THE STANDARDIZATION OF KILLED AGGLUTINABLE CULTURES OF *B. DYSENTERIAE.*

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(Report to the National Medical Research Committee.)

(With 2 Charts.)

Up to the present the value of the agglutination test in the diagnosis of bacillary dysentery has not been finally decided. Very numerous have been the attempts to settle the question, and as various have been the methods employed. It is more than probable that the variety and lack of uniformity of technique have been largely responsible for the wide differences in the conclusions arrived at by different experimenters.

The conviction that both improvement and, above all, uniformity of method are essential to the settlement of the question led Prof. Dreyer early in 1915 to consider the possibility of applying to cultures of *B. dysenteriae* the process of standardization that he had already put into use for the preparation of Agglutinable cultures of the Typhoid-Paratyphoid group of bacilli. At his instigation, and largely under his direction, I started the investigations set out in this paper, the practical result of which has been the issue of standard agglutinable cultures of three varieties of *B. dysenteriae* in Aug. 1916, by the Standards Laboratory on behalf of the National Medical Research Committee.

REASONS FOR STANDARDIZATION OF TECHNIQUE.

The following factors are the most important of those that are known to affect the degree or intensity of the agglutination—reaction given by a serum of a particular strength.

(1) Time.
(2) Temperature.
(3) Number or mass of the bacilli on which the serum acts.
(4) The sensitiveness of the bacilli to agglutination.

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Of less fundamental importance, but none the less exercising a very appreciable effect are:

(5) Motion or absence of motion of the fluid.

(6) Area of the surface with which the fluid is in contact.

Further, the recorded result will vary according to the method of observation adopted (e.g., microscopic or macroscopic) and in the arrangement of the light for reading. And, again, the precision of the result will depend upon the fineness of gradation of the dilutions employed.

It is evident that, in the absence of any recognized uniformity of method, uniformity of results is impossible. If one and the same serum be tested by two methods, e.g.,

(1) With a thick suspension of bacilli, at room temperature, readings taken at the end of 30 minutes microscopically.

(2) With a thin suspension, at 50° C., readings taken after 4 hours macroscopically.

The "titre" of the serum by method (1) may work out as one in 500; by method (2) as one in 5000.

Now there is little difficulty in determining what are the best conditions *of time and temperature for the agglutination of any group of* bacteria. The temperature at which the velocity of the reaction is greatest is clearly the ideal, and in this respect the dysentery bacilli behave in the same way as those of the enteric group, viz., a temperature of 50°–55° C. gives a much higher velocity of reaction than lower temperatures. The best temperature being determined, it remains to fix the period of time required at that temperature for the reaction to arrive approximately at its end-point. In the case of the typho-paratyphoid bacillus two hours has been found sufficient, but our experience with the dysentery bacilli confirms the observations of others that a longer period of time is required for their complete agglutination. As a result of many experiments four and a half hours at 50°–55° C. was fixed upon as the shortest time in which the reaction becomes sufficiently complete for the purpose of taking trustworthy readings.

It is well known that the number or mass of bacteria upon which the serum has to act is a factor of great importance (Gruber 1897, Winterberg 1899, Sacquépée 1901, Dreyer and Jex-Blake 1905). Great variations of titre may be found for a particular serum with bacterial emulsions of varying thickness. Constant results therefore can only be expected either when emulsions of constant thickness are used or when the variations of agglutinability of different emulsions due to this factor are determined by experiment and included in a numerical

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reduction-factor which expresses the true relative agglutinability of each emulsion.

Working with formalized broth cultures of bacilli of the typho-coli group Dreyer found that by far the best readings were obtainable with relatively thin emulsions. For with these not only is apparent end-titre of a serum greatly raised, but also it is possible to gauge the precise degree of agglutination in a given tube according to the size of the flocculi suspended in the fluid. With such a method of reading a number of serums which do not differ from each other in strength by more than 10 %, if tested under precisely the same experimental conditions, may be differentiated from one another with certainty, and that without employing any finer gradation of serum-dilutions than is used in the ordinary clinical test.

The optimum density for agglutinable cultures of *B. dysenteriae* is rather greater than for *B. typhosus*, *B. paratyphosus* A and B and *B. coli*, for the flocculi formed by the former group are always small, and they may be too small for good readings if the emulsion is very thin.

The standardization of the thickness of the emulsions used in all my experiments was done by the method described recently by G. Dreyer and the writer (1916).

The complete flocculation of dysentery cultures by a serum is assisted by movement or circulation of the fluid. This is also true of other bacilli, but it is of more importance in the case of *B. dysenteriae* owing to the slowness and fineness of the flocculation. Continual movement of the fluid in the agglutination tubes may be obtained by incomplete immersion of the tubes in the water of the water-bath, for the heated lower part of the fluid is continually rising, and the cooler upper part continually falling to the bottom. If two tubes be taken, each containing precisely the same mixture of serum and culture, and one of them be fully immersed (i.e., so that the whole column of fluid is beneath the surface of the water), and the other be only half-immersed; at the end of a given period of time the agglutination in the half-immersed tube will be found to be in advance of that in the fully-immersed tube. The difference, expressed numerically, is usually about 10 %. The differences in a series of tubes between "total," "standard" and "trace," and the finer intermediate gradations of flocculation are more constantly clear and easy to read when the fluid has been kept in motion by incomplete immersion.

Any standard technique must of necessity include the use of tubes of a standard size and shape. For the velocity of the reaction is

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distinctly influenced by the size and shape of the tube. The narrower the tube in which a volume of mixture is contained, the further will the reaction have progressed in a given time. In general it appears that within certain limits the greater the surface with which the fluid is in contact, the more rapid and complete is the agglutination.

The factors in agglutination-technique with which I have dealt so far are all easily controllable. Evidently the best technique is that which embodies the optimum temperature and time, the best density of emulsion for high sensitiveness and accuracy of readings, circulation of the fluid, and a uniform and suitable size and shape for the tubes. But there is one more factor which is of greater immediate interest than any one of the above, because it is far less easy to control—and that is the specific sensitiveness or degree of agglutinability of the bacillary emulsions.

If a number of emulsions of undetermined but approximately similar thickness be made from a number of subcultures of one and the same strain of a bacillus, each emulsion will have its own specific degree of agglutinability, which may be greater than, equal to or less than the mean agglutinability of the batch. The variation from the mean may be so considerable that two successive emulsions differ quantitatively to the extent of 100 % or more. If all the emulsions be made of precisely the same thickness, the variation in agglutinability is diminished, but it remains so considerable that it is impossible to devise a quantitatively accurate technique without controlling this variable factor. The necessity for this has long been recognized, by scientific workers on agglutination (Madsen and Jórgensen 1902, Dreyer and Jex-Blake 1905), but a large number of clinical pathologists have, until very recently, been content to work with uncontrolled and variable cultures. Dreyer (1906) elaborated and perfected a technique for clinical typhoid and paratyphoid agglutination tests, in which all the optimum conditions for the reaction were embodied, and the variable factor of the agglutinability of emulsions was fully controlled by the comparative standardization of all emulsions against an original arbitrarily chosen standard emulsion. This entailed the introduction of the agglutinin unit, and the reduction to units of readings first expressed as dilutions.

The essential qualities required in an emulsion that can be employed in this manner are: stability and durability, a sufficiently high agglutinability, and freedom from any tendency towards spontaneous agglutination.

The main object of my investigation was to discover whether emul-

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sions of this kind can be made from cultures of the different groups of *B. dysenteriae*, and whether these bacilli behave in the same way as the typhoid-coli group, or in a different way.

Since Dreyer's investigations had proved the superiority of broth cultures over saline suspensions in the case of the typhoid group, it was natural to expect a similar superiority when other, but not widely different, bacilli are in question. From the start, therefore, I have experimented with broth cultures, and I have only used saline suspensions in some comparative experiments with a microscopic technique.

An attempt has recently been made by O. Schiemann (1916) to prepare a stable emulsion of dysentery bacilli (*Ruhrdiagnosticum*) for agglutination. He treated saline suspensions of the bacilli with heat of various degrees and with a number of chemical substances, including formalin, phenol, thymol and chloroform, but he failed to prepare a satisfactory emulsion by any of these means. All emulsions began sooner or later to agglutinate spontaneously. His failure must be attributed to the use of saline suspensions, since by almost identical procedure it is quite easy to make from broth cultures perfectly stable and satisfactory standard dysentery cultures.

EXPERIMENTS WITH FORMALIZED BROTH CULTURES OF DYSENTERY BACILLI. TECHNIQUE OF PREPARATION.

The method of preparation was that recommended by Prof. Dreyer for the preparation of standard agglutinable cultures of typhoid and paratyphoid bacilli and described on a leaflet which is issued with these cultures on behalf of the National Medical Research Committee. The leaflet is reproduced here. Paragraphs 1 and 2 deal with the preparation of the cultures (see also Dreyer, 1906) and the method of opacity-estimation and dilution to a constant opacity (see also Dreyer and Gardner, 1916). In all opacity tests and in a large proportion of the earlier agglutination experiments I made use of graduated pipettes and dwarf test-tubes. But later on I adopted the drop measuring technique almost exclusively, on becoming satisfied that it is no less trustworthy, and more economic of material.

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DIRECTIONS.

Preparation and Standardization of Agglutinable Cultures.

B. typhosus, *B. paratyphosus* (A and B), *B. dysentericus* (Flexner, Shiga, and Y), *B. enteritidis* (Gaertner), *B. coli*, and *Vib. cholerae*.

1. *Preparation.*

The bacillus is grown for 24 hours at 37° C. in ordinary *veal* peptone bouillon[1] in large Erlenmeyer flasks partly filled (1 litre of bouillon in a one and a half litre flask).

Before use the flasks of bouillon are sterilized in the autoclave at 115° C. for *not more* than 15 minutes, and then tested for sterility by incubation at 37° C. for 48 to 72 hours.

They are inoculated with a few drops each from a 20 to 24 hours old bouillon culture of the bacillus (*B. typhosus*, *B. paratyphosus*, etc.).

The culture used should be one which has been subcultivated daily in bouillon for one or two weeks (or longer). This continued subcultivation has the effect of increasing its agglutinability and diminishing any tendency to spontaneous agglutination.

At the end of 20 to 24 hours' growth at 37° C. the flasks are well shaken, and to each is added 0·1 per cent. (1 c.c. per litre) of commercial (40 per cent.) formalin. They are again shaken and placed in a *cold chamber* in the dark at about 2° C.

At intervals on the same day and on subsequent days for four or five days the flasks are again thoroughly shaken and *replaced at once in the cold chamber.*

After three or four days they will be found to be absolutely sterilized. Should it happen that the bacterial suspension is not entirely homogeneous it may be shaken for some hours in a mechanical shaker, or may finally be filtered through sterile cotton-wool.

2. *Standardization.*

The process of standardization consists (*a*) in making up the killed culture to an *opacity* as nearly as possible identical with that of the standard agglutinable culture, (*b*) in measuring its *agglutinability* as compared with the standard agglutinable culture by the use of standard serum.

Dysentery cultures should after dilution to standard opacity be left to stand in the cold store for one or two months before they are standardized for agglutinability and used for agglutination tests.

(*a*) The *degree of opacity* (turbidity) of the new culture as compared with that of the standard culture is determined as follows:

Take two stands and place eleven agglutination tubes in each. The tubes must be chosen so as to be of very nearly equal internal diameter (for this purpose some kind of a gauge is almost essential), and their surface must be devoid of scratches.

Prepare

(1) a few c.c. of a 1 in 2 dilution of the new-killed culture.

(2) ,, ,, ,, ,, ,, standard culture.

[1] The bouillon is titrated against phenolphthalein, and two-thirds of that amount of sodium hydrate, which would render it neutral to phenolphthalein, is added before the final boiling and filtration.

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With the dropping pipette (held vertically) measure out

	Drops of water	Drops of culture	
Into tube 1 of each stand	0	20	Culture full strength
„ 2 „	4	16	
„ 3 „	8	12	
„ 4 „	10	10	
„ 5 „	12	8	
„ 6 „	6	14	Culture diluted 1 in 2
„ 7 „	8	12	
„ 8 „	10	10	
„ 9 „	12	8	
„ 10 „	14	6	
„ 11 „	16	4	

One series of tubes receives the new-killed culture, the other the standard culture. Mix the fluids in the tubes well, and take comparative readings with artificial illumination against a dark background. A frosted electric bulb with a large opaque shade and a piece of dull black board or paper propped up behind gives the best results.

Hold up the tubes to be compared against the edge of the lamp-shade, which is arranged so that the source of light is just hidden from the eye, while it illuminates the particles suspended in the fluids. A darkened room renders the reading easier and more accurate. The outside of each tube must be cleansed and wiped dry before a reading is taken.

A tube is chosen at random from one series, and it is compared with the tubes of the other series in succession, until one is found to match it in opacity. The identity of these two tubes is recorded as the first reading.

If the tube chosen fails to correspond exactly with a tube in the other series, but is seen to have an opacity intermediate between the opacities of two adjacent tubes, the reading is recorded thus:

e.g., Series I tube 3 = Series II tubes 4–5.

Several tubes in one series are thus matched in succession with tubes of the other series, so as to obtain a number of separate readings.

Suppose that two of the readings are:

	Standard culture series		New culture series
(1)	Tube 5	=	Tube 9.
(2)	Tubes 2–3	=	Tube 6.

(1) Reference to the table shows that tube 5 contains 8 drops of undiluted culture, tube 9 contains 8 drops of a 1 in 2 dilution, i.e., 4 drops of undiluted culture.

Therefore 4 drops of the new culture give the same opacity as 8 drops of standard culture, i.e., the new culture is twice as opaque as the standard.

(2) Tube 6 contains 14 drops of diluted, or 7 drops of undiluted culture. Tube 6 in the new culture series falls midway between tubes 2 and 3 of the standard series which contain 16 and 12 drops of full strength culture respectively. It therefore corresponds with 14 drops of standard culture.

Again it is found that the new culture has twice the opacity of the standard.

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In practice the readings are found to vary a few per cent. on either side of the mean, and therefore it is best to take at least five readings and calculate an average. From these data the degree of dilution necessary to bring the new culture to standard opacity is determined.

E.g., in the example quoted the new culture must be diluted one in two.

The diluting fluid used is normal saline solution to which 0·1 per cent. of commercial formalin has been added.

If a high degree of accuracy in the opacity-estimation is required, the measurements may be made with a graduated 2·0 c.c. pipette into larger tubes, tenths of a c.c. being substituted for drops.

(*b*) To measure the *agglutinability* of the killed culture thus diluted proceed as follows:

Take two stands[1] and place 12 agglutination tubes in each. Prepare (1) a dilution of standard agglutinating serum of such strength that each cubic centimetre contains from four to eight standard agglutinin units, and from this prepare (2) a second dilution of half that strength.

With the pipette measure out

	Drops of normal saline solution	Serum dilution
Into tube 1 of each stand	0	10 drops of dilution 1
,, 2 ,,	2	8 ,,
,, 3 ,,	4	6 ,,
,, 4 ,,	5	5 ,,
,, 5 ,,	6	4 ,,
,, 6 ,,	3	7 drops of dilution 2
,, 7 ,,	4	6 ,,
,, 8 ,,	5	5 ,,
,, 9 ,,	6	4 ,,
,, 10 ,,	7	3 ,,
,, 11 ,,	8	2 ,,
,, 12 ,,	10	0 ,,

To each tube of one stand is added 15 drops of *Standard Agglutinable Culture*, and to each tube of the other stand 15 drops of the *killed culture* under standardization.

At each stage of the procedure the pipette is carefully washed and dried out with successive quantities of absolute alcohol followed by successive quantities of ether.

The stands are placed for two hours (in the case of dysentery cultures 4½ hours) in a water-bath at 50° C.–55° C., then allowed to stand for 15 to 20 minutes at room temperature and a reading subsequently taken by selecting in the series made with standard agglutinable culture the tube which exhibits standard agglutination (the highest dilution in which marked agglutination, without sedimentation, can be detected with the naked eye), and ascertaining which tube in the other series shows the same degree of agglutination. This comparison is repeated and made more exact by selecting in succession from the first series one or two other tubes which exhibit less than standard agglutination and similarly matching them in the second series. Should the tube be the same in each series the agglutinability of the killed culture

[1] Stands, dropping pipettes, agglutination tubes, etc.. can be obtained from Messrs Baird and Tatlock, Hatton Garden, E.C., or from Messrs R. B. Turner and Co., Eagle Street, Southampton Row, W.C.

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is clearly equal to that of the standard. If not the same, the degree of agglutinability of the killed culture is now readily determined.

Thus suppose that tube 5 in the standard series corresponds to tube 2 in the other series. The standard agglutinable culture is twice as agglutinable as the killed culture under standardization, since only half the quantity of serum has been required to agglutinate it to the same degree.

Hence, if any given serum presented for examination is found to agglutinate this particular killed culture in a dilution of, say, 1 in 500, then 500 multiplied by 2 and divided by the figure given on the label of the standard agglutinable culture is the number of *standard agglutinin units*[1] in 1 c.c. of the serum examined.

Or again, if the killed culture were, say, 1·3 times as agglutinable as the standard agglutinable culture, then, in the same example as above, 500 divided by 1·3 and again divided by the figure given on the label of the standard agglutinable culture is the number of *standard agglutinin units* in 1 c.c. of the serum examined.

In order to increase the accuracy of the standardization the test of sensitiveness to agglutination should be repeated *ab initio* two or three times, and an average taken of the readings thus obtained.

Readings should always be made by artificial light against a dark background.

From the Department of Pathology, University of Oxford, on behalf of the National Medical Research Committee.

TECHNIQUE OF AGGLUTINATION TESTS.

In all essential points the technique followed paragraph 2 (*b*) of the reprinted leaflet. When graduated pipettes and larger volumes of fluid were used, the relative volumes of the different constituents were nevertheless identical with those given in the table. The actual quantities used in the graduated-pipette technique are—Serum (in c.c.) 1·00, 0·80 0·60, 0·50, 0·40, 0·35, 0·30, 0·25, 0·20, 0·17, 0·13, 0·10, 0·00. Saline is added to each tube to make a total volume of 1·0 c.c. Then 1·5 c.c. of culture is added to each tube. There is one more tube in this series than in the drop-pipette series, since fractions of 0·1 c.c. can be measured, whereas fractions of a drop can not. The additional tube increases the accuracy of readings when the end-point happens to fall among the higher dilu tions of the series. When two or more emulsions of a bacillus were being tested against a serum or against each other, a sufficient quantity of the desired serum-dilution for the whole experiment was always made up, and a number of rows of tubes, one for each emulsion, were filled

[1] The *Standard Agglutinin Unit* is that amount of agglutinating serum which when made up to 1 c.c. volume with normal saline solution causes standard agglutination on being mixed with 1·5 c.c. of a particular standard agglutinable culture and maintained at 50° to 55° C. for two hours (dysentery cultures 4½ hours) in a water-bath followed by 15 to 20 minutes at the room temperature.

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with the graduated quantities of the serum-dilution and then with the various emulsions. All were subjected to precisely the same conditions of time, temperature, etc., during the course of the experiment.

READINGS AND CALCULATION.

The system of reading degrees of agglutination followed in these experiments has been used by Prof. Dreyer for many years, and was described in a recent paper by Dreyer and Inman (1917). When properly prepared and formalized cultures are used whether of the enteric or of the dysentery group of bacilli, certain degrees of agglutination are easily recognizable in the neighbourhood of the end-point of the reaction, viz., *Total agglutination* (T), i.e., sedimentation of the flocculated bacilli, and clearing of the supernatant fluid; *Standard agglutination* (S), i.e., well-developed flocculation, the clumps all remaining suspended in the fluid; and *Trace* (Tr), i.e., the least degree of clumping visible to the naked eye. If a long finely graded series of dilutions of a serum be made to act upon a standard culture, each of the above-mentioned degrees of agglutination will be found, together with a number of intermediate degrees. Among these it is easy to recognize *Total minus* (T –), *Standard plus* (S +), *Standard minus* (S –), *Trace plus* (tr +), *Trace minus* (tr –); and for a doubtful reaction *Query trace* (? tr).

From a very large number of records of finely graded agglutination tests of cultures of the typhoid-coli group, it was possible for Prof. Dreyer to work out a reduction table for calculating the numerical value of standard agglutination from any reading between total and nothing.

For instance, if 100 parts of a serum give exactly "standard agglutination," 1·47 parts of the same serum will give "total," and ·68 part will give "trace."

The full table of readings, with their reduction values, is as follows:

Reduction Table.

TOTAL	T	1·47
Total minus ...	T –	1·29
Standard plus ...	S +	1·13
STANDARD	S	1·00
Standard minus ...	S –	0·88
Trace plus	tr +	0·77
TRACE	tr	0·68
Trace minus ...	tr –	0·60
Query trace ...	? tr	0·53
Nil	0	0·46

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The great value of this table lies in the fact that it enables us to calculate the serum-quantity or dilution that will give standard agglutination although we have not actually a tube in the series which gives a "standard" reading, e.g., if a dilution of 1/50 of a serum gives a reading of "tr +." by reference to the table, we can see that standard agglutination will be given by a dilution of

$$\frac{1}{50} \times \frac{1{\cdot}00}{0{\cdot}77} = \frac{1}{38{\cdot}5}.$$

This table, though calculated for the typhoid-coli group of bacilli, is fully applicable to dysentery-agglutination, provided that a considerably finer flocculation be taken as "standard agglutination" for the latter than for the former group of bacilli.

COMPARATIVE READINGS.

In comparing the agglutinabilities of two emulsions which were being tested in parallel with each other, comparative readings by Madsen's method were always taken. A tube showing standard agglutination or thereabouts was chosen from one series and matched in the other, and the process was repeated once or more times with tubes showing rather more or rather less than standard agglutination. In calculating the results of the comparison, the average of the readings was taken. Table IX on p. 492 of this paper is the record of two typical experiments, and it shows the method of recording and calculating results used in this Laboratory.

The experiments recorded in this paper have been selected from a large material collected during nearly three years of experimental and routine work. It has only been possible to present a few of the most illustrative records, and in most cases the actual readings and the process of calculation have been omitted.

EXPERIMENTS WITH FORMALIZED BROTH CULTURES OF *B. DYSENTERIAE*.

Has the addition of 0·1 % formalin any immediate effect upon the degree of agglutinability of a broth-emulsion of *B. dysenteriae*?

The following experiments show that a practically immediate loss of sensitiveness may be caused by the addition of 0·1 % formalin.

Experiment I.

A culture of *B. dys.* Flexner in a litre of broth after 24 hours in the incubator was put in the cold chamber, and specimens were taken from

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it at intervals. To each of these specimens 0·1 % of formalin was added. The opacity of the first specimen was determined in comparison with an old suspension of suitable thickness, and this sample and all subsequent samples were diluted to the right opacity with sterile formalized saline[1] and then put back at once in the cold chamber. The first specimen (A) was taken after the culture had been in the cold chamber for four hours.

The second (B) was taken fourteen hours later; the third (C) four hours after the second. The fourth (D) four hours after the third; the fifth (E) one hour after the fourth. Finally the remainder of the culture (F) was diluted with saline to the same opacity, and a parallel agglutination test with all specimens was put up just 24 hours after the taking of the first sample.

Thus, when the test was performed, (F) was unformalized, (E) had been formalized for two hours, (D) for three hours, (C) for seven hours, (B) for eleven hours, and (A) for twenty-four hours. All were tested with the same serum, and the figures given express the relative quantities of serum required to agglutinate the different emulsions to the same point (standard agglutination), the quantity of serum required by the unformalized culture being taken as unity. Each figure is calculated as the average of four comparative readings, two of which were taken after three hours in the water-bath, and two after a further 18 to 20 hours at room temperature.

TABLE I.

	Samples of Flexner culture and lengths of time since formalin (0·1 %) was added to them					
	F No formalin	E 2 hours	D 3 hours	C 7 hours	B 11 hours	A 24 hours
Relative quantities of Flexner serum required to give standard agglutination	1·00	1·35	1·41	1·42	1·38	1·45

The table shows that the formalized samples A to E all require more serum to agglutinate them than does the unformalized culture F. Their sensitivity, then, has been lowered by the addition of formalin by about 40 % of its original (F) value. Within the first two hours there is an increase of 35 % in the quantity of serum required to cause standard agglutination, and in the next hour the increase mounts up to 41 %. After this time, the figures give us no certain evidence of further change.

If, as might be suggested, the apparent loss of sensitiveness of the formalized specimens were in fact due to a real increase of sensitiveness

[1] In this paper "saline" means 0·85 % NaCl solution in distilled water. "Formalized saline" means the same solution, to which 0·1 % commercial formalin has been added.

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of the living culture during its 24 hours in the ice-chamber, then we should of necessity see a definite gradation of the figures; the most recently formalized being not far removed in sensitiveness from the untreated culture. This is, however, not the case.

Experiment II.

A similar experiment with a culture of *B. dys.* Shiga is given in Table II.

TABLE II.

	Samples of Shiga culture and lengths of time since formalin (0·1 %) was added to them				
	E No formalin	D 1 hour	C 2 hours	B 17 hours	A 22 hours
Relative quantities of Shiga serum required to give standard agglutination	1·00	1·04	1·03	1·11	1·12

Here we see a loss of sensitiveness similar to that in Table I, but not so great. Moreover the loss appears to have been more gradual in this case, since there is a pronounced difference between the samples that had been formalized for one to two hours and those that had been formalized for 17 and 22 hours respectively. A number of similar experiments, either with *B. dysenteriae* Shiga or with *B. dysenteriae* Flexner, were performed. In the case of *B. dysenteriae* Shiga in one experiment the addition of 0·1 % formalin did not give rise within eight hours to any measurable loss of sensitiveness. But in the experiments with *B. dysenteriae* Flexner, a pronounced loss always occurred during that period amounting in one case to over 50 % of its original value.

What is the further progress and duration of the change caused by the addition of 0·1 % *formalin?*

The experiments described below demonstrate the fact that the change is variable both in degree and in duration, that it comes to an end within a period of not more than two months from the addition of the formalin; and that after that point sensitiveness of the culture remains constant for an indefinite period (at least 9 to 12 months).

METHOD.

In investigating the question of change of sensitiveness of an agglutinable emulsion of bacilli over a period of time it is necessary to have some fixed standard or criterion by which the results of the successive tests may be judged and compared with one another. For this purpose

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the ideal criterion is a standard agglutinable emulsion of known and unchanging sensitiveness, with which the new emulsion may be compared at intervals.

But since it is the whole object of this research to establish the possibility of preparing such a standard, we are driven for the time being to other devices.

I. A specific agglutinating serum may be assumed to keep a steady titre over a period of three months or so if it be stored in the ice-chamber and not used too fresh.

Common experience with immune sera teaches that they nearly always undergo a progressive loss of strength. Many series of experiments with freshly made agglutinating sera done in the course of this work have shown that the loss of power is the more rapid the fresher the serum is. After two months or so the rate of degeneration is usually so low as to be inappreciable in a further period of three or four months. Many specimens of serum, in fact, seem to arrive at such a state of stability that for twelve months or more no diminution of their agglutinating power can be detected. If a serum gives with a particular agglutinable emulsion the same readings at the beginning and at the end of a considerable period of time, the simplest conclusion we can draw is that neither the serum nor the emulsion has altered during the time. For instance, an emulsion of *B. dysenteriae* Y (more than four months old) was tested with a serum specific for that organism on Dec. 30, 1916. The quantity of serum required to cause standard agglutination was found to be 0·00177 c.c. Both serum and emulsion were kept in the cold chamber for six months, and on June 28, 1917, the test was repeated. On this occasion the quantity of serum required was 0·00173 c.c.

Since almost exactly the same quantity of serum agglutinated the same volume of emulsion to the same degree, we must conclude either that both serum and emulsion have remained constant, or that while the serum has been losing in strength, the emulsion has gained in sensitiveness to an exactly corresponding degree.

A third theoretical possibility, that the serum may have increased in strength, while the culture lost sensitiveness, may be ruled out on our general knowledge that sera do not increase in agglutinating power.

The second alternative is only partially dismissed by the improbability that exactly equal and opposite changes should have occurred in the two fluids. It is, however, sufficiently discounted by the fact that two years of experimental work on this subject have failed to produce any evidence that formalized suspensions of dysentery bacilli ever undergo

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an increase of sensitiveness. So the balance of evidence is very strongly in favour of the first explanation, viz., that neither the serum nor the culture has undergone any appreciable change of its properties during the period of the experiment. The experiment just mentioned sufficiently demonstrates the complete stability of the materials (serum and emulsion) employed, and raises a presumption that other sera and emulsions, made and kept under similar conditions, will show a similar stability. Other instances of the same phenomenon will be found below.

For the purpose of testing the stability of agglutinable emulsions of dysentery bacilli, an old stock agglutinating serum may be used as a provisional standard and its stability over a period of some months may be provisionally assumed.

II. There is a second method of testing an agglutinable emulsion for changes in its agglutinability.

An old emulsion, whose stability has been provisionally demonstrated in the manner just described, may be taken as standard, i.e., may be assumed to be stable during the period of the experiment. The new emulsion which is being investigated is tested at intervals in parallel with the old emulsion. If the new emulsion be in process of losing sensitiveness, an increase will be seen in the relative amount of serum required to agglutinate it to the same degree as the old emulsion. In such an experiment it is not necessary to use the same specimen of serum in all the consecutive tests; any specimen of specific serum will do, because it is only the relation of the quantities required by the two cultures that interests us.

If the relative quantities of serum remain unchanged, we must conclude that the new emulsion has undergone no alteration.

III. The ideal method consists of a combination of the two methods just described.

EXPERIMENTS TO DETERMINE THE COURSE OF THE LOSS OF SENSITIVITY CAUSED BY THE ADDITION OF FORMALIN.

(1) A 24 hours bouillon culture of *B. dysenteriae* Shiga was diluted with saline to standard opacity, and its exact relative degree of sensitiveness to Shiga agglutinating serum was determined in comparison with that of a stock formalized emulsion (Sh. 1). Formalin (0·1 %) was then added, and the culture was put into the cold store.

Samples were taken out at intervals and tested, as before, against the stock emulsion, to determine and measure any change that might be occurring in the agglutinability of the new culture.

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Table III and Chart I give the results of these tests. In the right-hand column there are recorded a number of tests of the stock Shiga emulsion with a particular specimen of Shiga serum. It is seen that the quantity of this serum required to give standard agglutination has not changed during the period of the experiment, and therefore it may be assumed that the agglutinability of Sh. 1 (stock emulsion) has not altered.

TABLE III (Chart I).

Date	Relative quantities of serum required to give standard agglutination		Quantities of a single specimen required to give standard agglutination with the stock culture
	Stock culture	New culture	
11. xii. 15 Before adding formalin	100	57·8	0·00143 c.c.
11. xii. 15 After adding formalin	100	63·4	—
20. xii. 15	100	99·0	—
4. i. 16	100	115·0	0·00146 ,,
25. i. 16	100	114·5	—
3. iv. 16	—	—	0·00141 ,,
25. v. 16	—	—	0·00143 ,,

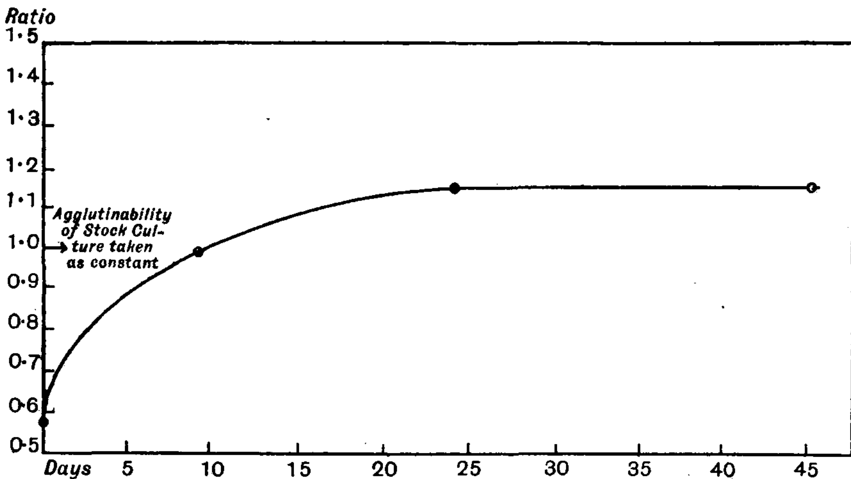

CHART I. Curve showing the increase of the ratio of the quantity of serum required to give standard agglutination with a new formalized broth culture of *B. dysenteriae* Shiga to the quantity required by an old stock culture of constant agglutinability.

Ordinates. Ratios of the quantities of serum required to agglutinate the two cultures to the same degree.

Abscissae. Time, in days.

Note. The rise of this ratio signifies a fall of the agglutinability of the new culture as compared with the old.

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This experiment demonstrates that the particular fresh formalized Shiga culture in question needed during the first 24 days of its existence a progressively increasing quantity of serum to agglutinate it to the same degree as the stable stock culture. In other words it underwent during that period a progressive loss of sensitiveness. Between the 24th and the 45th days no further loss was shown to have taken place. The third observation in the table (20. xii. 15) included also a test of the *remainder of the original unformalized and still living* fresh Shiga culture, which had been kept on ice. In contrast to the formalized specimen it showed, if any, only a slight alteration of sensitiveness, and that in the opposite direction, viz., the relative quantity of serum required to agglutinate it to the same degree as the stock culture had altered from 57·8 to 56·5. Increases of apparent sensitiveness in unformalized living cultures are always liable to occur owing to lysis of the bacteria and consequent thinning of the emulsion. This may or may not be over-compensated by the continued growth of the live bacilli, according to the degree of cold and other conditions under which the emulsion is stored.

(2) A fresh emulsion of *B. dysenteriae* Flexner was made and treated exactly in the way described in the foregoing experiment. Table IV and Chart II show the changes undergone by the freshly formalized emulsion as compared with old stock Flexner culture. A few tests of the unformalized living culture are included in a separate column of the table.

In this table and Chart II new formalized Flexner culture shows a decrease of sensitiveness, at first rapid and then gradually becoming slower, in the first 22 days of its existence, after which period it shows no further alteration of agglutinability during the subsequent 26 days.

The right-hand column of the table presents good evidence of the stability of the stock culture which was used as the basis of comparison.

The loss of sensitiveness of the unformalized, living culture may well have been due to a slow continued growth of the bacillus, and the consequent increase of the quantity of bacterial substance in the emulsion.

The foregoing experiments suffice to prove (1) that formalized cultures of *B. dysenteriae* may undergo an early loss of sensitiveness, and (2) that the change may run its complete course in some three weeks, after which period the agglutinability undergoes no further change. For how long this stability is maintained a later section of this paper will show.

It must be observed at this point that formalin does not invariably cause a decline in sensitiveness of dysentery cultures. Sometimes a culture is found to be stable from first to last; and this is more often

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TABLE IV (Chart II).

Date	Relative quantities of serum required to give standard agglutination with cultures of *B. dysenteriae* (Flexner): Stock culture	Formalized new culture	Unformalized new culture	Quantities of a single specimen of Flexner serum required to give standard agglutination with the stock culture
19. xi. 15	100	84·0	72·5	0·056 c.c.
29. xi. 15	100	182·0	96	—
30. xi. 15	100	172	102	—
11. xii. 15	100	311	—	0·054 ,,
6. i. 16	100	310	—	0·054 ,,

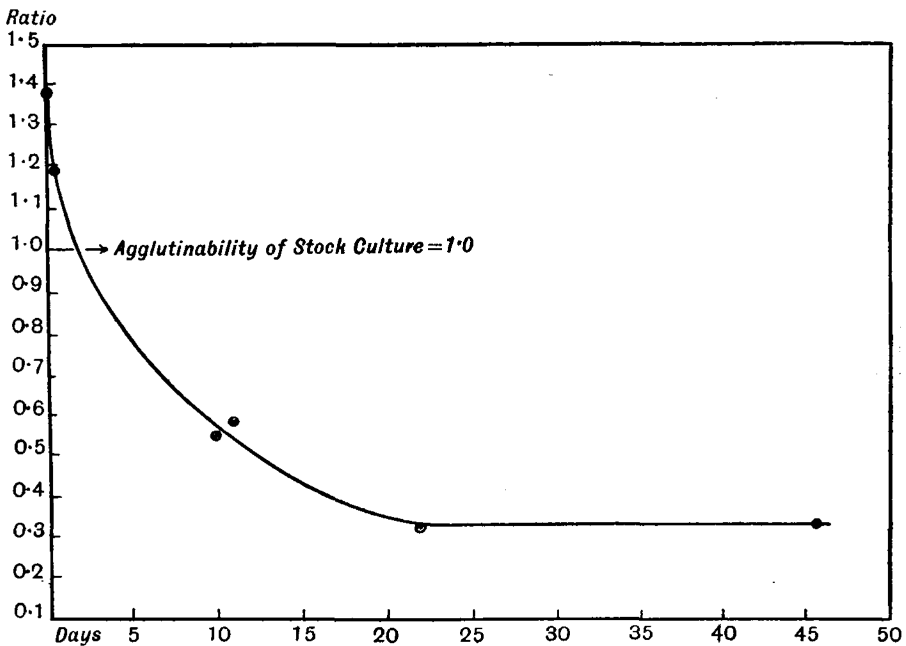

CHART II. Curve showing the fall of the ratio of agglutinability of a new formalized broth culture of *B. dysenteriae* Flexner to that of an old stock culture of constant agglutinability.

Ordinates. Ratios of agglutinability of new culture to that of old culture of the same bacillus.

Abscissae. Time, in days.

Note. The ratios of agglutinability are the reciprocals of the ratios of the quantities of serum required. A curve plotted with the latter values would have an upward direction similar to Chart I.

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the case with *B. dysenteriae* Shiga than with the mannite-fermenting group. Table VIII shows a series of tests of a Flexner culture which yielded no certain evidence of any loss of agglutinability from the moment after the addition of formalin to the last test nearly five months later. The mere addition of formalin to the fluid almost invariably lowers the agglutinability by some few per cent., but the progressive change seen in the experiments given above does not always ensue. The explanation may lie in the degree of cold in which the culture is stored during the early period while the formalin is taking effect. The cold store made use of for the purpose of this investigation was kept at an average temperature of 40° F., and at times was a little warmer, at other times colder than that. It is possible that a constant temperature only just above the freezing point of water as recommended in the Directions for the Preparation of Standard Cultures would appreciably reduce the frequency and degree of these changes.

DURATION OF THE LOSS OF SENSITIVENESS CAUSED BY FORMALIN.

Different batches of cultures show considerable variations in their period of stability. We have seen that in some cases no loss of sensitiveness at all occurs, while in others the culture takes a month or more to reach a constant level of agglutinability. No case has yet occurred within my experience of a continuation of loss of sensitiveness beyond the end of the second month. Nevertheless I have adopted the routine plan of considering every batch of culture as "unripe" until it is three months old.

THE STABILITY OF "RIPE" CULTURES.

When once the curve of sensitiveness has reached a constant level, it remains at that level for an indeterminate period.

Tables V–VIII give instances of a number of fresh dysentery cultures whose sensitiveness was determined at intervals in comparison with an old "ripe" culture. Table V gives a series of determinations of a fresh Flexner culture. The figures in the two middle columns may be read on this plan: If 100 parts of a serum are needed to give standard agglutination with the standard culture, 82 parts of the same serum are required to give the same degree of agglutination with the new culture, i.e., the new culture, at the first determination, was more sensitive than the standard culture, in the proportion of 100/82 = 1·22 : 1.

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TABLE V.

Periodical tests of a new B. dysenteriae *Flexner culture against standard culture of the same organism. Both cultures made with the same strain of* B. dysenteriae *Flexner.*

Age, in weeks of culture under standardization	Relative quantities of serum required to give standard agglutination with: Standard culture	Culture under standardization	Average figure for monthly periods
4	100	82	82
8	100	95	—
8—9	100	104	100
8—9	100	97	—
10	100	103	—
14—15	100	114	—
15—16	100	101	106
15—16	100	102	—
21—22	100	104	104
49—50	100	116	—
49—50	100	105	103
49—50	100	94	—
49—50	100	101	—

The standard culture was more than twelve weeks old at the date of the first test.

Considering next the average figures for monthly periods, we find that this ratio of sensitiveness has become 1·00 in the 8th–10th weeks—i.e., the new culture has lost sensitiveness (assuming that the standard culture has not gained). The three subsequent ratios are 0·94, 0·96 and 0·97, and it will be noted that the average for the 49–50 weeks is only 3 % greater than that for the 8th–10th weeks.

We cannot therefore conclude for certain that any loss of sensitiveness has occurred after the eighth week. From that point onwards, until the 50th week, the relative agglutinabilities of the two cultures remained unaltered.

Table VI presents a similar series of experiments with two cultures of *B. dysenteriae* Y. In this case no alteration of agglutinability was found in the new culture between the 4th and the 31st weeks. No further determinations were done, as the stock standard culture had all been used up.

Table VII contains the figures obtained by a series of tests of two Shiga cultures. Culture (A) was already six months old at the time of the first test, and culture (B) was slightly more than three months old. The period of the experiment is some six months. Since the variations in the relative observed values do not exceed 6 % on either side of the

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mean value (53) it is to be concluded that no significant alteration has taken place.

TABLE VI.

Periodical tests of a new "Y" culture against a standard culture of the same organism. Both cultures made with the same strain of "Y."

Age, in weeks of culture under standardization	Relative quantities of serum required to give standard agglutination with: Standard culture	Culture under standardization	Average figure for monthly periods
4—5	100	69	—
4—5	100	76	73
5—6	100	73	—
17—18	100	72	72
30—31	100	76	—
30—31	100	78	—
30—31	100	76	73
31	100	60	—

The standard culture was more than six months old at the time of the first test.

TABLE VII.

Determinations of the relative sensitiveness of two Shiga emulsions.

Date	Relative quantities of serum required to give standard agglutination with: Shiga culture (A)	Shiga culture (B)	No. of standard serum-units required to give standard agglutination with culture (A)
29. v. 17	100	56	1·9
9. viii. 17	100	52	1·7
31. x. 17	100	50	1·7
11. i. 18	100	54	1·8

Both cultures were made with the same strain of *B. dysenteriae* (Shiga) and both were more than three months old at the time of the first test.

The fourth column gives the number of standard serum-units which were found on the various occasions to give standard agglutination with culture (A). Different specimens of Shiga serum were used for the different tests, and the strength in units of each serum was controlled by testing them against a third and older batch of standard culture. The closeness of the figures obtained thus, provides an additional proof of the stability of the culture (A), which was used as standard.

Table VIII is in most respects quite similar to the foregoing tables. One point however must be mentioned. Two different standard cultures were used in this series of tests. Standard culture (X) was employed

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for the first two, and standard culture (Y) for the remainder (X having run out). But the two standard cultures had been thoroughly and repeatedly tested against each other, and a reduction-factor obtained

TABLE VIII.

Date	Relative quantities of serum required to give standard agglutination with		Averages of ratios
	Standard Flexner culture	New Flexner culture	
6. iv. 16	100	172	—
14. iv. 16	100	161	169·3
18. iv. 16	100	175	—
28. viii. 16	100	170	169·5
29. viii. 16	100	169	—

Note. The new culture was made on 5. iv. 16, and formalin was added on 6. iv. 16. Both cultures were made with the same strain of *B. dysenteriae* Flexner.

TABLE IX.

Two tests in parallel of two standard "Y" cultures. Different specimens of serum used on the two occasions.

Date	Readings		Quantity of serum		Ratio	
	Y 3	Y 4	Y 3	Y 4	Y 3	Y 4
7. iv. 17						
4½ hours	6—7	4 S	3·2	5·0	100	156
	7 S –	5	3·0	4·0	100	133
	8	6 tr +	2·5	3·5	100	140
	9	7 tr –	2·0	3·0	100	150
24 hours	10 tr –	9	1·5	2·0	100	133
					5) 500	5) 712
					100	142
Date	Y 3	Y 4	Y 3	Y 4	Y 3	Y 4
4. vii. 17						
4½ hours	4 S +	1—2	5·0	8·2	100	164
	5 S –	3—4	4·0	5·9	100	148
	5—6	4 tr	3·6	5·0	100	139
	7 tr +	5	3·0	4·0	100	133
24 hours	8	6 tr	2·5	3·5	100	140
					5) 500	5) 724
					100	145

Note. The first reading of the first test means: "Tube 4 of series Y 4 shows more agglutination than tube 6 and less than tube 7 in the other series. It is judged to be rather nearer tube 7." The quantity of serum to which this corresponds is less than 3·5 and more than 3·0; nearer the latter than the former, i.e., 3·2. So with the other interpolated readings. One to four marks under one of a pair of figures signify that the reading was judged to be slightly or very much nearer to the underlined tube.

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with which it was easy to translate the results with culture (X) into terms of culture (Y). It is in fact this operation that is the main purpose of the standardization of agglutinable cultures.

As additional illustrations of the stability of cultures over long periods the following may be mentioned.

A specimen of *B. dysenteriae* Y agglutinating serum, tested with a standard Y culture, was found to contain 538 standard units per c.c. Just over six months later the test was exactly repeated, and the serum showed an agglutinin constant of 545 standard units.

Evidently if the culture had undergone any alteration of sensitiveness in either direction, the number of serum units calculated could not have been practically identical on the two occasions.

An exactly similar experiment with a different Y standard culture (the same strain of Y) and a different sample of serum gave the following results.

In the first test it was found that 0·00085 c.c. of serum gave standard agglutination with the culture. Three months later the quantity was found to be 0·00082 c.c.; and again three months later 0·00087 c.c. Experimental error is quite sufficient to account for the differences between these figures, none of which varies so much as 4 % from the mean of the three.

Again, on 30. x. 16, 0·0020 c.c. of a Shiga serum was found to give standard agglutination with a Shiga culture. Nearly ten months later, on 10. viii. 17, the test was repeated, and 0·0021 c.c. of the serum was found to give standard agglutination with the same culture.

It is hardly necessary to multiply these illustrations, which are drawn from a large material. Those given are sufficient to show that formalized broth cultures of the various dysentery bacilli, after a period of "ripening," during which their agglutinability is diminished in varying degree, remain stable for at least ten months. There is, moreover, every reason to believe that the life of an emulsion is much longer than this, and it may in fact extend, as in the case of the best typhoid or paratyphoid cultures, to a number of years.

PRACTICAL SIGNIFICANCE OF THE ACTION OF FORMALIN.

The foregoing experiments prove that (1) stable standard agglutinable cultures can be made from the various dysentery bacilli, (2) such cultures cannot be standardized for about two months after they are made. For during this period a progressive loss of agglutinability may occur.

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Where agglutinable cultures are made on a large scale and stored until required, these considerations constitute no obstacle to the manufacture of standard cultures by the formalin method, for it is a simple matter to lay in stocks which will not come into use for three months or more.

Moreover if any bacteriologist should prefer to make his own formalized broth cultures and use them as soon as made the course is always open to him of determining their agglutinability at intervals of one or two weeks against a ripe stock standard culture during the period of change. Unless this is done the results of tests performed at intervals of more than a few days cannot be regarded as quantitatively comparable.

THE CONSTANT (K) OF AGGLUTINABILITY AND ITS SIGNIFICANCE.

Having determined the conditions under which formalized dysentery cultures reach and maintain a lasting level of agglutinability, we are in a position to apply to these cultures the whole system of standardization which was instituted by Prof. Dreyer for the typhoid and paratyphoid bacilli.

The principles may be stated thus.

(1) Every emulsion of bacilli has its own specific agglutinability. Differences of hundreds per cent. are possible in emulsions made with the same strain of bacillus.

(2) The observed end-point of an agglutinin reaction is conditioned by this factor, and therefore if the factor remains undetermined, the observed end-point is no real measure of the strength of the serum but merely a record of what happens with that particular emulsion.

(3) Emulsions made by the broth-formalin process have a long life of stable agglutinability. This makes it possible to choose an emulsion as an arbitrary standard, and to determine the relative agglutinability of any number of similar emulsions as compared with the standard. Each emulsion is thus provided with a numerical factor or constant of agglutinability, i.e., a figure which expresses how much more or how much less sensitive it is than the standard.

If a uniform technique be adopted in which a definite volume of serum in graded dilutions is made to act upon a definite volume of standardized culture at a definite temperature for a specified time, then the results of any number of tests performed with different standardized emulsions may be rendered quantitatively comparable by dividing the

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dilution-figure of the end-point of each reaction by the agglutinability-constant of the emulsion employed.

The figure so obtained is most conveniently expressed as "agglutinin units."

For instance, a Shiga serum, tested with two different Shiga emulsions, is found to give standard agglutination with the one at a dilution of 1/1000, with the other at 1/1800. The former emulsion is chosen as arbitrary standard, and its K of agglutinability fixed as unity. The number of agglutinin units contained in the serum is obtained by dividing the denominator of the dilution-fraction, i.e., 1000, by the agglutinability-constant, i.e., 1·0.

Thus the serum is said to contain 1000 standard agglutinin units per c.c. The second emulsion gave standard agglutination at 1/1800, which means that it is 1·8 times more agglutinable than emulsion No. 1. Since 1800 divided by 1·8 gives 1000, it is clear that 1·8 must be the agglutinability-constant of the second emulsion.

So, by the use of these constants, we obtain the same number of agglutinin units for the serum, whereas, by merely expressing our results as dilutions, we had arrived at quite different figures.

In practice the K of the original arbitrary standard culture is not fixed as 1·0, but at some higher figure. For with a K of 1·0, the unit is small, and the number of units in any given serum is rendered relatively large. Many normal and non-specific sera would thus be made to contain a considerable number of agglutinin units, whereas it is desirable that such sera should contain few or no units. By fixing the original K at 2·5, as was done by Prof. Dreyer in the case of typhoid and paratyphoid cultures, or at 3·0 (*B. dysenteriae* Flexner and Y) or at 2·0 (*B. dysenteriae* Shiga) the respective agglutinin units are made larger than they would have been if a K of 1·0 had been chosen, and thus the number of units contained by all sera are proportionally reduced.

Normal and non-specific human and rabbit sera are thus made to have a low unit-content, which is convenient when determining a diagnostic level for the specific rise of agglutinins in active infection, whether it be enteric fever or bacillary dysentery.

THE STANDARD AGGLUTININ UNIT.

Prof. Dreyer's standard agglutinin unit for the typhoid-paratyphoid group of bacilli is defined as "that quantity of an agglutinating serum which, when made up to 1·0 c.c. volume with normal saline solution, causes standard agglutination on being mixed with 1·5 c.c. of a particular

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standard agglutinable culture and maintained at 55° C. for two hours in a water bath, followed by 15 minutes at room-temperature.

It is to be noted that the method of measurement by drops is so arranged that the results can be read off directly as though the measurements had been performed in cubic centimetres. For ten drops of diluted serum always act upon 15 drops of culture, and the result is actually the same as that obtained with 1·0 c.c. of diluted serum and 1·5 c.c. of culture, provided that the size and shape of the tubes used in the two cases are proportional to the different volumes of fluid. For, as Ainley-Walker (1916) has shown, the differences in drop-sizes compensate for one another in this technique, so that the error from this cause is reduced to negligible proportions. It follows from the definition of standard agglutinin unit that the particular standard agglutinable culture referred to must be given a K of 2·5. For 1 c.c. of a serum which contained one unit per c.c. gave standard agglutination when mixed with 1·5 c.c. of the culture; i.e., standard agglutination occurred at a serum-dilution of 1/2·5. Now since the number of units per cc. in the serum

$$= \frac{\text{Dilution in which standard agglutination occurs}}{K \text{ of culture}},$$

we have

$$1 = \frac{2{\cdot}5}{K},$$

or

$$K = 2{\cdot}5.$$

Adopting for dysentery agglutination the definition of standard agglutinin unit given above, with the single alteration of two hours to four and a half hours, we must also give to our original basis emulsion of each strain the K of 2·5. But since, for the purely practical purposes already mentioned, we may wish to have higher or lower K's on an average for one or other strain of bacillus, we are at liberty to give our first real emulsion some figure higher or lower than 2·5, and make the theoretical assumption that the figure has been arrived at by standardizing this emulsion against a (fictitious) original standard culture whose K was 2·5. Such a procedure was adopted in the case of the various dysentery bacilli. A large number of normal human sera were tested by Prof. Dreyer and the writer (the results of which will appear in a separate communication) in order to determine the normal human limits of agglutination with standard cultures.

Since normal séra were found to contain more agglutinin for *B. dysenteriae* Flexner than for *B. dysenteriae* Shiga, the K for the former was placed high (3·0) and that for the latter low (2·0), which figures

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rendered the number of units present in normal sera for the two organisms approximately equal. This had the advantage of enabling a single inclusive figure to be given for the diagnostic level of agglutinin units for the two types of bacilli in clinical serum tests.

A suitable K for the first emulsion of a bacillary strain having been determined, all subsequent emulsions are standardized by repeated testing in parallel with the first.

In the routine standardization of dysentery cultures in the Standards Laboratory a new emulsion is always tested at least six times with the full series of tubes against two or more previous standard emulsions. A number of these tests, never less than four, are performed shortly before the emulsion is to come into use, at which time it is at least three months old. Thus the K is calculated as the average of anything from 24 single readings upwards; usually many more than this. By this means experimental error and errors due to sampling, etc., are overcome, and a correct K can be determined. One of the most troublesome sources of error under present conditions is found in the variable alkalinity of the glass of different batches of bottles, which sometimes causes an appreciable variation of agglutinability of different samples of the same emulsion.

In spite of the greatest care in the preparation of bottles, including prolonged treatment with acid, it has not yet been found possible entirely to eliminate this trouble, which seems to be one of those inherent in war-conditions. In the calculation of the K of an emulsion it can only be overcome by testing a number of different bottles of culture and striking an average. For the purpose of the clinical test the error is of no great significance, since it rarely exceeds a 10 % difference between individual bottles.

EXPERIMENTS WITH HEAT AND WITH ANTISEPTICS OTHER THAN FORMALIN.

Although the use of heat and of certain antiseptic substances other than formalin has been demonstrated to be greatly inferior to formalin in the preparation of agglutinable cultures of *B. typhosus* (Aaser 1905, Dreyer 1906), it does not follow that the same is the case with cultures of *B. dysenteriae*. For the latter, as we have seen, may undergo a diminution of agglutinability under the influence of formalin, while the former do not.

In order to discover whether there might be a better method of preparing stable emulsions than the formalin procedure, a series of experiments were carried out with emulsions prepared in the following ways.

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Broth cultures were always used, since saline emulsions from agar have been demonstrated by Dreyer in the case of *B. typhosus* to be greatly inferior, and since they failed to yield a satisfactory permanent "diagnosticum" in the hands of Schiemann.

METHODS OF PREPARATION EMPLOYED.

(1) Heating to 58° C. for 1 hour to 1½ hours.
(2) ,, ,, ,, ,, and adding thymol.
(3) ,, ,, ,, ,, ,, chloroform.
(4) ,, ,, ,, ,, ,, 0·1 % formalin.
(5) Simple addition of thymol to unheated culture.
(6) ,, ,, chloroform.
(7) ,, ,, phenol 0·5 %.
(8) ,, ,, ,, 0·3 %.

The thymol and chloroform were added in such quantities as to saturate the solution, leaving a visible excess in the bottle.

The various emulsions were diluted to the right opacity with salt solution, and were immediately tested for changes in agglutinability against a control untreated emulsion made from the same culture and diluted to the same opacity. One or more "ripe" formalized cultures of the same bacillus were included in the test, as a basis from which the agglutinabilities of the various cultures could be calculated. The cultures were (with the exception of No. 8) kept in the cold store when not being tested. The whole test was repeated at intervals, in order to ascertain what changes in agglutinability were in progress.

SUMMARY OF RESULTS.

(1) *Heating to* 58° C. *for* 1 *hour or* 1¼ *hours.*

A 24 hours culture of *B. dysenteriae* Flexner was treated in this way. A test immediately after heating showed the heated culture to have suffered a reduction in agglutinability amounting to slightly over 30 %. Repeated tests during the subsequent nine weeks showed no further alteration.

There was noticeable, however, an alteration in the appearance of the emulsion at the end of this period, a bluish translucency which suggested that some autolysis or disintegration of the bacilli had occurred. As we shall see, this change is constant in emulsions heated to this degree and not subjected to fixation with formalin.

A second Flexner culture was treated in the same way. The test immediately after heating showed that the heated culture had lost none

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of its agglutinability; and subsequent tests during the next five weeks showed an average loss of less than 5 %. But by this time clearing of the emulsion was becoming evident, as in the case given above.

Had further tests been possible over a longer period of time, there would doubtless have been a slow apparent increase of agglutinability, due to the disintegration of the bacilli, as we shall see in the next experiments.

(2) *Heating to* 58° C. *and adding thymol.*

A Flexner culture prepared in this manner was found to have suffered an immediate loss of sensitiveness amounting to 45 %.

No further loss was demonstrated in a series of tests during the following four weeks. Later tests during the second month showed a tendency towards an increase of sensitiveness, and in a final test performed four months after the preparation of the culture, a definite increase was established, the loss of sensitiveness being now reduced from 45 % to 13 %. Coincident with this change there was a very noticeable clearing and translucency of the emulsion.

It is worth while mentioning that the addition of thymol to a fluid has a profound effect on the surface tension, with the result that the size of the drop is greatly reduced. Drop-measurements of thymolized fluids are therefore inadmissible, unless corrective calculations are made.

(3) *Heating to* 58° C. *for* 1 *to* 1¼ *hours and adding chloroform.*

A 24 hours broth culture of *B. dysenteriae* Flexner was subjected to this treatment. The result was in every respect similar to that of the last-mentioned experiment, viz., an immediate considerable loss of agglutinability (40 %) followed by a short period of apparent stability, and later by a rise accompanied by a progressive diminution of the opacity of the culture.

Conclusion. Heating to 58° C. for 1 to 1¼ hours, with or without the addition of thymol or chloroform, is insufficient to fix the bacilli, and so to prevent their slow disintegration. Permanent and stable emulsions cannot be made in this way.

(4) *Heating to* 58° C. *for* 1 *to* 1¼ *hours and adding* 0·1 % *formalin.*

The Flexner culture used in this experiment showed a loss of 18 % directly after the treatment. Another specimen of the culture, treated with 0·1 % formalin only, gave evidence of very little loss (2·2 %). The observations were only continued for six weeks, at the end of which time

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no further change of agglutinability had been detected, and the appearance and opacity of the culture were unaltered.

Conclusion. The combined heat and formalin method is inferior to formalin alone.

(5) and (6) *Simple addition of thymol or chloroform to the unheated culture.*

A progressive loss of sensitiveness was found in the specimens of Flexner culture treated in this way. But it was probably due to continued growth of the bacillus, for sub-cultures taken two days after the antiseptics were added in both cases showed the presence of live bacilli, and even 17 days later the chloroformed sample gave a positive subculture. At the end of eight weeks very pronounced clearing of the culture betrayed the fact that disintegration of the bacilli had been taking place for some while, and the tests were on this account discontinued. These series of tests showed considerable fluctuations, which were difficult to interpret.

Conclusion. Owing to slow or incomplete sterilization and absence of fixation of the bacilli this mode of preparation proved to be totally unsatisfactory.

(7) *Simple addition of* 0·5 % *phenol combined with cold-storage.*

To a living 24 hours broth culture of *B. dysenteriae* Flexner 0·5 % phenol was added, and the culture was at once tested for its agglutinability against untreated sample which had been kept for a control.

The test showed a loss of sensitiveness amounting to 50 % of its original value (i.e., double the quantity of serum was required to give standard agglutination).

This culture was not tested further.

Another culture similarly treated, but whose initial loss was, by an oversight, not determined, showed a period of stability during four weeks of periodical tests. No examination was made until this culture had stood a further five months in the cold store. The tests then carried out demonstrated an increase of agglutinability amounting to 22 % of its previous value. There was no noticeable decrease of opacity. These facts suggest that 0·5 % phenol causes a partial, but incomplete, fixation of the bacilli, which disintegrate very slowly. Any clearing of the fluid due to this disintegration may be compensated by a continuous slow precipitation of dissolved albuminous bodies.

(8) *The addition of* 0·3 % *phenol* (culture stored in the cold for 24 hours thenceforward at room temperature).

A Shiga culture, treated in this way, underwent no immediate loss of

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sensitiveness. Nor were the subsequent changes of any great magnitude. They exceeded by little the experimental variations.

The figures however suggest a decrease of sensitiveness visible at the end of five or six weeks, and followed by a secondary increase. At the end of the experiment clearing of the culture had begun to be noticeable. So that, even if the agglutinability had been unequivocally steady up to that time, the culture could not be considered as satisfactory.

TABLE X.

	Quantities of serum required to give standard agglutination with			Relative serum quantities giving standard agglutination with	
Date	Standard Shiga culture	New Shiga culture untreated	New Shiga culture with 0·3 % phenol	Standard Shiga culture	New Shiga culture with 0·3 % phenol
15. xii. 17	·0022 c.c.	·0017 c.c.	·0017 c.c.	100	77
17. xii. 17	·0024 ,,	—	·0017 ,,	100	71
20. xii. 17	·0020 ,,	—	·0014 ,,	100	70
17. i. 18	·0019 ,,	—	·0014 ,,	100	74
18. i. 18	·0023 ,,	—	·0018 ,,	100	78
28. i. 18	·0023 ,,	—	·0019 ,,	100	83
21. ii. 18	·0023 ,,	—	·0017 ,,	100	74
22. iii. 18	·0022 ,,	—	·0016 ,,	100	73
22. iii. 18	·0022 ,,	—	·0016 ,,	100	73

Conclusion. Thus phenol by itself (0·5 % or 0·8 %) is insufficient for the preparation of standard agglutinable cultures whose agglutinability has to be guaranteed as constant over comparatively long periods of time. Unlike formalin, it fails to fix the bacilli and so to prevent their slow disintegration and solution.

SUMMARY.

(1) Killed agglutinable broth cultures of the various dysentery bacilli may be prepared by the formalin and cold storage process and may be standardized by the method elaborated by Prof. Dreyer for the typhoid and paratyphoid bacilli.

(2) Since formalin (0·1 %) usually causes a gradual loss of sensitiveness to agglutination during the early life of the emulsion, a certain period (two months) must be allowed to elapse before the emulsion can be used as standard.

(3) After such a period the sensitiveness of the emulsions remains unaltered for at least ten months, and probably much longer.

(4) By none of the other methods of preparation investigated could satisfactory standard agglutinable cultures be made.

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