarters of an hour. By means of a knife, which has been sterilized in a flame, they are then cut into halves and placed in a large moist chamber or suitable pan provided with a lid. The bottom of this vessel should first be covered with a piece of filter paper which has been moistened with water or with mercuric chloride. The cut and sterile surface of the potatoes can then be inoculated with the organisms to be cultivated, either by spreading the material over the potato with a sterile knife or by making parallel streaks. Inasmuch as there are several sources of contamination in this method, it has been largely displaced by the modified procedures.

In Esmarch's modification the potato is pared and cut into slices about a third of an inch thick, after which they are placed into small glass dishes about 7 cm. in diameter and 1 cm. high, known as Esmarch dishes. These are then sterilized by steaming in the usual way.

The best way of using potatoes for culture purposes is that introduced independently by Bolton and Globig. The cleaned potato is placed in boiling water for about half an hour. By means of a cork borer or a test tube, the end of which has been cut off, cylinders of potato may be punched out. The skin is removed from the ends of the cylinders, after which these are halved by a diagonal cut. The wedge-shaped semicylinders are now placed in sterile test tubes and sterilized by steam.

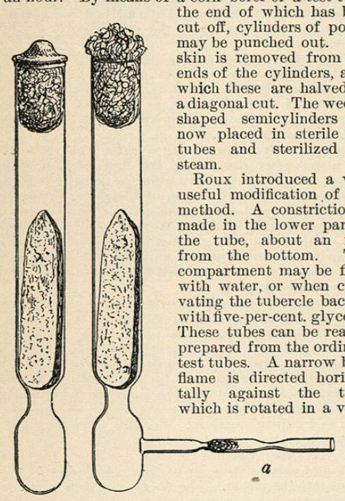

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FIG. 5058.—Roux Tube for Potato Culture.

Roux introduced a very useful modification of this method. A constriction is made in the lower part of the tube, about an inch from the bottom. This compartment may be filled with water, or when cultivating the tubercle bacillus with five-per-cent. glycerin. These tubes can be readily prepared from the ordinary test tubes. A narrow blast flame is directed horizontally against the tube, which is rotated in a vertical position. The Roux tube is shown in Fig. 5058. A good substitute for this tube may be made by placing on the bottom of the test tube a layer of absorbent cotton, which may be soaked with the glycerin solution.

Mashed potatoes spread over the bottom of a flask have been used, but they offer no special advantage over the methods given. The preparation of potato gelatin with or without potassium iodide has already been described

Bread Medium.—Ordinary bread is toasted to a crisp, then powdered, in which condition it may be kept in stock. For use the powder is placed on the bottom of small flasks and thoroughly moistened with water, then sterilized by steaming. This medium is particularly useful for cultivating moulds.

Plant Infusions.—These are useful for growing certain bacteria and also amœbæ. Infusions of hay, straw, fruits, grains, etc., take the place of meat extract. By the addition of agar or gelatin solid media may be prepared. Beer wort, either as such or as a gelatin, is valuable for the cultivation of yeasts.

Proteid-free Media.—With the exception of urine all the media described thus far contain some proteid matter. The latter, however, is not essential, for it is possible to grow bacteria on media which contain sulphur, nitrogen, and phosphorus in inorganic combination. Such a solution was used, for instance, at a very early date by Pasteur. It consisted of 1 part of ammonium tartrate, 10 parts of candy sugar, the ash of 1 part of yeast, and 100 parts of water. The botanist Cohn employed a similar solution, consisting of 0.1 gm. each of potassium phosphate and magnesium sulphate, 0.01 gm. of tribasic calcium phosphate, 0.2 gm. of ammonium tartrate, and 20 c.c. of distilled water. Naegeli's solution was made by adding 1 gm dibasic phosphate, 0.2 gm. magnesium sulphate, 0.1 gm. calcium chloride, and 10 gm. of ammonium tartrate to 1,000 c.c. of distilled water.

After the lapse of many years these non-albuminous fluids were again brought into use in a modified form by Uschinsky. His solution consisted of: Water, 1,000 parts; glycerin, 30–40 parts; sodium chloride, 5–7 parts; calcium chloride, 0.1 part; magnesium sulphate, 0.2–0.4 part; dipotassium phosphate, 2–2.5 parts; ammonium lactate, 6–7 parts; sodium asparaginate, 3–4 parts.

Fraenkel's modification of this solution contains 5 gm. of sodium chloride, 2 gm. of potassium diphosphate, 6 gm. of ammonium lactate, and 4 gm. of sodium asparaginate. These substances are dissolved in 1,000 c.c. of water and the solution is then rendered slightly alkaline.

Similar solutions have been used by Maassen and by others. Thus Proskauer and Beck cultivated the tubercle bacillus on the following solution: Commercial ammonium carbonate, 0.35 per cent.; monopotassium phosphate, 0.15 per cent.; magnesium phosphate, 0.25 per cent.; glycerin, 1.5 per cent.

For cultivating the nitrous and nitric acid organisms Winogradsky employed wholly inorganic solutions. The nitric-acid producers were grown in a solution consisting of 1,000 c.c. of water, 1 gm. potassium phosphate, 0.5 gm. magnesium sulphate, 0.01 gm. calcium chloride, 2 gm. sodium chloride. This is filled into flasks in portions of 20 c.c. each, together with a little freshly washed magnesium carbonate. To these flasks, after sterilization by steam, 2 c.c. of a two-per-cent. solution of ammonium sulphate is added, after which they are incubated to eliminate contaminations.

For the nitrous-acid organisms the solution consists of 1 gm. ammonium sulphate, 1 gm. potassium sulphate, and 1,000 c.c. of water. It is filled into flasks, magnesium carbonate added, after which they are sterilized by steam.

As a substitute for gelatin Winogradsky employed silicic-acid jelly, which was added to solutions of essentially the same composition as those just given.

Standardization of Media.—The procedure as introduced by Koch, and still followed in many laboratories, is to add a saturated solution of sodium carbonate, in portions of a cubic centimetre or more, to the nutrient medium, to be neutralized until a drop of the mixture, transferred by means of a glass rod, turns red litmus paper promptly blue. In some laboratories a strong solution of sodium hydrate is used in the same way. Obviously this method lacks quantitative precision, and the

duplication of the same reaction in several batches of material is out of question. Moreover, it is an established fact that the reaction of a medium has a very important influence upon the development of bacteria. For these reasons the bacteriological committee of the American Public Health Association, adopting Fuller's work, recommended the following method for the titration of nutrient media. The reagents necessary are:

1. Five-tenths-per-cent. solution of phenolphthalein in fifty-per-cent. alcohol.
2. Normal sodium hydrate (N. NaOH). A litre of this solution contains 40 gm. of NaOH.
3. Twentieth normal sodium hydrate ($\frac{n}{20}$ NaOH). A litre of this solution contains 2 gm. of NaOH.
4. Normal hydrochloric acid (N. HCl). A litre of this contains 36.5 gm. HCl.
5. Twentieth normal hydrochloric acid ($\frac{n}{20}$). A litre of this contains 1.825 gm. of HCl.

The preparation of these solutions requires some familiarity with the methods of quantitative analysis. The solutions can be built up by starting from a twentieth normal solution of oxalic acid, or, better, succinic acid.

The titration is carried out as follows: To 5 c.c. of the filtered medium in a six-inch porcelain evaporating dish add 45 c.c. of distilled water and 1 c.c. of the phenolphthalein solution; boil for three minutes to expel carbonic acid, then run in the twentieth normal alkali, drop by drop, with constant stirring, until a bright pink color results. The number of cubic centimetres of the twentieth alkali required to neutralize 5 c.c. of the medium gives directly the number of cubic centimetres of normal alkali (*i.e.*, percentage) required by 100 c.c. of the medium. Thus if 5 c.c. of the medium requires 2.8 c.c. of $\frac{n}{20}$ alkali, then 100 c.c. would need 56 c.c. $\frac{n}{20}$, or 2.8 c.c. of N NaOH.

The quantity of the medium remaining is now measured and the amount of alkali needed for neutralization is calculated and added. After the addition of the alkali the liquid is boiled and a portion is then titrated as before. It should be neutral, and if it is not, as often is the case on account of unknown changes, the requisite amount of alkali to make it so is added to the bulk.

The medium which is neutral with reference to phenolphthalein is very alkaline with respect to litmus. Thus a bouillon which is neutral to litmus will require about 25 c.c. of normal alkali per litre to make it neutral to phenolphthalein. In general the addition of 10 c.c. of normal alkali to a medium which is neutral to litmus imparts the most favorable degree of alkalinity. Hence the optimum reaction with reference to phenolphthalein is obtained by adding 15 c.c. of normal acid to the litre of neutralized medium. It is customary to use the sign + to indicate an acid reaction, and that of − for one that is alkaline. Thus +15 means that the reaction is acid with respect to phenolphthalein, and that one litre of the medium would require 15 c.c. of normal alkali for neutralization.

The titration with litmus as an indicator is best carried out in the following way: Portions of 5 c.c. of the medium are measured out into each of four or five large test tubes. In the case of bouillon the amount of $\frac{n}{10}$ alkali needed to neutralize this amount may vary from 0.3 to 0.6 c.c. Hence to tube 1 add 0.3 c.c.; to tube 2 add 0.4 c.c.; to tube 3 add 0.5 c.c., etc. The contents of each tube are then boiled for a minute, after which a slip of red and of blue litmus paper is dropped into the hot liquid and allowed to remain there for about a minute. The papers are then drawn out, side by side, on the walls of the tube when the colors can be compared. In this way the amount of alkali necessary to neutralize 5 c.c. with respect to litmus can be determined. Bouillon, as well as agar, usually requires about 5 c.c. per litre for neutralization, while gelatin needs about 30–35 c.c. Having determined the amount needed for neutralization, this amount, together with an excess of 10 c.c. per litre, to impart a suitable alkaline reaction, is then added to the medium.

For ordinary purposes it is hardly necessary to resort to these rather complicated methods. It is sufficient to add directly to bouillon and to agar 15 c.c. of normal alkali per litre. Gelatin will require about 40 c.c. In general sodium carbonate is preferable to the hydrate.

Preparation and Filling of Tubes.—The cheaper grades of test tubes should be avoided. They are very thin and therefore break easily, and, moreover, on heating they will often frost because of the separation of silicic acid. The best test tubes are the "blue-lined" or "resistant glass" quality, or those of genuine Bohemian glass. The size used varies with the purpose and the individual taste; 12 × 125, 15 × 150, and 20 × 150 mm. are convenient.

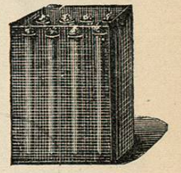


FIG. 5059.—Wire Basket for Sterilizing Tubes.

The new tubes of the better glass can be used after being swabbed out with warm water.

The cheaper grades are very alkaline, and for that reason should be first soaked in very dilute warm hydrochloric acid; after which they should be rinsed or swabbed thoroughly in clean warm water. The cleaned tubes are allowed to drain, and when dry are plugged. Used tubes should be sterilized by steaming for a half-hour, after which they may be filled with water and again heated, so as to bring the more or less dried contents into solution.

The simplest way of plugging is to place over the mouth of the tube a piece of cotton, about two inches square, which is then pushed within by means of a narrow glass rod or a pair of smooth forceps. Such plugs answer all ordinary purposes. They are, however, rather loose, and permit evaporation of the media, and cannot be used where the tubes are to be sealed with wax. A firm solid plug is made by taking a piece of cotton, about three inches square. This is folded into thirds and then rolled up from the end into as firm a cylinder as possible. By a twisting motion the plug is inserted into the tube, and only enough cotton is left on the outside to permit grasping of the plug. The plugged tubes are then placed in a wire basket, such as is shown in Fig. 5059. These baskets are made of heavy galvanized netting. The usual size is 24 cm. high and 18 cm. square. Smaller baskets, 10 × 12 and 18 cm. high, are very useful. Circular baskets, of a size to fit the sterilizer, are also used.

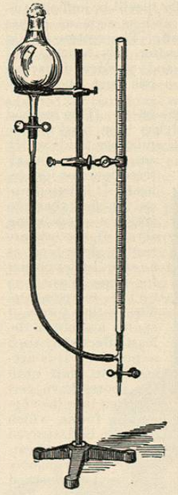


FIG. 5060.—Globe Receiver for Filling Media into Tubes, with Burette Attachment. (Novy.)

Flasks, bulbs, etc., should be prepared for sterilization in the same way.

After the tubes have been sterilized by heating in the dry-heat oven they are ready to be filled with the nutrient media. This can be done by the aid of a small funnel. Where large quantities of media are to be tubed much time can be saved by using a large funnel or globe receiver, such as is shown in Fig. 5060. The lower end of the bulb is connected with a drawn-out glass tube and is provided with a pinch cock. In this way the media can be rapidly filled into the tubes. Ordinarily the tubes are filled to the depth of one and one-half or two inches. In special cases in which definite quantities are desired, the simple apparatus, shown in

Fig. 5060, can be used. The containers, with the media to be filled, can first be sterilized by steaming, after which the media can be measured out into sterile tubes, which will not require further treatment. A less desirable apparatus is that of Treskow, shown in Fig. 5061.

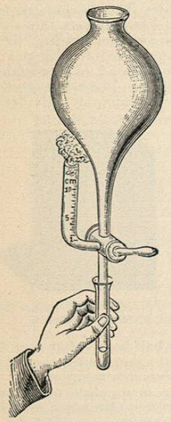


Fig. 5061.—Treskow's Apparatus for Measuring Media into Tubes.

Sterilization.—By sterilization is meant the total destruction or removal of all organisms in or about a given object. This can be accomplished in a variety of ways.

1. *By Direct Flaming.*—This method is applicable for the sterilization of glass rods, slides, cover-glasses, platinum wires, searing irons, and rough instruments. Valuable surgical instruments would of course be damaged by this procedure.

2. *By Means of the Dry-Heat Sterilizer.* — The form most often used is that of Koch, shown in Fig. 5062. This is made of sheet iron, is double walled, and the outer wall may be lined to advantage with thick asbestos board. The form as shown is designed to sterilize not only tubes and flasks but also glass tubing, pipettes, and the like. The oven is used for sterilizing only glass and metal ware. It must not be used for sterilizing media. A temperature of 150° C. should be maintained for one hour. Usually it will be sufficient to allow the temperature to rise, and as soon as it has reached 200° the gas is turned off. The cotton should show a slight tinge of yellow after this heating. If the plug browns considerably and powders it is due more to the fact that the cotton has been chemically treated than to the heat.

3. *Fractional Sterilization at* 56°–58° C.—This method was introduced by Tyndall, and has been used for the sterilization of liquid serum, milk, and other fluids which are liable to be altered more or less by heat. It is based upon the fact that the actively vegetating forms of bacteria are readily destroyed as a rule by exposure for some minutes to this temperature. The resting forms or spores are not in the least affected by such exposure. It is necessary to wait until the spores have germinated into the vegetating forms, which can then be destroyed by a second like heating. As ordinarily practised, the method is as follows: The tubes are placed in an apparatus, such as that shown in Fig. 5063, and are heated for one hour at the given temperature on each of seven or eight consecutive days.

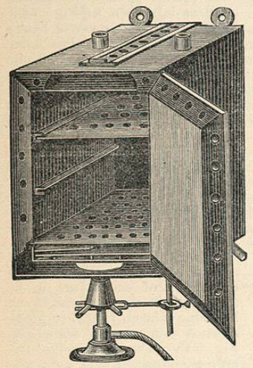


Fig. 5062.—Koch's Dry-Heat Sterilizer.

This method sometimes gives good results, at other times it fails. The reason for this lies in the presence or absence of the so-called thermophilic bacteria. These organisms actually grow best at the temperature employed, and hence, if they chance to be present, the method is inapplicable. A temperature of 70° C. may be used in like manner, but this causes coagulation of the serum.

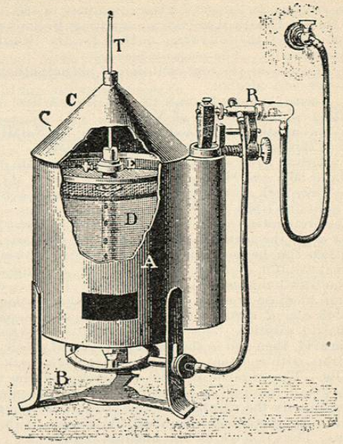


Fig. 5063.—Roux Water-Bath for Sterilizing Serum with Metallic Regulator.

By Pasteurization is meant the partial destruction of the organisms which are present in a milk. This is accomplished by exposing the milk for half an hour, or more, to a temperature of about 68° C. (155° F.). While this temperature does not destroy the spores which may be present, it does kill the lactic-acid and other bacteria, which do not produce spores. As a result, milk treated in this way will keep for several days without coagulating. If a higher temperature is used, the taste of the milk is likely to be impaired.

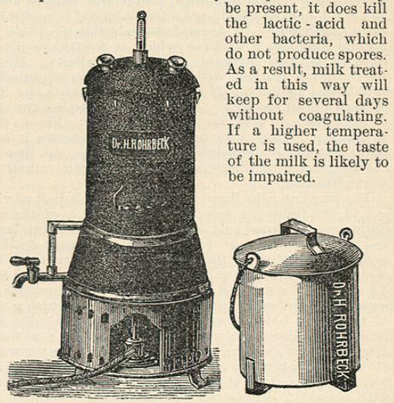


Fig. 5064.—Koch's Steam Sterilizer.

3. *Sterilization in Flowing Steam.*—Several forms of apparatus have been devised for this purpose. Among the earliest is the well-known form which bears Koch's name. This apparatus is used almost entirely in Germany, and to a considerable extent in this country. It is shown in Figs. 5064 and 5065.

It consists of a cylinder of galvanized iron, or better of copper, which can be given such dimensions as may be desired. Ordinarily it is about half a metre high and about 25 cm. in diameter. It is surrounded by a thick covering of felt *M*, to prevent loss of heat by radiation. In the interior of the cylinder at *R*, is placed a grate which serves as a support for the pail and other vessels to be disinfected. The water in the lower compartment is heated by one or more large gas burners. Above it is closed with a cover *D*, which is also covered with felt. A central opening permits the escape of steam, and can be used for the insertion of a thermometer. The pail shown to the right of the sterilizer has a grating for a bottom, to allow free access of the steam, and in it are placed the articles to be sterilized.

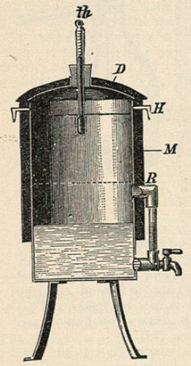
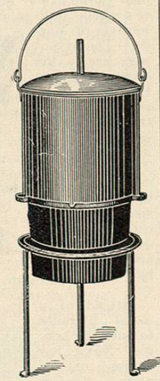


Fig. 5065.—Section of Koch's Steam Sterilizer.

The nutrient media are as a rule sterilized by steam. A single heating for one hour in steam at 100° C. is usually sufficient to render the media sterile. Prolonged heating, however, tends to alter the media, and for that reason fractional or discontinuous sterilization is resorted to. The latter has the additional advantage that it renders the medium more surely sterile. There are spores which can withstand steaming for one, and even five or six hours, and if such forms chance to be present, it is evident that the material cannot be sterilized by the single heating for one hour. In the other procedure the media are steamed for fifteen minutes or half an hour, according to the nature of the medium, on each of three consecutive days. The first heat serves to destroy the vegetating germs that may be present. In the interval which elapses between the first and second heating, the spores which are probably present will germinate and are thus converted into the much weaker form, which is then destroyed by the second steaming. The second interval allows any remaining spore, which may have failed to germinate the first day, a chance to do so, and the third heating is expected to dispose of these last organisms. As a rule all media should be incubated for one or two days to make sure that they are perfectly sterile. If any growth develops in the tubes or flasks these should be discarded, and only those which are free should be preserved for use.



Failure to secure sterilization by this procedure is due to one of the following conditions: The temperature which prevails during the twenty-four-hour period which elapses between the consecutive heats may be so high that the spores which are present not only germinate, but the vegetating forms in turn give rise to spores, so that a larger number of resistant forms are present on the second or on the third day than were present in the beginning. Again, it may happen that the temperature is too low, in which case the spores cannot germinate, and hence will be found to resist sterilization. Another source of error, though much less common, was pointed out by Smith. If the spores of anaërobic bacteria are present in a bouillon, they cannot from their very nature germinate under the ordinary conditions, that is, in the presence of the air, and may therefore escape destruction.

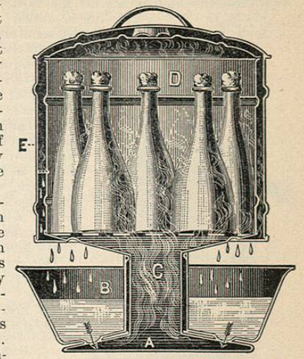


Fig. 5066.—Arnold Steam Sterilizer, Sectional View.

In this country, and even in Germany, the Arnold steam sterilizer has met with a very favorable reception. The apparatus is shown in Fig. 5066. It consists of (*a*) a flat, shallow boiler, holding but a small amount of water, and therefore requiring but a minimum amount of heat to produce steam; (*b*) a reservoir placed upon the boiler, which it constantly feeds and insures the constant formation of steam; (*d*) a covered steam chest or receiving vessel, placed above the reservoir and connected with the boiler by a cylindrical tube of large diameter (*c*); and (*e*) a hood, covering the receiver and enclosing an air space, which is constantly supplied with escape steam. The hood and the steam jacket which it encloses prevent variations in temperature in the receiving vessel so long as the heat applied to the boiler remains unchanged.

A cheap and thoroughly efficient steam sterilizer adapted for individual work is shown in Fig. 5067. This consists of an ordinary Hoffmann iron water-bath 10–20 cm. in diameter. On this is placed a copper pail (20 × 20 cm.), which is provided with a perforated bottom. Two perforated rings on the inside allow the passage of steam, and prevent the cotton of the tubes from coming into contact with the side of the steamer. The tubes filled with media are placed in the pail, and this is then set on the water-bath, the water of which has been previously raised to active ebullition. In a few minutes steam will issue from the tube in the top of the cover. It is always advisable to take the temperature of the vapor as it issues from a sterilizer and to count the time of exposure from the moment that the vapor actually shows the temperature of steam, that is 100°.

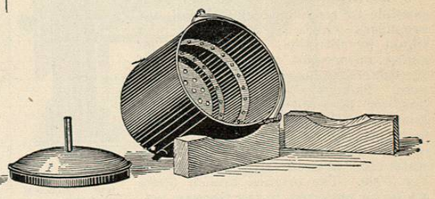


Fig. 5067.—Novy Steam Sterilizer.

4. *Sterilization by Steam Under Pressure.*—This procedure is used almost entirely by the French workers. Its usefulness is such as to merit a wide introduction into this country. The apparatus, which is designated as an