A TECHNIQUE FOR MEASURING THE EXCRETION OF BACILLI OF THE ENTERIC GROUP IN THE FAECES OF INFECTED MICE

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(With 1 Chart.)

In recent studies on experimental epidemiology, and especially in a series of reports by Webster (1922, 1923 *a*, *b*, *c*, *d*, *e*, *f* and *g*), attempts have been made to standardise certain of the factors concerned in the spread of bacterial infection among mice. One such factor is dosage, to which great importance has been attached by Amoss (1922 *a* and *b*) and also, though to a somewhat less extent, by Webster, whose later work leads him to emphasise the importance of host-susceptibility in determining the form of the epidemic wave. Webster would appear to regard variations in dosage as decisive, in the sense that an effective dose must be applied; but once the critical limit of dosage is exceeded he would give first place to host-susceptibility in determining the subsequent course of events.

The various factors which may play a part in determining the form of the epidemic curve of mortality are considered by one of us in a recently published report (Topley, 1923), and the factor of dosage is discussed at some length. Attention is called to the great difficulty in defining this term, when we turn from experiments in which graded doses of culture are administered by some controllable technique, and consider the spread of infection from host to host. A satisfactory definition will not, however, end our difficulties; we have still to determine in how far the methods at our disposal will enable us to give to the term, as defined, that quantitative expression which is essential if significant results are to be obtained.

It appeared reasonable to assume, in dealing with enteric infection in mice, that the most important path of infection was by the excretion of the causative bacilli in the faeces of an infected animal, and their passage to a fresh host in contaminated food. Acting on this assumption, the problem clearly resolved itself into the practicability of measuring the excretion of *B. enteritidis Aertrycke* (herein referred to as *B. aertrycke*[1]) in the faeces of mice, in a way that would allow continuous records to be obtained for each individual mouse, and so enable us to study the distribution of *B. aertrycke* among the population at risk.

[1] The organism referred to is of the "Mutton" variety.

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If we consider a mouse-population, among which enteric infection is spreading, we may ask whether the proportion of mice which are excreting the specific organisms at a given moment, or the number of bacilli which are being excreted by any given mouse, or by all the excreting mice together, forms the better measure of the effective dose to which a susceptible member of the population is submitted. A high proportion of excretors will give us a high proportion of the total daily collection of faeces with some degree of infective power, and thus the risk of any given portion of food being contaminated with such material will be high. Massive excretion by a few of the mice will be less effective in ensuring the contamination of a high proportion of the daily food, but may be of decisive importance by increasing the probability that any portion of the food which is contaminated will contain an effective dose of bacilli.

For these reasons, we were prepared, at this stage, to accept as adequate a technique which would serve to detect *B. aertrycke* in specimens of faeces with a considerable degree of certainty, and which would afford an approximate measure of the relative numbers of viable *B. aertrycke* present, when comparing one specimen with another.

THE TECHNIQUE EMPLOYED.

It would serve no useful purpose to outline the various methods and modifications which we tested during the earlier part of this investigation. The technique finally adopted depends upon the power of *B. aertrycke* to produce hydrogen sulphide from peptone, and perhaps from other constituents of the media employed. Lead acetate is added to an agar medium in suitable concentration, and the colonies of *B. aertrycke* are detected by the browning of the colony itself and of the surrounding medium. The medium actually employed differs little from that recommended by Amoss (1922 *a*) for the differentiation of colonies isolated on preliminary plates. The actual method of preparation is, however, a matter of the utmost importance. The addition of the lead acetate solution, during the later stages of preparation, may result in chemical or physical changes which render the medium almost useless as a nutrient material. We do not propose to discuss here the nature of the changes involved, but we would emphasise the importance of rigid adherence to the empirical formula which has sufficed to eliminate these disturbing variations, and which is given in full in the Appendix.

The specimens of faeces are collected by placing each mouse in a specially constructed funnel, with a short wide neck opening into a conical tube, and provided with a loosely fitting cover. The specimens are always collected in the morning, when the cages are changed, and the mice are placed in the funnels *en route* from the dirty cages to the clean. The general method adopted in putting up the cultures for counting follows closely the technique described by Wilson (1922), and we can entirely confirm his views with regard to the advantages of roll-tubes over plates, where large numbers of cultures have to be prepared.

The actual method of preparing the suspensions of faeces, and the subsequent dilutions is as follows:

The faeces are emulsified in a few c.c. of Ringer's solution, 2–10 c.c. according to the amount of faeces present, and the suspensions so prepared are allowed to settle until the coarser particles have subsided to the bottom of the tubes. Each tube of faecal suspension is now placed in a separate rack, and in the same rack are placed (*a*) a tube of standard diameter containing 10 c.c. of normal saline with 0·5 per cent. formalin, (*b*) a tube containing 3·96 c.c. of sterile Ringer's solution, and (*c*) three sterile test tubes, 6 by $\frac{5}{8}$ ins. in size.

For making the dilutions sterile capillary tubes are employed, calibrated to deliver 50 drops per c.c. With one of these about 1 c.c. of the faecal suspension is taken up, 10–40 drops are added to the tube of formalinised saline, the actual number added being noted, two drops are added to the tube containing 3·96 c.c. of Ringer's solution, and one drop is added to the first of the three empty tubes. The tube containing the 1/100 dilution in Ringer's solution is now shaken and, with a fresh pipette, ten drops of this dilution are added to the second empty tube, and one drop to the third. These three tubes are now placed in a rack in a water-bath kept at 40–45° C. and to each is added 2 c.c. of the special medium employed, which has been kept at hand in the melted condition in a second water-bath. The three tubes are immediately rolled under the tap in the usual manner, allowed to set firmly, and incubated for 24 hours at 37° C. in a sloping position with the plugged mouths of the tubes pointing slightly downwards.

The tubes containing the dilutions of the faecal suspension in formalinised saline are set aside until the following day, when the culture-tubes are counted. Counting is carried out with a hand lens, and only the brown colonies are enumerated. The typical *B. aertrycke* colony is sharply defined, deep brown in colour, and with a definite and sharply defined brown halo; but variations from the typical form occur and all brown colonies should be counted, except those which are greyish-yellow rather than brown, and have no halo. Colonies of this latter type are rarely present and do not in practice cause any real difficulty.

Each of the three tubes, containing 0·02, 0·002 and 0·0002 c.c. of the original faecal suspension, is examined. If brown colonies are present in the second or third tubes as well as in the first, only that tube is counted which corresponds to the highest dilution, unless the number of brown colonies in this tube is less than ten, in which case the tube containing the next lower dilution is also counted if the brown colonies are well-formed and distinct. In all cases the highest figure obtained after allowing for the dilution is taken as correct. Sometimes only one tube contains brown colonies, and these are then counted whatever the number may be.

When all tubes have been counted, the dilutions of the faecal suspensions in formalinised saline, which were put up at the same time as the roll cultures, are sorted out; and those are retained which correspond with the culture-

tubes which have given brown colonies, the rest being discarded. These tubes are well shaken, and their turbidity is compared with a graded set of turbidity tubes prepared according to the method of McFarland (1907), but adjusted so that tube 10 corresponds to a suspension of *B. aertrycke* containing approximately 1,000,000,000 bacteria per c.c. When adding the faecal suspension to the tubes of formalinised saline, the number of drops added is adjusted so that the turbidity may fall between 10 and 1 on the scale employed.

From the turbidity reading so obtained, and the number of drops which were added to the 10 c.c. of formalinised saline when the dilution was prepared, we calculate the factor required to reduce the actual count obtained for that specimen to the number of viable *B. aertrycke* per c.c. of a faecal suspension with a turbidity corresponding to 1,000,000,000 bacteria per c.c.; or shortly, and very approximately, we express our results as the number of viable *B. aertrycke* per 1,000,000,000 bacteria in the faeces, assuming the turbidity of the suspensions, after the settlement of the coarser particles, to be due entirely to the bacterial content.

The figure so obtained is based on the count of brown colonies, and it remains to verify the fact that these colonies are colonies of *B. aertrycke*. For this purpose five or ten brown colonies are picked from the positive tube, sub-cultured into small amounts of broth (1–1·5 c.c.) in small test-tubes, and incubated over-night at 22° C. Next morning, an equal amount of saline containing 0·5 per cent. formalin is added to each tube, and the tubes are then heated for one hour at 55° C. Each of these killed suspensions is then tested against high-titre agglutinating sera, agglutination being carried out at 55° C. for two hours. In the great majority of cases all the colonies tested from any one tube will be positive or negative when tested by agglutination. In such cases the figure already entered is retained if the agglutination results are positive, or discarded if they are negative. Where some of the colonies from a given tube are positive and others negative, the figure for the corresponding specimen is corrected accordingly. It is probable that this correction is often unnecessary, and indeed erroneous, the negative results being due to overgrowth of the brown-producing organisms by some other bacterium in the broth sub-cultures; for it is impossible to ensure pure sub-cultures from crowded tubes. On the other hand, such results are sometimes due to the presence of two kinds of brown colonies. It is probably better to correct all figures, where mixed agglutination results are obtained, rather than none; and one rule or the other must, of course, be rigidly adhered to.

To take an actual example:

A specimen of faeces was treated in the manner recorded above. The third tube of the culture series, containing 0·0002 c.c. of the faecal suspension, gave 13 brown colonies.

The corresponding turbidity tube, in which 15 drops (15 × 0·02 c.c.) of the faecal suspension had been added to 10 c.c. of formalinised saline gave a turbidity of 2, corresponding to 200,000,000 bacteria per c.c.

Thus, the number of bacteria producing brown colonies per c.c. of a suspension of this specimen of faeces having a turbidity corresponding to 1,000,000,000 bacteria per c.c., would be approximately

$$\frac{10 \times 15 \times 0{\cdot}02}{10 \times 2 \times 0{\cdot}0002} \times 13 = 9750.$$

Five of the 13 brown colonies were sub-cultured to broth and agglutinated as described. All gave good agglutination with a *B. aertrycke* serum. The figure 9750 was therefore entered unaltered in the records.

It will be noted that the figure taken for the dilution in the turbidity tube is not strictly correct, the quantity added to the total volume with the drops of faecal suspension not being allowed for. The technical error, as will appear later, is so much larger than any error involved by this omission in calculation, that no good purpose would be served by the additional labour involved in using the more accurate figure.

It will be noted also that the actual size of the drops employed is immaterial to the final figure obtained, provided it be kept constant during the whole operation.

Before considering the way in which this and similar figures should be treated in further calculations or arguments, it is essential to determine the errors to which they are liable. These unfortunately are very large, and quite indeterminable in any given case.

That this should be so is not surprising. We are not dealing with a simple bacterial suspension, but with a mixture containing enormous numbers of living and dead bacteria and other organisms of various kinds, and with unknown substances in solution or suspension which may be exerting potent but indeterminable effects on the particular bacteria we are trying to enumerate. Moreover the crowding difficulty, referred to in detail by Wilson, is here unavoidable. We are trying to count viable bacteria of one particular species, which are occasionally present in almost pure culture, are more often mixed with large numbers of viable organisms of quite other kinds, and in many cases form a tiny minority of all the organisms present.

But quite apart from the question of overcrowding, or actual crowding out of the organisms we are trying to isolate, there is another factor which needs consideration. We are relying on one particular phenomenon, the production of hydrogen sulphide and its subsequent interaction with the lead salt, to identify the colonies of *B. aertrycke*. Do such factors as overcrowding of *B. aertrycke* itself, or the crowding of the cultures with organisms of other types, modify this activity in such a way that the characteristic reaction may be suppressed, and colonies of *B. aertrycke* be missed?

If the crowding of other organisms masked the typical reaction, then we might reasonably expect that, if we were to take a considerable number of tubes, which did not show brown colonies but in which there was a chance of colonies of *B. aertrycke* having developed, and if from these tubes we were to sub-culture a series of colonies which, apart from the absence of browning,

might pass for colonies of this organism, we should find on applying agglutination tests that a proportion of such colonies would prove to be *B. aertrycke*.

We have sub-cultured in this way 620 colonies from 124 tubes, none of which showed any brown colonies, and all of which had been inoculated with faeces from mice fed on *B. aertrycke*. Subsequent agglutination tests were negative with each of the 620 cultures.

The question of the effect of overcrowding, where colonies of *B. aertrycke* are present, can clearly be studied by picking non-brown colonies from tubes in which brown colonies occur. Table I shows the result of such a proceeding. It may be noted that the brown colonies were in all cases proved to be *B. aertrycke* by the agglutination of broth sub-cultures.

Table I.

No. of brown colonies	No. of non-brown colonies agglutinating as		No. of brown colonies	No. of non-brown colonies agglutinating as	
	Positive	Negative		Positive	Negative
156	3	2	14	1	9
115	6	4	14	0	5
74	2	3	13	0	5
69	1	4	9	3	2
51	4	6	8	1	9
50	0	7	7	3	2
34	0	5	5	0	3
19	2	8	5	0	5

The answer is perfectly definite. The error due to the failure of browning in some of the colonies of *B. aertrycke*, in tubes where *B. aertrycke* is present and is producing brown colonies, is a very serious one; and it is increasingly serious with increase in the total number of brown colonies present. If we take the cases in which the brown colonies counted numbered 50 or over, we find that, of 42 non-brown colonies tested, 16 or 38·6 per cent. were actually *B. aertrycke*. If we take those cases in which the total brown colonies per tube numbered less than 50, we find that of 63 non-brown colonies tested by agglutination 10, or 15·9 per cent., were actually *B. aertrycke*.

There is no possibility of applying any correction in such cases, since we have no figure for the total non-brown colonies, nor could such a figure be obtained. To enumerate in each tube the colonies which, while not brown, might pass in other respects as colonies of *B. aertrycke* would be an impossible task.

It may reasonably be held that Table I displays the results in too unfavourable a light. The tubes examined in this way were purposely selected as showing relatively few brown colonies, compared to the total colonies present, or alternatively large numbers of brown colonies, sufficient in themselves to cause some degree of crowding. If similar estimations were made on a large sample of the tubes actually utilised for counting, the errors from this failure of browning in colonies of *B. aertrycke* would probably not be large, but the technique will always show a large and unknown error in defect, where the figure obtained is high.

It seems then that the technique adopted will enable us to detect small numbers of *B. aertrycke*, when these are present, with an accuracy at least equal to that obtained by any ordinary method of isolation of such organisms from the faeces. We have little doubt that the accuracy of detection is, in fact, considerably higher than is obtained in any of the ordinary plating methods, if only because of the three widely separated dilutions which are employed. In a considerable proportion of the counts, colonies of *B. aertrycke* have been found on one of the three tubes and not on the other two, and the tube in which they are present is by no means always that containing the largest amount of faecal suspension.

On the other hand the actual figures obtained are always liable to error, and the probable error increases rapidly as the count itself increases. Apart from any other considerations, therefore, we should hesitate to pay too much attention to the actual figure obtained in any one count.

In actual practice, very high figures, over 10,000,000 for instance, may be obtained for the count of viable *B. aertrycke* per c.c. of a faecal suspension of standard turbidity, and the question arises as to how we should treat such figures in considering the numerical results obtained from a considerable series of determinations. It is obvious that there are the gravest objections to the inclusion of such figures in a series in which average values are to be considered. In a series of 50 counts, for instance, which included one count of 10,000,000 it would be quite immaterial, so far as average results are concerned, whether 40 of the remaining 49 counts gave figures of 100 apiece, or whether all were negative.

This overwhelming effect of single large counts appears to us to be so obviously undesirable, when the biological possibilities are kept in mind, that we have thought it best to adopt a purely arbitrary method of recording and charting the results of our counts. This method actually consists in using a logarithmic scale. It should, however, be regarded merely as an arbitrary method of scoring the rate of faecal excretion of any given mouse at any given moment. Any figure greater than 0 and not greater than 10 is scored as 1; any figure greater than 10 but not greater than 100 is scored as 2; any figure greater than 100 but not greater than 1000 is scored as 3, and so on. A count of 10,000,000 would thus be scored as 7, while a count of 10,000,010 would be scored as 8.

When dealing with any considerable number of counts this procedure simply means that we are adopting an arbitrary series of class-intervals, and are using the class-frequencies, instead of the absolute or average numerical values of the individual determinations, in subsequent arguments.

It may, perhaps, be doubted whether quantitative results with so wide a margin of error have any real advantage over simple records of presence or absence of *B. aertrycke*. We shall deal more especially with this question in later reports, where we hope to consider the effect of variations in the distribution of the excretion of *B. aertrycke* among the members of a population,

in which this infection is spreading. For the moment, we will content ourselves with pointing out that the time expended in obtaining roughly quantitative results is very little in excess of that needed for qualitative determinations. With the help of one assistant, two workers can easily put up the necessary dilutions, add the media and roll the tubes, at the rate of 80 specimens an hour. The time taken in preparing the dilution tubes and the suspensions is not great, and the counting is rapidly accomplished, unless an unusually high proportion of the specimens give brown colonies. We doubt if any qualitative method, giving equally accurate results as regards detection, would take less time.

Although it is necessary to confirm the nature of all brown colonies by agglutination tests, and indeed these tests are eminently desirable for quite other reasons which are referred to in a subsequent paper, yet it may be pointed out that the work involved in eliminating brown colonies which are not *B. aertrycke* is not very considerable.

In going over records of the testing of 3795 brown colonies, we find that 2786 of these proved to be *B. aertrycke*; 77 gave negative agglutination results, although other colonies from the same tubes reacted positively; while 932 colonies gave negative results, and no colonies of *B. aertrycke* were identified in the tubes from which they came. It may be noted that 345 of these 932 colonies came from five mice which persistently excreted organisms, other than *B. aertrycke*, which gave rise to brown colonies. We have tested the fermentation reactions of large numbers of such colonies, but the only organisms so far encountered have been *B. proteus*, and an organism which has given the more important reactions of *B. coli communior*, but which appears to be very rarely present in mouse faeces.

We may note, at this stage, the results of examining specimens of faeces from our normal stock.

We have so far examined 692 specimens obtained from 632 normal mice. Of these, 691 specimens were negative, while one specimen gave colonies of *B. gaertner*. *B. aertrycke* was never isolated. These results are in close agreement with those cited by Webster (1923 *a*). It may be added that brown colonies of any kind were exceedingly rare, being noted in less than 0·5 per cent. of all the specimens examined from normal mice.

In conclusion, we would note a few of the results obtained by repeated examinations of the faeces of individual mice, which have been fed on cultures of *B. aertrycke* on a single occasion. In such cases we have taken counts on the second day following feeding, and on the one or two succeeding days; three counts have been made during each of the two succeeding weeks, and two counts during each of the three weeks following. We have ended our period of observation by making two successive counts on the 41st and 42nd day. These time-intervals have been modified slightly in individual cases, but have not been departed from in any essential respect, except where a mouse has died during the period of examination, or has been kept alive beyond it for some special purpose.

In charting our results we have recorded time-intervals as abscissae and the score of the *B. aertrycke* counts as ordinates. The area between the curve of excretion and the base-line has been blackened to increase clearness of representation. The actual days of observation have been marked below the base-line in the case of each individual mouse.

In Chart I are shown the records of six mice.

Mouse *A* affords a good example of persistent carriage and excretion lasting over several months and then ceasing, the animal remaining in apparently perfect health.

Mouse *B* affords a good example of persistent excretion followed by death from typical enteric infection.

Mouse *C* illustrates a common type of result in which massive excretion for a few days is followed by death with typical lesions.

Mouse *D* shows a not uncommon result in which a mouse excretes *B. aertrycke* on a single occasion only, immediately preceding death from enteric infection.

Mouse *E* shows that an animal may die of typical enteric infection without ever excreting the specific organism in its faeces.

Mouse *F* is an example of the numerous occasions on which administration of *B. aertrycke* by the mouth has been followed neither by the appearance of this organism in the faeces, nor by any other evidence of infection.

Results of each type are discussed more fully in the succeeding papers. We include them here partly as an illustration of our methods of recording results, partly because it seems to us that they afford in themselves some evidence that the technique is reliable within certain limits. We must avoid the circular argument of approving the technique from a consideration of the results, and then accepting the results because of our approval of the technique; but we may legitimately attach importance to the fact that the results, in a considerable series of observations, tend to be consistent among themselves, and that the general picture presented by our records fits in with our general knowledge of the processes of infection.

APPENDIX.

The composition of the medium employed for the bacterial counts is as follows:

Beef extract (Lemco)	5 gms.	Salicin	10 gms.
Peptone (Witte) ...	10 ,,	Andrade's Indicator ...	10 c.c.
Sodium chloride ...	5 ,,	Agar	25 gms.
Lactose	10 ,,	Water	1000 c.c.
Saccharose	10 ,,		

The beef extract, peptone and sodium chloride are added to the water in a flask and placed in the steamer till dissolved. The agar is then added and the flask replaced in the steamer till this has dissolved, when it is again removed and white of egg is added for clearing. This is coagulated in the

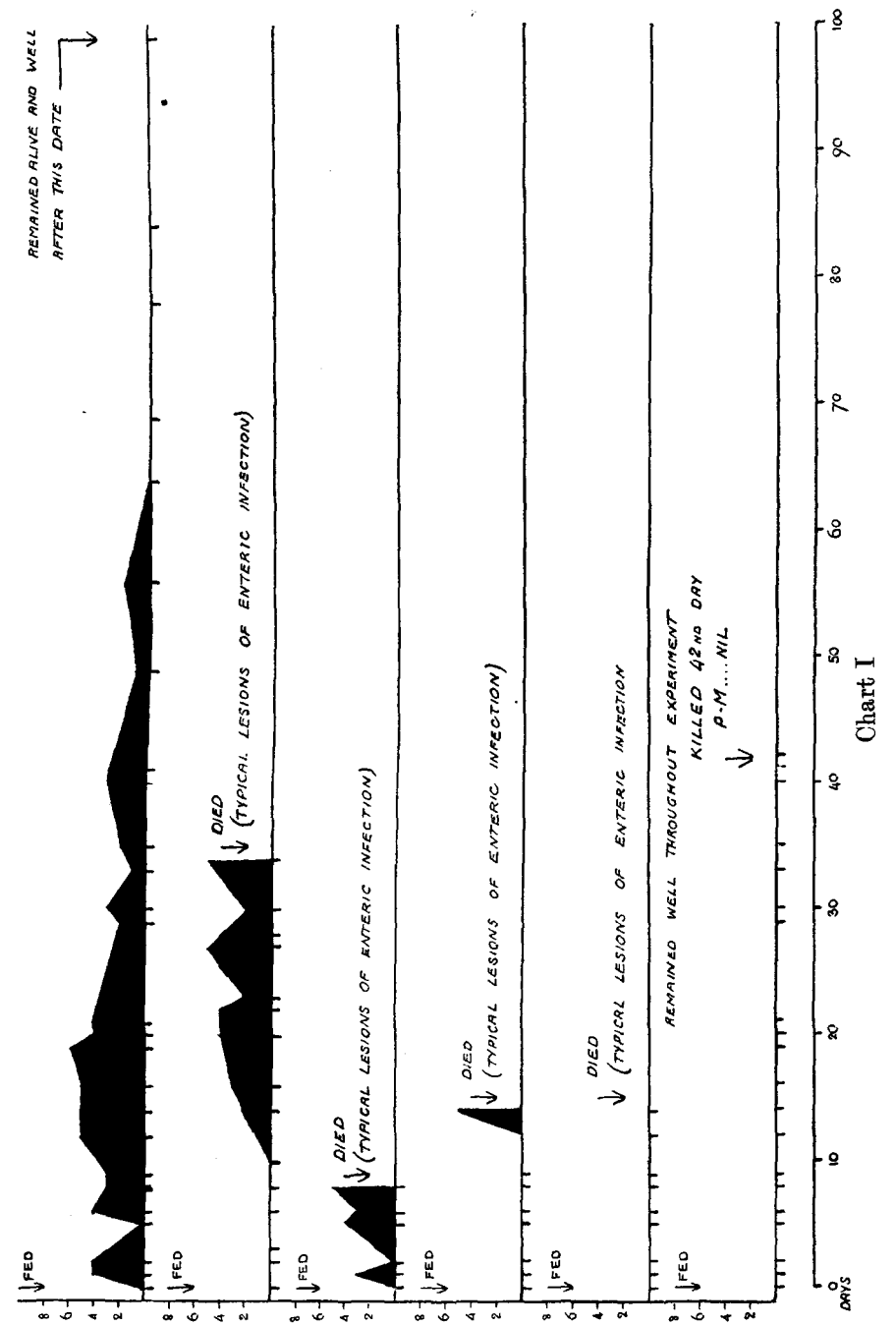

Chart I

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steamer and the medium is then filtered. The *p*H is determined and is adjusted to 7·1. The Andrade indicator is then added and the medium is autoclaved at 107° C. for 20 minutes. Meanwhile the requisite amount of lactose, saccharose and salicin have been dissolved in the minimal amount of distilled water (about 30 c.c.) and placed in the steamer for 20 minutes. As soon as the medium is removed from the autoclave this concentrated solution is added to it, and stirred with a sterile rod. The medium is immediately transferred to small flasks and tubes, with due precautions as regards sterility and these flasks or tubes are then placed in the steamer for 10 minutes, after which they are stored until required. At this stage the *p*H of the medium is again determined, and should be found to be in the near neighbourhood of 7·4.

When the medium is to be employed the necessary amount is melted in the steamer and placed in a water-bath at 55° C. A solution of lead acetate, approximately 1 per cent., is prepared by taking a small flask containing 30 c.c. of sterile distilled water, raising this rapidly to the boil and boiling for 2 minutes. The loose wool-plug is then removed and 0·3 gm. of neutral lead acetate (lead diacetate), previously weighed out and kept in a small sterile tube, is dropped into the flask; the wool-plug is at once replaced. This procedure ensures the removal of the dissolved CO_2 and prevents the precipitation of lead carbonate. The solution so prepared is at once added to the melted medium, by means of a sterile pipette, in the proportion of 5 c.c. for every 100 c.c. of medium, and the flask or tube is quickly and vigorously shaken. With a separate sterile pipette 5 c.c. of a sterile 1 per cent. solution of disodium hydrogen phosphate is added, for every 100 c.c. of medium, and the flask or tube is again shaken, and is then placed in the water-bath at 55° C. The medium is now ready for addition to the culture tubes.

The addition of the lactose, saccharose and salicin to the medium is probably optional, though the presence of small amounts of some fermentable carbohydrate is certainly not a matter of indifference. The presence of these fermentable substances, however, sometimes yields useful information with regard to the general character of the bacteria present[1].

What the exact effect may be of the addition of sodium phosphate in the last stage of preparation, we are not in a position to say. The exact change which occurs in the complex agar medium from the addition of the lead salt is at present unknown. We were led to test the effect of the addition of the phosphate for reasons which need not be entered into here. As a matter of experience we have never had any difficulty with this medium since we made this addition to our previous procedure.

[1] In recent experiments we have replaced the lactose, saccharose and salicin by dextrose (1%), since this gives better results when *B. aertrycke* greatly outnumbers other organisms in the faeces.

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