# SOME THEORETICAL CONSIDERATIONS UPON THE NATURE OF AGGLUTININS, TOGETHER WITH FURTHER OBSERVATIONS UPON BACILLUS TYPHI ABDOMINALIS, BACILLUS ENTERITIDIS, BACILLUS COLI COMMUNIS, BACILLUS LACTIS AEROGENES, AND SOME OTHER BACILLI OF ALLIED CHARACTER.[1]

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[1] This article having been written without access to a library, I have taken the liberty of inserting in foot-notes the references to some of the authors cited in the text.—Editor.

Owing to my participation in an expedition for the study of certain tropical diseases, it is improbable that I shall be able to continue, or to render so complete as would be desirable, an account of the studies which have been made during the past five years. However, although these studies have been interrupted, the following notes may be of some interest to those working in the same field, especially since many points require further elucidation. The work upon groups of bacilli to be considered was begun in November, 1894, in connexion with the question of serum tests. These studies, together with those upon vibrios, initiated the method of testing by the clumping or agglutinating action of sera of immunised animals upon appropriate organisms.[2]

## I.—AGGLUTINATING SERA.

### THEIR SPECIAL ACTION UPON CERTAIN BACTERIAL RACES.

Further experience with the differential actions of sera of treated animals tends to confirm the conclusion that so far as bacteriolytic and

[2] Since many writers continue to use the name of Widal in connexion with some of these reactions, it may be pointed out that so far as priority of publication is concerned, there can be no doubt that Gruber was the first to publish the fact that human typhoid-fever patients acquire clumping power of their blood serum towards the typhoid bacillus. The whole principle of the test was established from Prof. Gruber's laboratory, and from the historical side the question of the exact period of the illness at which the reactive power is perceptible, is a mere matter of detail. Grünbaum has pointed out that some of the original cases referred to by Gruber at the Wiesbaden Congress in April, 1896, were as early as the 10th or 11th day of the fever. It may also be noted that a Fellow of the Royal Society of London was interested in the paper which I presented to the Royal Society (published in January, 1896) and which, owing to the delays in the printer's hands, was not published in full in the *Journal of Pathology and Bacteriology* until July, 1896; and he made a long abstract of this contribution which he sent to the editor of a French medical periodical. This abstract, I understand, neither was acknowledged nor was it published. Not long afterwards the application of the clumping test to the diagnosis of typhoid fever was published in France, apparently without much reference to Gruber's remarks made in April. I understand that the editor of the above-mentioned periodical was then F. Widal. Anyhow, from the point of view of priority of reference to the matter in print, if any name should be attached to the reaction it should be that of Gruber. I feel impelled to call attention to these points, since, although I personally was concerned with working out the prime foundations of the agglutination test for artificially prepared serum (and Grünbaum in the case of human typhoid fever), yet, so far as I am concerned, the inspiration was derived from my respected teacher Professor Max Gruber, as, indeed, I have already recorded in my paper in the *Journal of Pathology and Bacteriology*, 1896.

agglutinating actions are concerned, the word "specific" is inapplicable. In my previous paper[3] it was suggested that the word "*special*" would be a better one to employ. It will be remembered that Prof. Gruber and I found that within the "species" Vibrio choleræ asiaticæ the serum reactions were not uniform, in that the serum obtained by immunising with one race did not necessarily give more than a trace of reaction in vitro and none whatever in vivo when tested with another race, although it was capable of giving complete clumping and positive Pfeiffer reaction when tested upon the first race. At that time we ascribed this to differences in virulence of the races, for the serum obtained by the use of non-virulent cultures had little or no effect upon the most virulent stocks; on the other hand, the serum obtained by the use of virulent cultures affected both the non-virulent and the virulent stocks of cholera vibrios. Since then, however, I have found that certain less virulent strains of Bacillus enteritidis derived from the same source were less affected by a given serum (both in vitro and in vivo) than the more virulent strain. The matter is one which possesses considerable complexity, and we (and Pfeiffer in following us) were probably incorrect in ascribing the difference to a mere difference in virulence.

Further, in discussing the "specific" value of the test, I found that two clearly differentiable vibrios—the "Massowah," with its four flagella (not the "Massaouah" used by Bordet and others in the Institut Pasteur, which reacts as a cholera vibrio with serum, as well as in its cultural characters), and the "phosphorescent Elwers" vibrio, with its single flagellum and other special characters—were, so to speak, *serum-identical*, that is, the serum of either affected the other not only by the clumping test, but also by the Pfeiffer bacteriolytic test and the protection afforded to animals. It seemed from these observations that the serum test, both in the test-tube and in the animal, could not be considered a final criterion for the diagnosis of species; for, first, there was some want of uniformity in the action of serum tests within the "species" cholera vibrio (especially when the serum

[3] H. E. Durham, On a special action of the serum of highly immunised animals. *Journal of Pathology and Bacteriology*, 1896, iv, p, 13.

identity of V. "Berolinensis" of Rubner and V. "Versailles" of Sanarelli and V. "Iwánoff" are remembered), and secondly, there was mutual serum identity when apparently perfectly distinct "species" (such as V. "Massowah" and V. "Elwers phosphorescent") were investigated.

Since then I have made a number of agglutination tests upon members of the B. enteritidis group. So far as cultural and morphological tests are concerned, I could not certainly diagnose these races from one another, it being understood that comparative observations were made not only at a single time, but now and again in some cases over a period of years and in others of months. Temporary variations do occasionally occur, but these are not constant and, after all, are insignificant in that they do not affect main characteristics. So far as these characters go we have a well-defined group, yet, when tested by serum for agglutination, marked differentiation may be found. Thus the races "Gärtner" and "Morseele" are both strongly affected by "Gärtner" serum, whilst the races "Hatton," "morbificans bovis," "psittacosis," "Aertrycke," "Calmpthoult," "Gand," "Sirault," "hog cholera," "typhi murium," "Sheffield" (kindly sent to me by Dr. Robertson), are not very markedly affected by this serum. To some extent the race "Günther" would appear to be intermediate; still it is more markedly affected by the serum of the Hatton type. Taking the reverse view, we find that the serum of "Hatton" has comparatively slight effect upon the races "Gärtner" and "Morseele;" thus a serum which gave good reaction at 1 in 200,000 upon "Hatton," practically gave but a minimal reaction upon "Gärtner" and "Morseele" at 1:2000. It may also be mentioned that Gärtner serum, though efficient against "Gärtner," has practically no protective effect against the living Hatton bacilli; so that here within a group of bacilli, the characters of which can be more closely studied than is the case with the vibrios, the serum test does not give material aid in defining its limits. At the same time the Gärtner type has a distinct tendency to be affected by the serum derived by the use of typhoid bacilli. Here again we find differences between the typhoid bacilli, for instance the serum of the race

" Weichselbaum " has much more effect than that of my race " HS," though here it is far less than that produced upon any of the typhoid races (22) with which I have directly compared them. To put the matter in a short way, it might be said in the formula of a proportion sum that " Hatton " is to " Gärtner " as " Gärtner " is to typhoid " Weichselbaum."

Two very interesting cultures from the Pathological Laboratory of the Johns Hopkins University, " Gwyn " and Bacillus " O," for which I have to thank Prof. Flexner and Dr. Harvey Cushing respectively, have also yielded evidence that the clumping test has only a limited value in the diagnosis of species. Both of these were obtained from cases clinically resembling typhoid fever.[4] They are both distinguishable from the typhoid bacilli and also from the enteritidis group in their cultural reactions, but I hardly think that I could distinguish one from the other with certainty, so much do they resemble each other. Both of them fail to give the slightest clumping reaction with typhoid serum (potency 20,000-100,000) or with various enteritidis sera (potency 50,000-500,000); of the latter, " Gärtner," " Hatton," " Günther " and " morbificans bovis " have been tried. I prepared a " Gwyn " serum, active up to 1:20,000 upon " Gwyn," but this has not the slightest effect upon Dr. Cushing's Bacillus " O " at 1:100 dilution. Here the serum reaction confirms the notion obtained from cultural tests that these two bacilli are different from the typhoid and the enteritidis bacilli, but from their similarity it might be expected that they would shew some mutual reaction; this, however, is not the case.

One more example may be given, and it is of some value in shewing that distinctly differentiable bacilli of the colon group may simulate one another by their mutual serum reaction. Bacillus " W," a member of the B. coli communis verus race (vide, p. 371), has no power of fermenting sucrose (cane-sugar), Bacillus " G " will readily ferment this sugar; both bacilli have been under my observation for fully four years, and they have retained these characters and can be differ-

[4] Gwyn, *Bulletin of the Johns Hopkins Hospital*, 1898, ix, p. 54. Cushing, ibid., 1900, xi, p. 156.

entiated from each other by culture with ease. Now, both "W" and "G" are mutually susceptible to the clumping serum test, so much so indeed that one sample of "W" serum acted on a culture of "G" up to about 50,000 dilution, whilst upon "W" itself it fell out about 30,000.

Next it was found that apparently indistinguishable true B. coli communis races were not mutually affected by highly potent sera (none of which was less than 50,000 potency). This is a point which was foreshadowed in my earlier paper, and which, indeed, has been worked at by a number of observers with a like result. I think, however, that they have not worked with such highly potent sera, and that they thereby have saved themselves the time and patience which in some respects I have wasted. So far as my experience goes, it is rather unusual to find two otherwise indistinguishable colon bacilli from different sources, which give any mutual serum reaction in test dilutions. I have now tested a good many different cultures during the past four years and have almost always obtained completely negative results, although the sera were highly potent for their own races. In the light of the oft-repeated statements of Prof. Baumgarten, that the serum of rabbits readily agglutinates colon bacilli, I may state clearly that this is not my experience. I rarely test in lower dilutions than 1 in 200, and at this or higher dilutions of rabbit's serum (whether normal or immunised) the results are practically always negative. It is possible that his results may be partly due to the use of cultures in broth containing muscle sugar, which, when mixed with fresh broth or with serum, may give some precipitation of proteid matters and thus carry down the bacilli, giving rise to their apparent agglutination. In order to avoid such fallacies, I use agar cultures rubbed into a suspension with sterile 1 per cent NaCl solution; this is a standard method when a given parallel-sided loop and equal amounts (say about 2 mgrm.) of bacilli per 1 cc. are used. In sedimentation tubes, I find that the reading after 18-20 hours is generally the same as that taken after 48 or 72 hours, there being little or no multiplication of the bacilli.

THEORETICAL CONSIDERATIONS UPON THE CONSTITUTION OF SERUM OF IMMUNISED ANIMALS.

It should be understood that mutual reactions may be partial or complete, but in speaking of these I do not include reactions which are obtained with test sera below 1:1000. The effects produced upon bacilli by the serum of apparently normal animals in less dilute conditions can only be considered to be of a special nature, when it is *not a constant peculiarity* of the species of animal, and then to avoid fallacies dilutions not less than 1:100 must be employed. Just as we are at present unable to evaluate the position of the antitoxic action of the sera of some "normal" animals, so we cannot yet assign a position to these clumping actions of the sera of normal animals in low dilutions. Personally, I believe that both antitoxic and clumping characters in such sera owe their origin to the presence and absorption of appropriate bacillary products, but it is also possible that mutually reacting substances, *i. e.* bacillary product and serum constituent, are present, the latter of which is not necessarily identical with the true agglutinins. I may instance the precipitating effect of quillaic acid upon peptone solutions, as a case where two organic substances interact, whilst other glucoside constituents of Quillaia bark do not have this effect.

But to point my moral more aptly, an illustration may be taken from the precipitating actions of ricin and abrin upon sera. Solutions of the proteoses (albumoses) obtained from the seeds of Ricinus and Abrus (like the globulins obtainable from the seeds) form a precipitate when a drop of serum is added; the serum of normal rabbits, guinea-pigs, rats, fowls, horses and hedgehogs all produce this effect. But working with rabbits I find that the amount of precipitate obtained by adding 10 cmm. of serum to 1 cc. of a solution of Ricinus albumose is far more copious when the serum is taken from an animal which has been immunised with the Ricinus albumose than when taken from a normal or an abrin-treated animal. Moreover, the precipitation can be obtained in dilutions of the albumose solution with anti-ricin-albumose at which the normal or anti-abric serum fails to give any precipitate. Only abrin albumose precipitates both kinds of serum

(anti-ricin and normal) in like degree. I may add that the anti-ricin serum has a protective effect, whilst normal serum has none, when the serum and ricin are given in recently made mixtures.

I made some experiments with abrin, which shew that in the act of precipitation by normal serum much of the toxicity of a given solution may be removed. Thus 0.01 cc. of a given abrin solution killed 300-gramme guinea-pigs in about 60 hours, 0.02 cc. in about 48 hours, and 0.05 cc. in about 30-40 hours. To some of the same solution normal rabbit serum was added (0.05 serum to 3 cc. abrin), this was allowed to settle for a few hours and the clear supernatant fluid tested; it was then found that 0.01 cc. of the original solution failed to kill, whilst 0.05 cc. killed only after about 60-70 hours. The nature of this action is not clear and I do not propose to discuss it here. The point is that by dilutions in vitro at any rate a quantitative difference between the "coagulins" can be detected, and the protective action of the special serum is suggestive that there is a qualitative difference also.

It will be noticed that I have been comparing the bacillary agglutination process with the action of precipitating agents, more or less after the theory propounded by Kraus.[5] I do not think that bacillary agglutination is due purely to an entanglement of the bacilli in coagula formed in the free fluid. The microscopical observation of bacilli mixed with very dilute special sera is most suggestive of some alteration of the surfaces of the bacilli in the direction of increased stickiness. It may be that this surface alteration is due to a precipitation or more or less "nascent" precipitation upon the surfaces of the individual susceptible bacilli; such bacilli as are secreting more of appropriate substances will be more susceptible to the action of the serum and become more profoundly affected. It is always a striking phenomenon that all the bacilli are not equally influenced in a given dilute mixture.

In order to explain the somewhat perplexing partial and mutual reactions of agglutinating sera upon different groups or races of bacteria the following more or less graphic method may be suggested.

[5] *Wiener klin. Wochenschr.*, 1897, x, p. 736.

I suppose that a given " agglutinin " is not a single substance, but a complex one, the constituent elements of which I will designate by capital letters, whilst the bacillary components which are capable of giving rise to the formation of the agglutinins (when introduced into an animal) may be represented by corresponding small letters. Thus if we take typhoid and enteritidis sera which give some mutual reaction, they will be graphically represented thus:

| Elements concerned with agglutinins | B. typhi. | B. enteritidis (Gärtner type). | B. enteritidis (Hatton type). |
|---|---|---|---|
| Serum constitution | A, B, C, D, E | C, D, E, F, G, H | E, F, G, H, J, K |
| Bacillary constitution | a, b, c, d, e | c, d, e, f, g, h | e, f, g, h, j, k. |

When typhoid serum containing (A + B + C + D + E) is added to typhoid culture (= a + b + c + d + e) the maximum effect of clumping is produced where each substance reacts upon the other; when it is added to Gärtner bacilli (= c + d + e + f + g + h) it will only produce an effect when the substances C, D, and E are able to affect a certain proportion of the bacilli or rather their constituents c, d, and e to a sufficient degree; still greater must be the concentration of the typhoid serum to produce an effect upon the bacilli containing only the susceptible substance e.

The matter is further complicated; for instance, it may be supposed that although two typhoid races both consist of a + b + c + d + e, these substances are not present in equal quantities. Thus one typhoid race or individual bacillus may be represented by the formula 20a + 10b + 5c + 2d + 1e, and another by 1a + 2b + 5c + 10d + 20e. When tested by a third serum containing all the constituents in equal quantity (*i. e.* A = B = C = D = E) they may give identical dilution limits, but they will not do so when tested by means of their own sera. Moreover, any given race does not necessarily produce the same quantities of the different constituents at different times, and hence the variations of agglutinability, virulence, etc. Further, I suppose that the bacteriolytic, inhibitory and protective or preventive substances have a similarly complex constitution, the amount of each unit being to some degree independent of the other, though all the substances tend to be grouped together more or less

dependently. These hypothetical considerations appear to be simple and at the same time a fairly satisfactory explanation of intricate phenomena of the various actions of the sera of immunised animals.

### METHOD OF PREPARING SERA FOR AGGLUTINATION TESTS.

It seems from numerous experiments that the most satisfactory method of producing clumping sera is to give considerable quantities of killed bacilli by means of intraperitoneal injections. It is possible to get moderate potency by giving sterile filtrates of cultures, as I shewed in my paper [6] in 1896, a discovery which has been variously claimed by or ascribed to Levy and Bruns or Widal, all of whom, however, described the fact about a year or so later. It is also possible to induce some power (about 1:4000 is the highest I have obtained) by giving killed cultures by the mouth; were evidence wanting that the agglutination is no "reaction of infection" this might be cited as such.

Rabbits are the most satisfactory animals to use, chiefly because a good supply of blood can be obtained from them from time to time. About one-tenth of an agar culture killed at 60°-65° C. is a good dose to begin with for a young rabbit of about 900 grammes (I prefer to begin on young animals) and this is increased gradually according to the gain in weight at intervals of a few days at first and of a few weeks later. The injections should be given on the left side of the abdomen in the inguinal region; if a blunt needle is used and the skin perforated by means of a small cautery there is no risk of wounding the intestine, for this is the region of the small intestine. The sites of the cæcum and stomach should be noted and avoided. In the guinea-pig the right side, about midway between ribs and pelvis, is the proper site for intraperitoneal injections. I have had several rabbits under treatment for periods of 2-3½ years, and they have received very many injections without causing any adhesions of the viscera.

When the dose of bacilli from 3 or 4 agar tubes has been successfully passed, it is economical and convenient to smear the surface of agar which has been allowed to set in Petri dishes. In order to smear the surface rapidly, I have made use of the turn-tables which are used for ringing microscopical specimens with cement; by removing the spring clips and putting 3 small pellets of modelling wax an excellent revolving support is made for the purpose. A quantity of culture is scraped from a gelatine or agar slope and smeared on to a broad platinum spatula

[6] *Journal of Pathology and Bacteriology*, 1896, iv, p. 13.

made of a piece of foil soldered to a wire and mounted on a holder. The dish is set spinning and the bacteria are rapidly spread.

When the culture has grown the bacteria are scraped up with a loop and emulsified with saline solution and then killed by exposure to a temperature of 60°-65° C. for about half an hour.

It should be noted that broth is not a suitable substance for making the injection. I discarded it at first for reasons of economy, but the recent work upon the precipitins and coagulins, especially that of Dr. Walter Myers,[8] shews that in animals treated with peptone injections substances capable of precipitating these bodies are produced in the serum. Such a serum, when mixed with a peptone broth culture of bacilli, may cause some apparent agglutination of the bacilli by virtue of an entanglement with the precipitated proteid. A parallel effect is seen in the clumping of mica particles in a mixture of typhoid serum and filtrate of typhoid culture, as in the experiments of Kraus, or by the clumping action on indifferent blood corpuscles, as Myers has shewn.

In the treatment of the animals it is best to give injections increasing up to about four Petri-dish cultures. A series of injections of smaller quantities every day for a week, followed by an interruption and then another series of injections, is not quite so good, I think, for the preparation of highly potent sera. It appears probable that a smart reaction should follow the injection; in fact, for the highest potency the final injections should be near the maximum recoverable dose. Thus two rabbits, which had been under treatment for rather more than two years, and which had received equal doses at the same intervals for the last 12 months, were each given a considerable dose of the same material; one of the rabbits died in about 24 hours and had a clumping potency of about 1:200 at death; the other was very ill for a time but developed a potency of over 1:2,000,000. With this mode of treatment it is easy to obtain sera of a potency of 1:200,000, but it is more difficult to increase the potency up to and above 1:500,000.

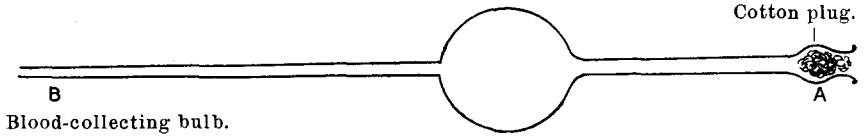
To obtain blood, the ear of the rabbit is shaved; the skin is cleansed with lysol (2 per cent) and soap and thoroughly dried with sterile cotton wool; the basal vein of the ear is compressed, and when the minute vessels are quite turgid, a small cut is made in the marginal vein by means of a sharp lancet. The blood will flow rapidly from quite a small puncture, and, indeed, will often "pump" as if from an artery. It is taken

[7] The same method is useful for separating impure cultures, the same spatula or a comb-like instrument (made by cutting a number of cuts in a piece of platinum foil) being used to smear successive plates whereby discrete colonies may be obtained.

[8] On immunity against proteids. *Lancet*, 1900, ii, p. 98.

up by means of sterile bulbs, with suction if necessary, from the pool of blood upon the skin.

Many forms of tube have been tried, but that shown in the diagram seems the most useful apparatus. The blood is then blown out into sterile test tubes, which are inclined to the maximum limit allowed by the tube in order to get a thin layer of blood clot. The tubes should not be touched until the blood has thoroughly well clotted, otherwise some blood-corpuscles will be liberated and become mixed with the serum. After an hour or so the tubes are stood up vertically. By these means some 10-15 cc. of blood and a corresponding quantity of serum may be obtained with ease from a rabbit without any operation beyond a mere prick in the vein; no pain is induced and no anæsthetic is required, the animal sitting perfectly still and shewing no signs of discomfort. The process may be repeated from day to day if desired and a considerable amount of perfectly *sterile* blood-corpuscle-free serum easily obtained.[9]




A. Mouth-piece with cotton plug.
B. Collecting capillary about 2 mm. diameter.

When it is desired to bleed the animal to death, I do so from the carotid artery, under an anæsthetic. In order to obtain a maximum quantity of serum, it is necessary to allow the blood to clot in as thin a layer as possible, and then to stand the vessel so that the clot is vertical. The most convenient means of doing so is to use flat bottles (ordinary medicine vials) into which a number of thin pieces of drawn-out glass rod are placed. These thin glass rods hold the flat thin clot so that it will not slip down when the bottle is stood up. After 24 to 48 hours the serum is pipetted off and stored in sealed glass tubes or otherwise as desired.

By this technique more, sterile, clear serum is obtained from a given amount of blood than by any other means with which I am acquainted; and I would recommend its adoption on a larger scale by those who are interested in the preparation of serum. For the preservation of the

[9] It may be added that as much as a cubic centimetre or so can often be obtained from the marginal vein of the guinea-pig's ear. This vein bleeds better than the larger veins of the ear.

serum I have never found occasion to add antiseptics, but it would appear that there is no reason against doing so, should it appear to be advisable, since for testing purposes it is rarely, if ever, necessary to use less than thousandfold dilutions, and also since, so far as clumping is concerned, the death of the bacteria to be tested is not a factor which introduces fallacies.

From numerous observations upon the course of the development of agglutinins in the blood, which I hope to publish in detail when I again have access to the records of my experiments, I am disposed to think that the effect of a given injection reaches its height (when killed bacilli are given) about 10 days or a fortnight after an injection. In following out this matter I have taken both slightly and rather highly immunised animals and removed samples of blood-serum at various periods after giving a further immunising injection. In all cases at least two samples have been taken previously to the injection so as to have ample control; samples were then taken either hourly or daily or at rather less frequent intervals and all were subjected to test with the same emulsion of bacteria. In order to gain some idea of the dilutions required for the estimation, four samples or so were tested at a number of dilutions in order to know what dilutions will be required for the final test. The tests are made by means of sedimentation in plain bulbous capillary tubes after the method devised by my friend Prof. Wright[10] of Netley. With the large number often required it is impossible to use the microscope, whilst in an afternoon several hundred sedimenting preparations can be put up. The capillary sedimentation method has the further advantage of putting all the effects before the eye at once, so that the different tubes may be put side by side and compared with one another. Moreover, for testing the potency of a serum, the microscopic method has proved very unsatisfactory as well as laborious.

After using 10 pounds of glass tubing within a period of 3 months in the form of capillaries, I came to the conclusion that it would be advisable to adopt some means of cleansing them and putting them to further use. The following method has given satisfactory results and has not been guilty of giving rise to any accidental infection to my laboratory attendant: Two sufficiently deep wide jars, one filled with solution of potassium permanganate and the other with dilute (about 10 per cent) hydrochloric acid, are required. The attendant cuts off the lower (sealed[11]) end of the capillaries with a file over the pot of permanganate,

[10] *British Med. Journal*, 1897, i, p. 1214.

[11] It is not necessary to seal the upper end of the sedimentation capillaries.

into which each one is dropped vertically, whereby it becomes filled from below upwards with the disinfecting solution. After a day or so the tubes are removed, placed similarly in the acid and left there for a few days until the brown material is removed. They are then placed in a jar with syphon arrangement in which a constant slight flow of tap water gives them an automatic washing. When all the acid has been thoroughly removed they are shaken out and placed in a jar of distilled water (to remove lime salts) for a day or two, then they are again shaken out and allowed to dry. Lastly, they are put through the hot-air steriliser, after which they are ready for use.

For supports I keep a number of strips of sheet aluminum (about 1 inch broad and 18 inches long), each with a row of small holes (some 30-35 in number) sufficiently large to pass the capillary but not the fusiform bulbous part of the sedimentation tube. Each support is marked with a letter and each hole with a number, whereby the different specimens may be identified. When filled, the racks or supports are rested by their two ends upon a stand which formerly did service to support an incubator.

For making dilutions of small quantities and for making the mixtures of diluted serum and culture, the small porcelain pans, which are used for moist water colours, are extremely serviceable and, to my mind, more convenient than watch glasses.

Another small technical process which I have found most convenient may be mentioned. Given a sample of serum sealed in a glass tube, how is it most conveniently opened without contamination? Heat the end which it is desired to open to redness or nearly so in the flame and grasp it with the points of a pair of forceps which have been wetted with some antiseptic solution (*e. g.*, 2 per cent lysol); the end is immediately cut off and a sterile capillary tube, a number of which should be kept at hand in a sterile plugged test-tube, may be passed in and the required quantity of serum withdrawn. In this way I have removed samples of serum from the same tube over and over again without any accidental contamination; of course, after removal of sufficient serum, the point of the stock tube is again sealed.

## II.—ON OTHER MEANS OF DIFFERENTIATING THE GROUPS OF BACILLI UNDER DISCUSSION.

From what has been already stated it is clear that the clumping reaction is of little value for differentiating and classifying these

bacilli in a satisfactory manner. It appears that we can only find whether the products of bacilli which are capable of giving rise to agglutinins are the same in two or more cases. Even then, although there may be the same substance or substances *qualitatively*, these may not necessarily be present *quantitatively* to the same extent. Again, by taking the same race of bacilli and its own serum, we find that the susceptibility of cultures made at different times is not necessarily the same. It seemed necessary, therefore, to enquire into other means of differentiation, but before discussing these, I propose to give a sort of classification of the types which have been studied, then to consider the ordinary characteristics which are used in laboratories, and, lastly, to give some account of media which may be useful for further work.

SUMMARY OF CLASSIFICATION.

Division I. Typhoid-like morphology: motile.

Order i. *Non-saccharid-fractors, i. e.,* do not ferment any saccharids.

Group A. Type, B. fæcalis alcaligenes (Petruschky).

Order ii. *Dextroso-fractors; non-lactoso-fractors:* ferment dextrose and certain other saccharids, but not lactose.

Group B. B. typhi abdominalis: no evolution of free gas bubbles; $CO_2$ is formed by action on dextrose.

Group C. Type, Bacillus "Gwyn"; the gas liberated under favourable conditions from dextrose not limited to $CO_2$.

Group D. Type, B. enteritidis: gas liberated from dextrose not limited to $CO_2$, even in comparatively unfavourable conditions.

Division II. Colon-like morphology: motile.

Order i. *Dextroso-non-lactoso-fractors.*

Groups E, F, and G (not to be confused with B. coli communis): differentiable by nature of growth in lactose media especially.

Order ii. *Dextroso-lactoso-non-sucroso-fractors.* Group H. B. coli communis verus: do not ferment sucrose, but cannot be subdivided by present tests except serum-reactions.

Order iii. *Dextroso-lactoso-sucroso-fractors.* Group J. B. coli communior: differ from Group H in being able to ferment sucrose.

Division III. B. lactis-aërogenes-like morphology: non-motile.

*Polysaccharid-fractors.* Includes B. lactis aërogenes, B. pneumoniæ Friedländer, etc. Subdivision into groups requires further work. Ferment polysaccharids, such as starch.

DIVISION I. TYPHOID-LIKE MORPHOLOGY.

*Group A.* Type: *B. fœcalis alcaligenes* (Petruschky). Culture kindly sent to me by Prof. Lehmann, who obtained it from Dr. Dieudonné. Under my observation about one year.

Rate of growth typhoid-like; does not ferment nor form acid in any sugary media; not agglutinated by highly potent (*i. e.*, thousandfold dilutions) typhoid, enteritidis or coli serums, which are otherwise efficient dilutions.[12] Morphologically typhoid-like.[13]

*Group B.* *B. typhi abdominalis.**[14] Types: A number of different races have been utilized and some have been kept under observation for several years.

Forms abundant acid in dextrose media but no free bubbles of gas. Growth comparatively slow. Does not form any acid with lactose or sucrose. Gives clumping in high dilutions with potent typhoid serum. Sparse growth with characteristic acid formation in litmus milk whey.

[12] All the samples of human serum, typhoid or otherwise, which I have tested, clump this organism even up to hundredfold dilutions. It or some ally is probably a common inhabitant of the intestine of man, and its products are probably absorbed.

[13] B. fluorescens nonliquefaciens has been taken for this organism, to my certain knowledge. B. alcaligenes, however, gives no trace of fluorescent pigment.

[14] Serums were made from all types marked with asterisk *.

*Group C.* Types: *Bacillus "Gwyn"** and *Bacillus "O"* of Dr. Cushing; kindly sent me by Prof. Flexner and Dr. Cushing.

Form abundant acid in dextrose media, but free gas only when the other constituents of the medium are favourable (p. 384). No acid or gas from lactose or sucrose. Rate of growth typhoid-like. In milk whey typhoid-like. Morphologically typhoid-like. No reaction with clumping typhoid, enteritidis, colon, etc., sera.

*Group D.* *Bacillus enteritidis group.*[15] Types: Cultures of "Gärtner"* (see foot-note 14), "Günther,"* "Hatton"* (own), "Morseele," "Gand," "Sirault," "Calmpthoult," "Aertrycke," hog cholera, B. typhi murium, Psittacosis, morbificans bovis (of Basenau),* "Sheffield," "A" (own). (Unfortunately I was not able to procure cultures of Sanarelli's B. icteroides to compare with the others.) None of the above have been under observation less than one year.

Form acid and gas from dextrose. No gas or acid from lactose or sucrose. Rapidity of growth greater than typhoid; growth on gelatine in general distinguishable. Milk whey, characteristic turbidity, preliminary typhoid-like acidity (2-3 per cent), alkaline about fourth day. Serum reactions not universal within the group; three main subgroups are recognisable (see p. 356): ($\alpha$) "Gärtner," "Morseele;" ($\beta$) "Hatton" and other races mentioned except ($\gamma$) B. morbificans. These subgroups run more or less into one another, thus "Günther" is more or less intermediate between ($\alpha$) and ($\beta$); but there is no advantage in complicating too much.

Slight reactions with sera from certain races of typhoid, especially in the case of subgroup $\alpha$. No reaction with colon, etc., sera. Morphology typhoid-like.

[15] Owing to the confusion which has been made amongst the bacteria of swine diseases, I think it well to avoid speaking of a "Hog-cholera" group.

DIVISION II.—COLON-LIKE MORPHOLOGY.

*Group E.* Dextrose, but not lactose, fermenter. Type: "Urethra." Given to me as a typical B. coli communis; obtained in a culture made from urethra; under observation 5 years.

Forms acid and gas from dextrose, none from lactose or sucrose. Milk whey never goes acid but becomes turbid. Morphology colon-like. Rate and appearance of growths colon-like. No reaction with any of the sera mentioned; it is capable of reaction to its "own" serum, but none sufficiently potent was prepared for trial on other bacilli on an extended scale.

*Group F.* Dextrose, but not lactose, fermenters. Type: "425"* (see foot-note 14). Obtained by me from fatal case of perforative peritonitis; under observation 4 years.

Growth colon-like. Morphology colon-like. Forms acid and gas from dextrose. Slow and slight formation of acid with lactose but no gas. Milk whey becomes turbid, and acid corresponds (2.5 per cent) to that of a typhoid or enteritidis culture in milk, this, however, gradually increases instead of diminishing; milk is generally loosely clotted from 10th day. No acid or gas with sucrose. No reaction with sera of other groups mentioned. Reacts well with its own serum; no other type found which reacts with its serum.

*Group G.* Dextrose, but not lactose, fermenters. Types: "+ A,"* "$FE_3$,"* and 14 others; many obtained from contaminated water near Maidstone.

Acid and gas from dextrose; acid, but no gas, in peptone-lactose solutions; no acid or gas with sucrose. Morphology and rate of growth colon-like. In milk whey, after 24-48 hours, only about 5-6 per cent acid, milk whey does not become very turbid. By serum reactions fairly well-marked group, but some races which resemble the types fail to give serum reaction; members do not react with other sera than those of the group.

*Group H.* *B. coli communis verus.* Types: "Escherich 1"* and "2,"* "$CN_2$,"* "W"* and others. I have taken this to be the standard for Bacillus coli communis, from the fact that the type species were kindly especially examined for me in Prof. Escherich's laboratory and kindly sent to me by Dr. Pfaundler. "$CN_2$" and "W" were types from Prof. Gruber; so far as cultural and morphological characters go I am unable to distinguish them from Escherich's type.

Acid and gas from dextrose and lactose; *none* from sucrose. Milk whey turbid, with much acid (10-15 per cent), never becomes alkaline. Mutual reactions of sera absent. No reaction with other sera tried except type "G" of the following group, which reacts with "W" mutually.

Under this heading I may mention that several observers have kindly sent me cultures. One from Prof. Flexner agrees with the types; but one "Tübingen," used as a type for class purposes in Prof. Baumgarten's laboratory and brought to me by Dr. Fawcett in 1895, is certainly no B. coli communis. In general appearance on culture media and in fermentation tests it agrees fairly with the type, but in morphology it tends to grow in threads and the flagella are quite unlike those of any other colon-like bacilli; they are quite short and very closely coiled. One peculiarity is the tendency to aggregate in flocculi in broth cultures. If this is the culture used by Baumgarten as the foundation of his statement that a high clumping power of normal rabbit's serum upon B. coli is common, it gives some explanation of his statement by insufficient controls. "Tübingen" serum, which is potent to 200,000, has no effect upon B. coli ver., nor do any of the serums tried affect "Tübingen" cultures when carefully controlled against the flocculi formation. The bacillus has been examined from time to time during 5 years, and retains its original peculiarities.

*Group J.* Acid and gas from dextrose, lactose and sucrose. Types: "G,"* "TK,"* "CaV,"* "CaVI"* and many others—B. coli communior.

Characters and morphology like those of group Bacillus coli communis verus except that sucrose is fermented and acid freely formed from it. Mutual serum reactions not frequently met with within the group. I am inclined to think that this is a commoner inhabitant of human fæces than members of the last group, but have not made any direct experiments. Should this prove to be the case, it might be distinguished from the Escherich type as *B. coli communior.*

*Note on non-motile colon bacilli.*—Besides the bacilli directly conforming to Classes H and J, there are many that I have met which differ in apparent absence of motility and also want of flagella; in general, the majority I have studied are able to ferment sucrose. It is difficult to evaluate the true worth of this, to my mind slight, morphological difference or, indeed, to be perfectly certain that there is this difference. It may be that the actual "B. coli immobilis" is not real, for the conditions necessary for the full manifestation of motility are by no means determined. It sometimes happens that after long searches in hanging drops one or two really motile individuals may be found, although the vast majority are devoid of any true locomotor power. In searching for motile power it is necessary that the medium should not contain substances capable of giving rise to directly injurious matters (such as fermentable saccharids or alcohols) by the induction of acidity. It is generally stated that colon bacilli are sluggish, and this is no doubt true for ordinary media; I once succeeded in preparing a medium (containing horse's serum and human ascitic fluid) in which the types $CN_2$ and G whizzed across the field of the microscope fully as rapidly as a cholera vibrio; unfortunately typhoid and enteritidis bacilli were not tried in it. The matter was not followed out, but it appears to be evident that there is a good field for research in this direction.

Then, again, there are possible fallacies in the direction of the demonstration of flagella. The flagella of colon-like bacilli are far more difficult to demonstrate than those of other allied organisms. It seems that they are more readily shed and broken off than in the case of typhoid, enteritidis, etc., bacilli, especially when staining methods necessitating much washing and many transferences from one fluid to others are employed. Notwithstanding this defect, van Ermengem's silver method is, on the whole, to be preferred. To illustrate the fracture and disappearance of flagella by this method, I once made a batch of coverslip smears from an emulsion (race $CN_2$) and stained some by van Ermengem and others by a method resembling that of Pitfield, but in

which saturated stanneous chloride was used in place of alum. (This gives very intense staining with the single fluid, but is apt to give very much precipitate, although I have sometimes obtained quite clear specimens. Formula: sat. sol. $SnCl_2$, 1 pt., 10-15 per cent tannin 1 pt., sat. alcoholic methyl violet few drops; warm; put in the coverslips and allow to cool; wash in distilled water. I may note here that in methods like this all precipitate and indeed all stain may be removed by treatment with warm tannin solution and the specimen restained.) In the van Ermengem specimen the flagella were short and many of them broken off, whilst in the tin-method specimen the flagella were mostly retained and fully twice the length of those in the van Ermengem one. It is erroneous to state that the flagella of B. coli are shorter than those of typhoid bacilli; when complete they are fully as long. By repeated observations of apparently non-motile cultures I have occasionally succeeded in finding isolated flagella-bearing individuals. I am inclined to think that the absence of conditions favourable for the development and demonstration of flagella may lead to a false conclusion as to motility and the presence of flagella.

DIVISION III.—B. LACTIS-AËROGENES-LIKE MORPHOLOGY.

*Division of B. lactis aërogenes.*—Fermenters of polysaccharids, such as starch. Type: B. lactis aërogenes, "Kosseck" obtained from Prof. Escherich's laboratory, "Brad 1,"* "Brad 2,"* "Aërogenes"* from typhoid stools and many others.

Besides possessing the power of fermenting dextrose, lactose and sucrose, these groups can ferment also substances like starch and inulin. Some have very strong reducing power, and also great power of surviving and overcoming considerable amounts of acid which they have produced. They are non-motile, without flagella, and plumper than B. coli. They do not form chains or threads and are not often seen even in pairs. B. mucosus capsulatus, Friedländer's pneumobacillus, and also the "Schweinepest" bacillus used by Voges and Proskauer, are closely allied to this division. All of those which I have tested give the pink-red reaction of Voges and Proskauer,[16] and this possibly may be regarded as a group colour-reaction; it is *not* given by any of the members of the other divisions which have been classified above. The bacilli are grown in a peptone-sugar-salts solu-

[16] *Zeitschr. f. Hygiene*, 1898, xxviii, p. 30.

tion for a day or two and then about 1 cc. of strong caustic potash is added; an eosin-like colour appears after a while near the surface and lasts several days; the pink colour may appear as early as an hour after the caustic is added; Voges and Proskauer only describe its appearance after a day or so. The red colour may be quite intense and slowly fades away generally after several days.

There is no mutual serum reaction between the members tried nor with the other sera (typhoid, etc.) which have been prepared.

This group ("lactis aërogenes" of Escherich) is divisible into subgroups by the aid of fermentation tests, thus the "Kosseck," which, as coming from Escherich, may be regarded as "B. lactis aërogenes verus," is able to produce acid and gas from peptone-starch but not from peptone-inulin media. Other types will ferment both starch and inulin; others again inulin only. The power of fermenting starch no doubt has led to the description of the frothy culture on potato.

On gelatine plates the colonies of this group are bulky, white and moist, without the tendency to spread which characterises the colon-like organisms; consequently they are circular in outline. In slope cultures there may be a tendency for the growth to slide down when the surface is vertical.

### BACILLI NOT TO BE CONFUSED WITH THE FOREGOING GROUPS.

One not infrequently meets with organisms which simulate the thin, spreading, irregular colonies with brown translucency of the colon and of the somewhat colon-like groups in gelatine plates. These, so far as I have observed them, are distinguishable either by morphological characters, such as chain and thread formation, or in cultures. Some of them are incapable of producing acid or gas with the three sugars, whilst others have some power in this direction. Some observers have gone so far as to include bacilli which are capable of liquefying gelatine under the colon group. This seems to me to be a childish disregard for one of the prime criteria of Bacillus coli communis. I have met with organisms which have the power of slowly liquefying gelatine media and which at first on gelatine plates had some resemblance both to B. coli communis and to B. lactis aërogenes. Any cultures about which there is some doubt should be kept on sugar-free gelatine media for 8-10 weeks, by

which time liquefaction will probably set in if it occurs at all. In general, I find these types do not produce acid in milk whey nor do they produce acid from lactose in broth. One culture of such an organism was kindly sent me by Dr. Mervyn Gordon; in his description of it in his paper,[17] he states that it is agglutinated by typhoid serum. I have tried cultures of it with typhoid sera of potency 1:20,000-1:50,000 without the slightest trace of positive reaction at 1:100 and I have also prepared a serum with it of potency 1:200,000, which has not the slightest action upon several typhoid or enteritidis races even at 1:100 dilution. It is to be surmised that the technique adopted by him was faulty, as indeed is suggested by his description of his experiment.

### REVIEW OF SOME TESTS ORDINARILY APPLIED.

*Morphology.*—Speaking generally, morphological characters are not of much value for subdivision of these bacteria. Among the organisms with typhoid-like morphology, I find that some individuals, as B. morbificans bovis, are not described as forming threads in cultures; in my hands this bacillus readily forms threads in broth and in agar.

The characteristic staining of the enteritidis group when grown at room temperature, especially upon gelatine, I have not met with in any other group or division. There is perhaps a tendency for the appearance of stained middle and unstained ends with the Bacilli of Gwyn and of Cushing, though I have not obtained very characteristic preparations such as are obtained with the enteritidis group.

It may be noted that flagella are of no value as a classifying test; it is not possible to differentiate between groups A, B, C and D by their means. The numbers of flagella given by many observers are not reliable, and are usually too low. The number of flagella will also not differentiate between the different groups of the more colon-like organisms. They are, therefore, of no value for ultimate classification.

*Cultural Tests. Normal Agar and Gelatine Media.*—The former does not afford any particular aid to differential diagnosis, although differences, such as the free growth of B. aërogenes, should be noted. The latter may give some idea of probabilities between the main

[17] *Journal of Pathology and Bacteriology*, 1897, iv, p. 446.

divisions. By continued transference upon gelatine for many months or several years, as in keeping the collection growing, I find that the tendency to spread and form thin layers is frequently lost by the typhoid and sometimes by the more colon-like races; in general, the enteritidis has not much tendency to spread, although each of the races I have had does so from time to time. It should be noted that the gelatine for a slope culture must always be melted and sloped within a few hours or minutes of the time of sowing; gelatine which has been sloped for days becomes dry on the surface and this tends to prevent the spread of all but the most hardy races. Gelatine does not avail for discrimination between many of the groups.

*Broth and "Peptones."*—The normal broth I use is made from stale meat (beef or ox-heart) which has been hung for 3 or 4 days, infused for about 24 hours, strained, and heated after addition of 1 per cent Witte's "peptone" and 0.5 per cent pure NaCl. This is the basis of the gelatine and agar media. The neutralisation is done by caustic soda until a decided pink tinge is obtained with *rosolic acid* either on a porcelain plate or by cautiously dropping the dilute almost colourless indicator on the surface of a sample. The end reaction is practically at litmus neutral point. Phenolphthalein appears to me to be a clumsy indicator and the growths of the bacilli which I am dealing with do not appear to thrive nearly so well in media in the preparation of which this indicator is used. I have received several cultures from the Johns Hopkins laboratory and these, as well as others which have been sent to me from elsewhere, have given markedly more luxuriant growths in my hands than the original cultures from which they were planted; whether this is due to the reaction or the general constitution of the media is more than I can say.

In broth cultures of the enteritidis group, I have always found general turbidity of the medium and that a pellicular growth formed on the surface. In such broth no pellicle is formed by typhoid, "Gwyn" or colon groups. No pellicle occurs when simple "peptone" (Witte) and salt solutions (with or without sugars) are planted with any of the groups.

By digesting meat with pancreatic ferment (the "zymine" of

Burroughs and Wellcome was used), the resulting broth being made faintly alkaline (as above) to rosolic acid, most luxuriant growths occur with all the groups; and (except B. lactis aërogenes, which was not tried) abundant pellicle formation occurred. In this medium I find that typhoid races grow more freely than colon races do in "normal" broth. On the other hand, broth (and gelatine media made from it) made by digesting meat with pig's stomach and acid (L. Martin) is a very unfavourable medium for all these organisms; in general (including B. lactis aërogenes) they grow in floccular masses and leave the broth almost clear.

When broths for sugar tests—either the normal or pancreatic varieties—were made, they were first inoculated with B. lactis aërogenes and then with B. coli communis until no trace of acid or of gas was generated.

*Milk.*—Milk is not a satisfactory differentiator. It fails to distinguish groups A, B, C, D and E from one another, although these are so widely different. It also fails with H and J, early clotting occurring in both cases. Even when litmus is added it does not become much better, since owing to its initial alkalinity it does not shew differences markedly between A, B and E. Owing to alkali formation (after a preliminary diminution in some cases) a certain amount of clearing may occur (due to saponification?). This is especially the case with the enteritidis group, where it may occur after about 2 or 3 weeks; it may also happen with the typhoid group after a few weeks, as I have not infrequently seen, but it has generally occurred in tubes which have been left untouched in the incubator. Since the alkali formation is dependent upon growth in contact with the air, slight jarrings by removal may interfere with the phenomenon (compare the effect of movement in the preparation of diphtheria toxins).

If dextrose (1 or 2 per cent) is added, the milk becomes highly acid (*e. g.*, 10-15 per cent to normal alkali) with typhoid and enteritidis, but is not clotted. The clotting caused by B. coli is commonly ascribed to the acidity, but in this case it must be due to some special acid. According to Blachstein,[18] B. typhi produce a lævo-

[18] *Arch. d. sciences biol.*, St. Petersb., 1892, i, pp. 199 and 299.

rotatory lactic acid from dextrose whilst B. coli gives a dextrorotatory one.

*Potato.*—Potatoes are too uncertain in their constitution (especially their acidity) to be reliable as a test. Moreover, it is impossible to distinguish by cultures on potato such widely different groups as E, F, G, H and J. Potato is therefore useless for purposes of discrimination.

*Test Media of Capaldi and Proskauer.*—The two media ((1), 2 per cent Witte peptone, and 0.1 per cent mannite, (2) asparagin medium with salts and glucose or mannite) recommended by Capaldi and Proskauer,[19] are also of slight differential value. I find the peptone medium remains acid with groups B and C (typhoid and Gwyn) for several days, whilst it fails to differentiate between D, E, F, G, H and J. The other medium does only what is claimed of it for the typhoid group. After 24 hours' incubation, according to the vigour of growth, it soon becomes acid. I do not think that it is to be accorded any very high position as a test.

*Indol.*—The presence or absence of indol in cultures of these groups of bacilli is not of value for differential methods. Thus group G forms but a trace of indol, much like the amounts formed by typhoid and enteritidis. The most abundant indol production I have met is with Bacillus "425" (group F). There does not appear to be any relation between the amounts formed by the sucrose-fermenters and the non-sucrose-fermenters, nor between the lactose- and the non-lactose-fermenters, if we compare type "G" with "$CN_2$," or "Urethra" with a true B. coli communis of Escherich, in which the quantities of indol are about the same.

*Semi-gelatinised Media.*—These I have not tried. Since the designers have merely claimed that their action is due to the diffusibility of more highly motile organisms, it is not to be expected that groups A, B, C and D would be differentiable by this means.

### *Litmus Milk-whey.*

Up to this point I have acted the part of a destructive critic, but I cannot speak too highly of this medium. It is largely upon it as a

[19] *Zeitschr. f. Hyg.*, 1896, xxiii, p. 472.

basis that the present grouping or classification of these organisms has been made. In fact, according to my experience, more can be learnt from the course and appearance of cultures in this medium (titrated for acid if necessary with one-tenth normal NaOH) than *with any other single medium.* It is easy to prepare, but the method originally described by Petruschky,[20] who devised it, is not to be recommended in that he advocates the use of mineral acid, whereby some change of the lactose into dextrose and galactose might occur.

The following method gives admirable and apparently very constant results: Fresh milk (free from antiseptic adulterations) is slightly warmed and clotted by means of essence of rennet. The whey is strained off and the clot hung up to drain in a piece of muslin. The whey, which is somewhat turbid and yellow, is then cautiously neutralised, neutral litmus solution being used as an indicator, with 4 per cent citric acid solution. When it gives a good neutral violet colour with the litmus, it is heated upon a water-bath at 100° C. for half an hour or so; thereby nearly the whole of the proteid is coagulated. It is then filtered clear and neutral litmus is added to a convenient colour for titrations or rougher observation. Lastly, it is measured off by a burette into quantities of 10 cc. per tube or else sterilised at 100° C. in bulk. If difficulty is found in obtaining it perfectly clear it may be sterilised in bulk and allowed to settle for a few days or it may be passed through a Berkefeld filter; this, however, is rarely necessary to the practised laboratory attendant. *It must never be heated above 100° C.*

The value of this medium is partly due to the almost complete absence of proteid, to the presence of lactose and to the presence of a small quantity, perhaps about 0.1 per cent, of a sugar of the nature of dextrose or galactose (vide infra). Theobald Smith [21] has recently called attention to the presence of the latter constituent in milk, and I may say that I came to the conclusion some while back that there was some other saccharid in milk besides lactose; the exact determination must be left to the chemist. The contained salts (which by the

[20] *Centralbl. f. Bakter.*, 1889, vi, p. 657.

[21] *Journal of the Boston Society of Medical Sciences*, 1898, ii. p. 236.

way do not interfere with the colour or titration accuracy of the fluid) may also be of value in determining the results.

Group A. B. fæcalis alcaligenes clouds the whey and produces alkali without previous formation of acid, and is thereby at once distinguished from members of groups B, C and D.

Groups B and C are much alike; after 24 to 48 hours at 37° C. the medium is hardly perceptibly turbid but has become distinctly acid (= about 3 per cent normal soda); the reaction after this remains about the same.

Group D begins with an acidity of from 2.5-4 per cent, the medium being made quite turbid. About the 4th day, at 37° C., it commences to become alkaline and may soon reach about 16 per cent normal $H_2SO_4$.

Group E, type "urethra," never forms acid in whey. It is distinguished by growth in glucose media from A.

Why this organism does not produce any acid is not clear; possibly the second sugar is not dextrose or possibly alkali is formed too rapidly for the manifestation of acid.

Group F produces an enteritidis-like culture at first, but the acidity continues and increases.

Group G produces 5-6 per cent of acid; the culture is only slightly turbid in 24-48 hours and never becomes alkaline.

Groups H and J give at least 10-15 per cent of acid in 24-48 hours; the fluid is turbid and never becomes alkaline.

Group B.lactis aërogenes makes acidity and turbidity; in vigorous growths the colour of the litmus is completely reduced except a thin film on the surface; upon shaking and aëration the red colour appears.

These short notes will give some idea of the value of the medium. No artificial medium that I have tried has given such good results.

### FERMENTATION TESTS.

*Methods.*—The apparatus for determining the production of gas bubbles which I described some time ago [22] has enabled me to carry

[22] *British Med. Journal*, 1898, i, p. 1387.

out a very considerable number of fermentation tests. The apparatus consists merely of a small test-tube inverted within an ordinary one; the medium is poured in and the process of discontinuous sterilisation causes the inverted tube to become completely filled. It is preferable to use fairly recently sterilised media, for when kept for a few weeks air becomes dissolved and may appear as a bubble in the collecting tube if acid is produced by the bacillus under observation, giving a false impression of gas production. In all these tests it is advisable to do considerable batches at a time, both of known and unknown organisms, in order to be sure that the medium reacts towards the known in the way it should. It is also not a bad plan to do a double set and leave one undisturbed in the incubator whilst the other is examined day by day. The routine of examining after 24 and 48 hours, and then on the 7th, 14th and 21st days, seems to be ample.

When only $CO_2$ is formed by destruction of a sugar (as in the case of glucose with typhoid) no gas collects in the collecting tube as a rule, but when the evolution is caught in full vigour some may sometimes be seen in the inverted tube. When both H and $CO_2$ are given off, some $CO_2$ collects in the tube; this may be absorbed by caustic solution if desired, but I find that a sojourn of about a week in the incubator has a precisely similar result in that all the $CO_2$ has disappeared. I have not made any estimations of the relative quantities of H and $CO_2$ in the fermentation tubes, since these would be of no value whatever. To obtain ratios of value, the *total gas* formed must be collected and extracted by vacuum over mercury before estimation. The fad of recording the ratios $CO_2$ to H, obtained with ordinary open fermentation tubes, appears to me to be a sheer waste of time and trouble.

### FACTORS TO BE CONSIDERED IN APPRECIATING FERMENTATION TESTS.

Several factors have to be considered in appreciating the results of growths in media containing sugars, fermentable alcohols, etc.

It is of prime importance to know that the sugar employed shall not have been changed in its constitution during the process of making the medium. It is of no less importance to ensure that other con-

stituents of the medium are unable to give rise to fermentations. To ensure these points:

(*a*) No sugar-containing test medium should be exposed to the action of free acids (especially mineral acids) or caustic alkali.

(*b*) No sugar-containing test medium should be exposed to temperatures above 100° C.; indeed, the lower the sterilising temperature used the better.

(*c*) No sugar-containing test medium should be heated at all, except it be in a *neutral* condition, some such neutral point as that of litmus being used.

(*d*) No sugar-containing test medium should become yellower or browner than the stock solution to which the sugar has been added for the preparation of the medium; the change of colour shews that some change has taken place. (The medium called dextrose-broth, or dextrose-gelatine, seen in most laboratories is manifestly browner or darker than the plain media. This only means that the medium is not truly a dextrose-medium; the addition of dextrose to a medium should produce no change of colour if it is to be used for test purposes.)

(*e*) The stock solution to which the test sugar is to be added must be tried with appropriate cultures to prove the absence of fermentable matter. It should be ascertained that no acid, as well as no gas, is produced. This is especially necessary where meat-broths are used. It may be necessary to plant broth several times with enteritidis, colon, or aërogenes cultures before it is finally made up with sugar.

(*f*) Eggs should not be used for clearing media destined for fermentation tests, the ovomucoid and other constituents being capable of being split up.

(*g*) When tests are made upon sugary or fermentable media, they should be efficiently controlled by means of cultures of known bacilli in the same batch of medium.

(*h*) It is well to see that tested cultures are really pure and also that any given test gives the same result on repetition as it did on first trial.

Especially in the trial of media of unknown constitution with bacilli

of known proclivities, it is necessary to pay heed to the following facts, which are also not without importance for general purposes:

1. *The result depends on the nature of the sugar, etc., employed.* By means of the groups of bacilli we are dealing with it is possible to determine that certain sugars may be present or that they are absent. But they will not shew differences between certain sugars. Thus dextrose, lævulose, mannose, arabinose (a pentose), galactose and maltose (a disaccharid) give much and apparently fairly equivalent quantities of acid with races which are able to decompose dextrose (*e. g.*, typhoid) or acid and gas with races (enteritidis and coli) which are able to ferment dextrose. This statement is founded on titrations of 1 per cent solutions containing 1 per cent Witte's peptone; very exact titrations with equimolecular solutions have not been tried.

Mannite and dextrin also react like dextrose, in that those bacilli which will make acid or gas with one of the former will do so with the other; less acid appears to be formed from the dextrin, which was carefully tested with Fehling's solution to see that it was sugar-free.

The groups under discussion fail in a general way to distinguish between these different substances; group A forms no acid from any of them. This being the case, it did not appear to me to be advisable to follow the suggestion kindly given me by Dr. Ruhemann to compare samples of these sugars of different configuration or rotatory power. It may be that the finer discrimination or greater differentiation of these or other bacilli may permit of less coarse work and greater discrimination.

With dextrose, lactose and sucrose, and starch, inulin and glycerin in conjunction with the use of milk whey, much may be done in classifying the bacilli which belong to these groups; the morphological characters, the rate of growth in normal media, the non-liquefaction of gelatine and appearances thereon and the decolorisation by Gram's method not being omitted.

2. *The result depends upon the amount of the sugar present and the power of overcoming initial acid formation.* Capaldi and Proskauer[23] have shewn that some bacillary cultures tend to remain acid

[23] *Zeitschr. f. Hyg.*, 1896, xxiii, p. 452.

when small quantities of glucose or mannite are present. Thus typhoid cultures in 1 or 2 per cent peptone with 0.1 per cent dextrose, and, I might add, with maltose, etc., tend to remain acid; eventually, however, this bacillus is capable of overcoming the acid and the culture then becomes neutral and eventually alkaline. I find, similarly, with 0.1 per cent lactose and a Bacillus coli communis, or 0.1 per cent sucrose and a Bacillus coli communior, after 24 hours' incubation there is no acidity, the acid formed being rapidly neutralised. On the other hand, 1 per cent of either sugar with the appropriate bacillus yields a permanently acid culture. It is evident, therefore, that the amount of the fermentable substance present must be duly considered.

3. *The result depends upon the favourableness of the other constituents of the medium to which the fermentable substance is added.* It is not merely necessary to have a given fermentable substance in a medium, the remaining constituents must be sufficiently favourable to the growth of the bacillus to be tested.

For most of the groups a plain solution of Witte's peptone 1 per cent and sugar 1 per cent is sufficiently rich in nutritive material for gas production, given a bacillus which has the power of fermenting the sugar. In the case of organisms which are not able to flourish well in such simple media no gas may collect, although the sugar is fermentable. I have repeatedly tested Bacillus "Gwyn" at different times (and also Bacillus "O" of Cushing less often) for gas formation in 1 per cent Witte's peptone and 1 per cent dextrose. It appears from these oft-repeated tests that, although the medium is acidified, no gas bubbles collect; in fact, so far as appearances go, the cultures might well be of typhoid bacilli. Very different, however, is the result if 1 per cent of dextrose is added to sugar-free "normal" or "pancreatised" broth; here in both cases, besides the abundant acid, much gas is formed.

The action of B. enteritidis (a hardy race) upon glycerin may be in the same category. On repeated observation I note that only acid and no gas is produced by growth in 1 per cent Witte's peptone and 1 per cent glycerin. On the other hand, all the groups from E on-

ward have the power of producing gas from this simple medium. I find, however, that Voges and Proskauer,[24] in their paper on the bacteria of hæmorrhagic septicæmia, say that the hog cholera bacillus ferments glycerin added to their peptone and salt solution. While I have not observed gas formation with this bacillus growing in simple peptone-salt solutions containing glycerin, it may be that the more nutritive meat broth may enable the bacillus to produce gas from glycerin. This is a point which I was about to determine when I left England on the present expedition.

Further experiments of a like nature were contemplated to determine the simplest media compatible with gas formation by more and less hardy groups. Some preliminary experiments were made with proteid-free media, such as asparagin solutions and sodium-urate solutions. The latter give very pretty results if saturated solutions are used with sugars, for where acid is engendered the uric acid comes down in beautiful acicular crystals.

4. *Is the sugar altered by growth of a bacillus which does not produce acid or gas?* I have made experiments, for instance, by growing B. fæcalis alcaligenes in glucose, and, after awhile, planting the medium with B. typhi; abundant acid is then produced. Or after growing B. enteritidis in lactose media, subsequent planting with a B. coli gives rise to acid and gas. These and other experiments shew that even if the sugar is altered, the resulting bodies are fermentable by those bacilli which are ordinarily capable of so doing.

*Preliminary Search for other Sugars and Fermentable Substances.*

It seemed possible that there might be many substances of carbohydrate nature which would serve for further differentiation of these and other bacillary groups. After consulting Tollens' "Handbuch der Kohlehydrate" and Landolt's "Das optische Drehungsvermögen," the most useful mode of attacking the question appeared to be an empirical haphazard one.

Various substances, such as fruits, seeds, etc., were collected and extracts of these were made in the cold state when starch, inulin, etc.,

[24] *Zeitschr. f. Hyg.*, 1898, xxviii, p. 20.

were present or suspected. The extract was then tested for its power of reducing Fehling's fluid both before and after hydrolysing with a trace of dilute sulphuric acid. Plain neutral litmus solutions [25] were made according to the indications of the amount of sugar present. These were put into my fermentation tubes and tried with beer yeast and two members of each of the above groups for acid and gas formation. Great care was taken that no extract was heated except in a litmus-neutral condition. Again, other media were first planted with yeast and thoroughly freed from any substances which this could destroy before testing with the bacilli.

Among the sources of sugars which have been tried the following may be mentioned: Truffles, yeast, potato juice (starch-free), juice of Jerusalem artichoke, milk of ripe cocoanut, acorns, madder root, mangold-wurzel, carrots, fruit of Cratægus oxyacantha, C. pyracantha, C. punctata, Rosa, medlar, Solanum nigrum, S. dulcamara, tomato, egg plant (brinjal), Physalis, Cytisus laburnum seeds, Digitalis leaves, privet berries, holly berries, mistletoe berries, ivy berries, fir cones, Abrus seeds, bananas and many other sources. In several instances the hydrolysed, as well as plain, extracts were tested. A few glucosides (*e. g.*, Quillaia) have also been tried.

It was intended to go more fully into those substances which appeared to promise results, and to purify the fermentable substance. This I have as yet been unable to do. After purification it was proposed to retest in media made of favourable constituents.

I shall not go fully into the results obtained up to the present. The work upon this line was commenced early last year (1899), but was much interrupted by other calls upon my time. But anyhow, this mode appears to open up an enormous field for research both by the bacteriologist and the chemist. A few points of interest may be noted. In the first place the plain extract from the fruits, seeds, etc., may not have sufficient pabulum for fermentations by the bacilli; thus a plain extract of hawthorn gave no gas with group C and one of medlar gave the same result with groups C and D, although acid

[25] Much credit is due to my laboratory attendant, Mr. W. Mitchell, for the preparation of these media; he managed to produce beautifully clear media from the most unpromising materials.

was formed. When peptone or broth (sugar-free) was added to the medium free gas evolution took place when the same bacilli were cultivated in it. In fact, all through the work with many of the extracts the difference between groups C (Gwyn and Cushing) and D (enteritidis) was exemplified time after time, the former giving cultures like those of typhoid. A few substances deserve particular mention:—

*Yeast-broth.*—The first lot tried was made from baker's yeast on Spronck's formula.[26] Much $CO_2$ was formed by all the group types: in only Cushing, Gwyn and aërogenes, however, did permanent (non-absorbable to NaOH) gas result. This appeared to promise well as a differential test between C and all the other groups except aërogenes. However, on repetition, I was unable to obtain exactly the same result, although the first test gave a most striking and apparently unequivocal result. This requires further work.

*Cocoanut milk.*—This was obtained from the ripe cocoanuts we get in England. According to Tollens, it should contain the disaccharid, cane-sugar or sucrose. On testing with Fehling it gives no reduction until it has been hydrolysed. If this non-reducing sugar were really sucrose, it should not be attacked by any of the groups but " J " and B. lactis aërogenes. As a matter of fact, however, even the typhoid bacillus produces much acid and all the other cultures much gas as well. It is clear, therefore, that the sugar is different from the ordinary sucrose; it reacts towards these bacilli like dextrose or more accurately the disaccharid maltose.

*Jerusalem artichoke.*—The filtered juice contains a similar sugar in that it gives no reduction of Fehling till inverted. With the cultures it acts like the cocoanut sugar. The same is true of tomato, mangold-wurzel, Solanum dulcamara, etc.

*Glucosamin,* prepared from decalcified crab-shells by sulphuric acid, reacts like dextrose with the bacilli. With group C the addition of something more than plain peptone is necessary for gas production. I need hardly add that this substance is not attacked by pure cultures of yeast.

[26] *Annales de l'Institut Pasteur*, 1898, xii, p. 702.

*Potato juice*, starch-free, is not without interest. It contains a substance capable of reducing Fehling, but it is attacked only by B. lactis aërogenes.

*Acorns* contain a body which is fermented by the sucrose-fermenters, but is not attacked by the other groups. This substance is destroyed by English beer yeast so that no fermentation occurs with the sucrose-fermenters after the action of the yeast.

*Gums*, such as arabic, tragacanth and agar-agar, are not fermented by any of these groups until hydrolysed.

It would be idle to pretend to do more at the present time than to call attention to the wide field for research which is thus opened. Much of the information upon sugars in books on chemistry, especially in regard to the fermentations by yeasts, seems most unsatisfactory. It does not appear at all clear (vide Tollens) that in general pure yeast cultures were used nor indeed what species of yeast was used.

If this contribution should stimulate further and more accurate work, both bacteriological and chemical, in this interesting field, it will indeed be more than this incomplete account of my preliminary work on the subject can claim to merit.

*Pará, Brazil, August, 1900.*