EXPERIMENTS ON THE AGGLUTINATION OF WATERY EXTRACTS OF *B. TYPHOSUS.*

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I. *Introduction.*

THE following observations on the agglutination of bacterial extracts were made in the hope of finding out whether the substances agglutinated by a specific serum and by acid were identical, and also with a view to throwing light on the nature of the process of agglutination by acids.

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The conglomeration and subsequent sedimentation of the more or less invisible colloidal particles in a bacterial extract is often spoken of as a precipitation and distinguished from the agglutination of an emulsion of bacteria. Since, however, there appears to be no essential difference between the two processes whether they are brought about by specific serum or by acid, the term agglutination has been used in this paper to include the precipitation of clear bacterial extracts.

It was shown by Kraus (1897, B) that the phenomenon of agglutination by a specific serum still occurred when a filtered bacteria-free extract was used instead of an emulsion of the bacilli. He used filtrates of old broth cultures of *B. typhosus* and *V. cholerae,* and specific sera which were only slightly diluted.

Ch. Nicolle (1898) showed that a filtered watery extract of *B. typhosus, B. coli* or *V. cholerae* grown on agar was agglutinated by specific serum diluted 1/10. He also showed that in a watery extract to which specific serum had been added, suspended bacilli of quite a different kind from that used in preparing the extract were completely clumped. When for instance he mixed an extract of *B. coli* and an antiserum to this strain of *B. coli*, and to this mixture of antigen and serum he added an emulsion of *B. typhosus,* he obtained complete clumping of the latter. Further he showed that inorganic particles, *e.g.* finely divided talc in suspension were clumped in a mixture of bacillary extract (antigen) and its specific serum (agglutinin).

He did not use highly diluted sera nor dilutions of extracts, as the agglutination reaction was comparatively newly discovered at that time.

Paltauf (1897) and Kraus (1897 A) also put forward the view that the agglutination of bacteria was due to the formation of a kind of coagulum in the liquid, in which the bacteria were entangled and drawn together into clumps.

Kraus and von Pirquet (1902) produced further evidence of the identity of bacterial agglutination and the precipitation of bacterial extracts by serum. They used a specific serum, which in some cases was diluted as much as 1/150, in order to obtain the agglutination or precipitation of an extract, but a dilution of serum of 1/10 or 1/50 was more commonly used for this purpose.

Dean, H. R. (1912), showed that a watery extract diluted 1/160 would in some cases give a precipitate with specific serum 1/10. He also showed that on addition of a small quantity of the mid-piece (euglobulin) of guinea-pig's complement a precipitate was obtained with extract 1/640 and specific serum 1/10, whereas with this high dilution

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of extract no precipitate was obtained on the addition of specific serum alone. He considered this enhanced result was due to the mid-piece increasing the size of the precipitate mechanically, and thus making it visible, and suggested that the action of the mid-piece in this case was similar to that of the conglutinin of ox-serum which was used by Bordet and Gay (1906) and Streng (1909) for enhancing or producing agglutination in mixtures of specific serum and red blood corpuscles, or specific serum and bacteria.

Michaelis (1911) described the agglutination of bacteria by acids. He used for this purpose regulator mixtures of acetic acid and acetate of sodium, lactic acid and lactate of sodium, etc., affording solutions of known hydrogen-ion concentration, and he showed that the kind of acid was unimportant so long as the correct hydrogen-ion concentration was present. He records the observation that at the optimum hydrogen-ion concentration for agglutination and even in slightly stronger acid mixtures no change in the electric charge of the bacteria could be observed by means of U-tube cataphoresis experiments. In commenting on the difference of behaviour in this respect of bacteria and suspensions of various proteins which have been examined, he suggested that the peculiarity of bacterial emulsions was due to the complex composition of bacteria and that the different constituents had iso-electric points corresponding to different concentrations of hydrogen-ions. Beniasch (1912) and Beintker (1912) extended Michaelis' agglutination experiments, corroborating his views, and maintained that the substances agglutinated by acid and by serum were identical, on the ground that the two reactions ran parallel in a series of strains tested. Michaelis and Davidsohn (1912) agglutinated a watery extract of *B. typhosus* with acid, removed the precipitate by centrifuging and redispersed it by neutralising. They found that the clear "solution" of the precipitate thus obtained was precipitable by serum like the original extract. They maintain that this proves the identity of the substances in a bacterial extract or emulsion which are agglutinable by serum and acid respectively. They assume that the optimum hydrogen-ion concentration for precipitation of the extract is also the isoelectric point, on the analogy of the proteins and other substances which they have previously examined, and they conclude that the substances which are agglutinable by serum and acid are identical and are precipitated by acids at their isoelectric point.

However, they appear only to have shown that the substance agglutinated by serum is carried down with the acid agglutinable substance,

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when the requisite [H˙] is present for the precipitation of the latter. Also they record no direct experiment to demonstrate the charge on the particles in an extract in solutions of varying [H˙].

The work of Krumwiede and Pratt (1913) and of Sgalitzer (1913) must be mentioned, since their observations have a bearing on the agglutination by acid of mixtures of bacillary bodies and normal serum and of bacillary bodies and extract recorded in Section 7 of the following paper. Sgalitzer also records a large number of experiments on acid agglutination under different conditions. These workers describe methods of combined agglutination by acid and specific serum which they claim are more delicate than the use of acid or serum separately. Both sets of experiments were carried out by adding acid in the presence of the serum proteins. The effect therefore of the acid on the protein as well as on the bacilli makes the test a complicated one. Sgalitzer uses hydrochloric acid and therefore the [H˙] cannot be accurately calculated. Krumwiede and Pratt use acid regulators and the effect of the salt (acetate or lactate) in the regulator on the sensitised bacilli must further complicate the action of the acid on the sensitised bacilli and on the serum proteins.

Michaelis and Davidsohn (1912) record experiments on the action of acid regulators on sensitised bacteria (after removal of the serum protein), which do not support the view that acid affects sensitised differently from unsensitised bacilli.

II. *Writer's experiments.*

(1) *Method of preparing bacterial extracts.*

The observations here recorded were all made with strains of *B. typhosus.* In order to be able to observe serum agglutination and acid agglutination with the same bacterial extract the bacilli from a 24 to 48 hrs. agar slope were washed off with 10 c.c. of distilled water and the resulting emulsion was centrifuged after standing for half an hour to an hour at the temperature of the laboratory. The supernatant liquid was again centrifuged once or twice till quite clear. In some cases a second active extract could be obtained by making up the deposit to its original volume with distilled water, allowing it to stand at room temperature for one or two days and again centrifuging. Chloroform was added to the emulsions and to the extract to prevent decomposition, this addition being found by preliminary experiment not to perceptibly influence the agglutination reactions.

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It was observed that, independently of the age of the culture, different strains and different races of the same strain, and also the same strain on different occasions, yielded extracts of very different values.

Sometimes the bacteria in the first deposit obtained by centrifuging when re-emulsified no longer agglutinated with acid or serum and the extract obtained by centrifuging this emulsion after it had stood for a day or longer, gave no precipitate with either reagent. On other occasions the bacilli were still agglutinable by serum after washing two or three times in the original volume of fresh distilled water. On two or three occasions extracts were obtained bacteria-free by filtering through a porcelain filter, but it was usually considered sufficient to centrifuge the extract after it had stood for some hours, as by this means clear extracts could be obtained which showed only six or eight bacilli in a field of the microscope when a drop was examined with a Zeiss D objective.

The results with filtered extracts were quite similar to those with extracts obtained by centrifuging.

(2) *Agglutination of extracts by salts.*

It was found in the case of a strain of *B. typhosus*, an emulsion of which agglutinated without serum in sodium chloride, calcium chloride or lanthanum nitrate solution, that a watery extract was also agglutinated by these salts and it was found that the optimum concentration of salt for agglutination was approximately the same whether an emulsion of the bacilli or an extract was used. For this purpose only weak solutions of salts were used. In the cases of those strains which were examined the optimum concentration of sodium chloride was N/4 to N/8, of calcium chloride N/16 to N/32 and of lanthanum nitrate N/400 to N/800. See Table I.

(3) *Agglutination of extracts by acids.*

The regulator mixtures of known hydrogen-ion concentration recommended by Michaelis were used as a rule, but experiments were also made with dilute hydrochloric acid or sulphuric acid.

Regulator mixtures of acetate of sodium and acetic acid or of lactate of sodium and lactic acid were prepared in such a way that each acid solution contained 5 c.c. of normal acetate or lactate in 210 c.c. The solutions were numbered 1 to 10. The hydrogen-ion concentration or $[H^{\cdot}]$ in each solution was twice that in the preceding solution of the series. See Table II.

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TABLE I.

Agglutination of emulsion and extract of a "spontaneously" agglutinable strain of B. typhosus *by salts.*

NaCl	N/1	N/2	N/4	N/8	N/16	N/32	N/64	N/128	N/256	D. W.
Emulsion	−	−	+	−	−	−	−	−	−	−
Extract	−	+	+	−	−	−	−	−	−	−
$CaCl_2$	N/1	N/2	N/4	N/8	N/16	N/32	N/64	N/128	N/256	D. W.
Emulsion	−	−	⧺	⧺	⧻	⧻	+	−	−	−
Extract	+	+	+	⧺	⧻	⧻	+	−	−	−
$La(NO_3)_3$	N/200	N/400	N/800	N/1600	N/3200					D. W.
Emulsion	⧻	⧺	⧻	⧺	−					−
Extract	⧻	+	+	−	−					−

TABLE II.

Agglutination by acid regulator mixtures of emulsions of three different strains of B. typhosus.

Regulator solution	10	9	8	7	6	5	4	
[H˙]	$1{\cdot}1\times10^{-3}$	$5{\cdot}6\times10^{-4}$	$2{\cdot}8\times10^{-4}$	$1{\cdot}4\times10^{-4}$	$7{\cdot}2\times10^{-5}$	$3{\cdot}6\times10^{-5}$	$1{\cdot}8\times10^{-5}$	
B. typhosus, Strain L	−	−	−	+	⧺	⧺	−	37° 15 mins.
	+	−	⧺	⧻	⧻	⧻	−	37° 30 mins.; room temp. 30 mins.
	⧺	⧺	⧺	⧻	⧻	⧻	−	37° 30 mins.; room temp. 20 hrs.
B. typhosus, Strain G	−	−	−	−	−	−	−	37° 15 mins.
	−	−	−	−	−	−	−	37° 30 mins.; room temp. 30 mins.
	−	−	−	−	−	−	−	37° 30 mins.; room temp. 20 hrs.
B. typhosus, Strain S	−	−	−	−	⧺	⧺	−	37° 15 mins.
	⧺	+ s	+ s	⧻	⧻	⧻	−	37° 30 mins.; room temp. 30 mins.
	⧻	⧺	⧺	⧻	⧻	⧻	−	37° 30 mins.; room temp. 20 hrs.

− = no agglutination, + = distinct agglutination, ⧺ and ⧻ = marked and complete agglutination, + s = slight agglutination.

If a series of tubes is put up containing the different solutions and an emulsion of *B. typhosus* in distilled water is added in equal quantity it is found that the optimum [H˙] for agglutination is almost invariably that corresponding to tube 5 or 6. The best time for making this observation varies but is usually after about 15 minutes at 37° C. and 15 minutes at the temperature of the laboratory in the case of

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emulsions, but in the case of extracts two or three hours at room temperature after a short time in the incubator are often required to allow the formation of a visible precipitate. On standing for longer than the time required to show the optimum, *i.e.* for agglutination to appear in the first tube in which it occurs, further agglutination is observed in some of the tubes containing stronger acid, but seldom in any tube containing weaker acid than tube 5.

These effects were described by Michaelis and his co-workers, but they laid stress only on the optimum [H˙] for agglutination.

As a rule agglutination is soon seen in tube 6 and about the same time it is also evident in tube 10 and sometimes to a less degree in tube 9. The agglutination in tubes 9 and 10 is usually of a different character, the clumps being in the form of fine granules, whereas in tubes 5 and 6 loose flocculi are seen. Eventually agglutination may occur in all the tubes from 5 to 10, but if they are observed from hour to hour a zone of absent agglutination is generally to be seen corresponding to tubes 8 or 9.

Table II shows the agglutination by acid of the emulsions of three strains of *B. typhosus.* Three observations at different intervals of time are recorded in each case. The later agglutination which occurs in higher [H˙] than the optimum is of interest and shows the zone with diminished agglutination in tube 9.

The two optima and the zone of absent agglutination are perhaps seen best with bacilli which have been centrifuged down and re-emulsified (first deposit). When bacilli have been repeatedly washed with distilled water the emulsified deposit no longer agglutinates in acid solutions Nos. 5 and 6, but agglutination still occurs in solution No. 10. (Certain other bacteria including staphylococci and some strains of *B. coli* agglutinate first in tube 10, and later in tubes 9 and 8.) On the other hand a clear watery extract of *B. typhosus* as a rule agglutinates in solution No. 5, but not at all in No. 10. These observed facts appear to indicate that two substances, both agglutinable by acid, are present in whole emulsion of *B. typhosus,* one of which is soluble in water and has its optimum [H˙] for agglutination in tube 5, and the other, which is closely adherent to the bodies of the bacilli, is agglutinated best in tube 10.

As a general statement it may be asserted that, although there is much variation in the behaviour of emulsions and extracts with acids, if a watery extract and well-washed bacilli from the same emulsion are compared, the substance whose optimum for agglutination is in No. 5

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is most evident in the extract, while that whose optimum is in No. 10 is found more in the washed bacilli.

The original emulsion is sometimes not agglutinated in tubes 9, 10 and 11, although the usual agglutination occurs in tubes 5 and 6. This behaviour suggests the presence of a substance in the original emulsion which inhibits agglutination in tube 10. The substance may be merely a trace of agar or may be a real constituent of the bacilli. It is no doubt present in the first extract and may be in part that bacillary substance which is agglutinated in tube 5. It cannot be entirely composed of this agglutinable substance, since in the case of a strain of *B. typhosus*, *e.g.* strain "G," an emulsion of which is not agglutinated in any of the acid solutions 5 to 10, the bacilli after they have been washed are agglutinated in solution 10.

TABLE III.

Comparison of agglutination by acid of extracts and re-emulsified deposits.

Regulator acid solution—		10	9	8	7	6	5	4	D. W.
B. typhosus L,	Extract 1	–	–	–	++	++	–	–	–
,,	Extract 2	–	–	–	–	+	+	–	–
,,	Deposit 1	+++	++	++	+	+	+	–	–
,,	Deposit 4	++	+++	++	+	–	–	–	–
,,	Extract 3		–	–	+	+	+	–	–
,,	Deposit 3		++	–	–	–	–	–	–

Table III shows the agglutination by acid of successive extracts and repeatedly washed bacilli of *B. typhosus* strain "L." Extract (1) and Deposit (1) were the clear supernatant fluid and the re-emulsified deposit obtained by centrifuging the original emulsion. Extracts (2) and (3) and Deposits (3) and (4) were obtained by re-centrifuging Deposit (1), removing the supernatant, re-emulsifying the fresh deposit, etc. The two different optima, for the extracts in tube 6, and for the washed bacilli in tube 9 or 10, are well shown.

Table IV also shows the different zones of agglutination by acid obtained with the original emulsion, the first and second extracts, and the first and second deposits (*i.e.* bacilli free from extract). Three observations on the agglutination are recorded after the tubes had stood for different lengths of time in the case of the emulsion and Deposit (1), and two observations on Extract (1). The last line in the table shows the reappearance of agglutination in tube 6 when Extract (2) is added to Deposit (2), and also shows the two optima with a zone of absent

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agglutination in tubes 7, 8 and 9. Some observations on the electric charge carried by the bacilli and precipitate are also recorded and will be referred to later.

TABLE IV.

Agglutination by acid of the "whole emulsion," extracts and deposits from a strain of B. typhosus *"S."*

Acid solution—	12	11	10	9	8	7	6	5	4	D. W.	
B. ty. "S" Em.	–	–	–	–	s+	+	s+	–	–	–	Agglutination.
	–	–	+	–	++	+++	+++	++	–	–	
	+	++	++	+	++	+++	+++	+++	-	–	
	0	0	–	–	–	–	–	–	–	–	Electric charge.
,, Ex. 1	–	–	–	–	–	–	+s	–	–	–	Agglutination.
	–	–	–	–	+	+++	+++	–	–	–	
	–	–	–	–	–	–	–	–	–	–	Charge.
,, Dep. 1	+	–	–	–	–	–	–	–	–	–	Agglutination.
	+	+	+	–	–	+s	+	+s	–	–	
	+++	++	++	+	+s	++	+++	+++	++	–	
	0	–	–	–	–	–	–	–			Charge.
,, Ex. 2	–	–	–	–	–	–	–	–	–	–	Agglutination.
Dep. 2	+	++	+++	+s	–	–	–	–	–	–	
,, Ex. 2 + Dep. 2	–	+s	+	–	–	–	++	–	–	–	

(4) *Agglutination of bacterial extract with specific serum, and its relation to agglutination by acids.*

In experiments with watery extracts and emulsions sufficient salt must be added to enable agglutination to take place after sensitisation by the agglutinins. It was found that 0·42 % of sodium chloride was about the optimum salt concentration for the agglutination of *B. typhosus* by specific serum. If ordinary salt solution (0·85 %) was used to dilute the serum and the watery emulsion or extract was added to dilutions of serum in equal parts sufficient salt was present. A smaller percentage of salt was however amply sufficient, and in some experiments only 0·2 % or 0·3 % of NaCl was used.

If a strain of *B. typhosus* is selected which agglutinates readily with a specific serum and an active clear watery extract is prepared, on the addition of dilute anti-typhoid serum a delicate fleecy precipitate is produced. If a powerful anti-typhoid serum (titre 1/50,000) is used, this agglutination or precipitation reaction can be obtained when the serum is diluted as much as three thousand or nine thousand times.

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Kraus and most other workers appear to have obtained a precipitate only when comparatively strong serum, *e.g.* diluted to 1/50 or 1/150, was used. With a perfectly clear extract, the precipitate is very small when the serum is highly diluted, for the size of the precipitate depends in part on the amount of serum proteins precipitated. There is reason however to think that the specific substances may be present in sufficient quantity to produce a precipitate under favourable circumstances, even when the extract and serum are both diluted considerably beyond the point at which a visible precipitate ceases. (See Section 7.)

In Table V the results with the original emulsion, the extract and the deposit are compared. Two strains of *B. typhosus* were used. "L," a strain of medium agglutinability, and "G," which agglutinated less well with serum than "L" and never agglutinated at all with

TABLE V.

Agglutination of emulsion, extract and deposit of two strains of B. typhosus *by serum and by acid.*

	Antityphoid serum					Hydrochloric acid				
	1/1600	1/3200	1/6400	1/12800	Salt sol.	N/200	N/400	N/800	N/1600	D. W.
B. ty. "G" Em.	⧻	⧻	+	+	–	–	–	–	–	–
,, Ex.	⧻	⧺	–	–	–	–	–	–	–	–
,, Dep.	⧺	+	–	–	–	⧻	⧺	–	–	–
B. ty. "L" Em.	⧻	⧻	⧻	+	–	⧻	⧺	+	–	–
,, Ex.	⧺	⧺	⧺	⧺	–	⧻	⧻	+	–	–
,, Dep.	⧺	⧺	⧺	+	–	⧻	⧺	+	–	–

acid regulators Nos. 5 to 7. The deposit of "G" showed agglutination in the absence of extract in the stronger acid solutions which probably corresponded to No. 10 or 11 regulator, but the [H˙] of the tube marked HCl N/200 was not determined. The two optima for the case of "L" are not shown; probably because the gradations in successive dilutions of HCl are separated by too large intervals.

It has usually been found that if an emulsion agglutinates in a high dilution of serum, an extract obtained from this emulsion also agglutinates well, *i.e.* when the serum is highly diluted or the extract is diluted several times.

It is unusual to obtain a visible precipitate on the addition of acid or highly diluted serum to a clear extract which has been diluted more than four times. A precipitate is sometimes seen on addition of dilute specific serum (*e.g.* 1/3000) to a diluted extract although no precipitate is visible on addition of acid (No. 5). This may happen in the case of

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an extract which undiluted readily agglutinates with acid. Specific serum therefore appears to be a more delicate test of the presence of extract than is acid.

TABLE VI.

Ex. *B. ty.*		*B. ty.* "L"				*B. ty.* "G"				
		1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	Dilution of ext.
Acid mixture No. 6		+	–	–	–	–	–	–	–	
Anti-ty. serum	1/3200	+	+	+	+	+	+	+	–	

Table VI shows the greater sensitiveness of serum than acid as a test for the presence of agglutinable substance, but in this experiment the difference between the two reactions was unusually great in the case of strain "L." This was probably due to the use of rather strong serum and also to the fact that only one acid solution was used which may not have been the optimum. Strain "G" never agglutinated with acid No. 6.

A variant strain of *B. typhosus* was met with which did not agglutinate (either extract or emulsion) with serum or acid. The strain was however a descendant through agar cultures of a normal culture of *B. typhosus* and resembled the parent strain in its cultural and fermentative reactions. It is probable that in this case the failure to agglutinate was due to the absence of soluble bacillary substance from the liquid part of the emulsion. This suggests a probable explanation of the inagglutinability of some strains of *B. typhosus*. On the other hand some strains, *e.g.* strain "G," which do not agglutinate with acid (No. 5) are readily agglutinable by specific serum, though usually not quite so readily as other strains which are also agglutinated by acid. The writer has met with two such strains which were agglutinated by serum but not by acid.

Strains of *B. typhosus* also occur which are not agglutinated by specific serum, but which agglutinate in acid solution of the strength characteristic for this bacillus. (McIntosh and McQueen, 1914.) The record of such a strain completes the proof that the two properties of agglutinability by acid (Nos. 5 and 6) and by serum may vary independently. Probably this variability is concerned with at least two factors:

(1) The total amount of soluble bacterial substance in the liquid of the emulsion or extract; the bacilli of some strains appear to give up soluble material to water more readily than in the case of other strains.

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(2) The relative amounts of the two substances agglutinable by acid or serum respectively (if these two substances are really distinct), or the relative degree of the property of agglutinability by acid or by serum possessed by the agglutinable substance (if there is only one agglutinable substance whose properties vary in different strains).

(5) *The effect of heating extracts and emulsions of* B. typhosus.

Porges and Prantschoff (1906) showed that if an emulsion of *B. typhosus* was heated to 80° C. it ceased to be agglutinable by serum and by certain electrolytes. After further heating to 100° C. however the agglutinability was restored. Porges (1905) also found that bacilli whose agglutinability had been destroyed by heating to 80° again became agglutinable if they were washed with salt solution, and he maintained that by this means some inhibiting substance was removed.

Beniasch (1912) found that if an emulsion of *B. typhosus* in distilled water was heated to 100° C. agglutination subsequently could be obtained with acid, but that the optimum [H˙] was altered from $3{\cdot}6 \times 10^{-5}$ to $1{\cdot}1 \times 10^{-3}$ or $2{\cdot}2 \times 10^{-3}$. He also found that *B. coli*, *B. dysenteriae* and certain other bacteria showed the same optimum after being boiled.

The present writer has repeated these experiments, and has also tested the effect on agglutination by acid of heating extract and washed bacilli to 80° and 100° with the following results:

Heating the whole emulsion for 20 or 30 minutes at 80° completely abolished the agglutination in acid solutions Nos. 5 and 6, *i.e.* [H˙] $3{\cdot}6 \times 10^{-5}$ and $7{\cdot}2 \times 10^{-5}$, whereas the agglutination in No. 10, *i.e.* [H˙] $1{\cdot}1 \times 10^{-3}$, was either absent or much diminished. After heating to 100° for 5 minutes good agglutination occurred in No. 10 but not in Nos. 5 or 6.

After being heated to 80° or 100° the extract was completely inagglutinable at either optimum. Heating the washed bacilli to 80° or 100° removed any agglutinability which the unheated re-emulsified deposit showed in tubes 5 and 6, but the effect on the agglutination in No. 10 was very slight.

Table VII shows the effect of heat on a "whole emulsion," an extract and a re-emulsified deposit of washed bacilli (Dep. 2). The bacilli, as in the other experiments, were from a culture of *B. typhosus*. Two observations at 5 hours and 20 hours are recorded in the case of the unheated "whole emulsion," as the two optima for agglutination are best shown in this way. Otherwise the observations were all made

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TABLE VII.

The change in agglutinability by acid after heating emulsion, extract and re-emulsified deposit of B. typhosus.

Acid regulator mixtures—	11	10	9	8	7	6	5	4	D. W.	Time at temp. of room (preceded by 15 mins. at 37°)
Whole Emulsion (unheated)		–	–	–	+ s	+	+++	–	–	5 hours
,, ,, ,,		+++	++	+	++	+++	+++	–	–	20 hours
,, ,, 80° 30 mins.		+	–	–	–	–	–	–	–	,,
,, ,, 100° 5 mins.		+++	++	–	–	–	–	–	–	,,
Extract (unheated) ...	–	–	–	++	++	+++	+++	–	–	,,
,, 80° 30 mins. ...	–	–	–	–	–	–	–	–	–	,,
,, 100° 5 mins. ...	–	–	–	–	–	–	–	–	–	,,
Dep. (2) (unheated) ...	+	++	+	+	–	–	++	–	–	,,
,, 80° 20 mins. ...	+	++	++	+	–	–	–	–	–	,,
,, 100° 5 mins. ...	+	++	++	+	+	–	–	–	–	,,

at the end of 20 hours at the temperature of the room preceded by 15 minutes at 37° C. It appears that the effect of heating to 100° C. for four or five minutes is to destroy or render inactive the substance whose optimum [H·] for agglutination is in tube 5 or 6, whereas the effect on the agglutination in No. 10 of the substance peculiar to the washed bodies (Dep. 2) of the bacilli is very slight or nil. Heating to 80° for 20 or 30 minutes alters the substance whose optimum [H·] is in tube 5 or 6, so that it is no longer agglutinable by acid. It may be that this substance is so changed by heating to 80° that besides being rendered inagglutinable it also prevents agglutination of the bacilli in tube 10. In this case it would represent the inhibitory substance of Porges. The effect of this inhibitory substance is seen in Table VII in the case of the "whole emulsion" which has been heated to 80°; only slight agglutination confined to tube 10 occurred. The source of the inhibitory substance suggested above is made more probable by the very slight evidence of this inhibitory effect when the bacilli were washed before being heated to 80°. Table VII also shows that further heating to 100° renders the inhibitory substance inactive and good agglutination of the whole emulsion in tubes 9 and 10 again takes place.

It is also seen that heating the substance contained in the washed bacilli to 80° or 100° has little effect on their subsequent agglutinability by acid, which remains as before.

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(6) *The part played by the agglutinable substance in the extract in the agglutination of ordinary bacillary emulsions.*

(*a*) *Interbacillary substance in the clumps.* If an extract which has been centrifuged till very few bacilli remain in suspension (*e.g.* four or five in the field of an oil-immersion objective, in a dried drop on a slide) is agglutinated with acid or serum and the process watched in a microscope cell with dark ground illumination, a very delicate, loose, fleecy cloud is seen to appear in which are entangled any bacilli or particles of dust present. The whole "agglutinum" appears to contract slightly after it has been formed. It is stained slightly if suspended in a weak watery solution of fuchsin and then appears as a delicate network or tangle with highly refractile and more deeply stained points corresponding to the nodes in the network. If a drop is allowed to dry on a slide and stained as a dry film the characteristic appearance is lost, but a very finely granular background is stained by fuchsin. If Leishman's stain is used the granules are stained a faint pink and a very few blue bacilli are seen amongst the granules. Only three or four bacilli each about two or three microns long may be seen in a mass of granules corresponding to an "agglutinum" about 20 × 15 microns. The frame-work of the "agglutinum" corresponds to the interbacillary substance described by Löwit (1913) as occurring in a clump of bacilli agglutinated with specific serum.

(*b*) *Agglutination of indifferent particles with extract, and conglutination.* If a clear watery extract of *B. typhosus* is mixed with an emulsion of well-washed *B. typhosus* (rendered inagglutinable by washing) and acid solution No. 5 or dilute anti-typhoid serum is added, the bacilli are agglutinated in the same way as if the original emulsion had been used. If the bacilli in the mixture are too numerous then agglutination is incomplete. The same effect is produced if a thin emulsion of *B. coli* or *B. acidi lactici*, etc. is added to an extract of *B. typhosus* instead of washed *B. typhosus*.

It was also found that a suspension of staphylococci, kieselguhr or animal charcoal was agglutinated in the same way, but washed *B. typhosus* or *B. coli* were agglutinated better than staphylococci or inorganic particles.

If an active extract is used the anti-serum required to produce agglutination may be highly diluted (see Table VIII). If the extract is diluted with distilled water till no precipitate is visible on addition of acid or weak specific serum, agglutination and a visible precipitate

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can be obtained with the same strength of extract, etc. if in addition washed bacilli are present, although these latter alone are quite inagglutinable with serum or in No. 5 solution.

In Table VIII is shown the agglutination of a *B. coli* emulsion, (1) by a mixture of diluted *B. typhosus* extract and anti-typhosus serum in three different dilutions, and (2) by diluted *B. typhosus* extract and acid solution No. 5. Equal parts of the three reagents extract, *B. coli* emulsion or distilled water and in addition a similar quantity of serum or acid were placed in each tube. The extract was quite clear and did not contain constituents for a precipitate in sufficient quantity to enable the reaction to become visible unless *B. coli* was

TABLE VIII.

The agglutination of varying dilutions of B. typhosus *extract by serum and acid. The use of* B. coli *as an indicator.*

Dilutions of *B. ty.* Extract—	1/3	1/6	1/12	1/24	1/48	1/96	D. W.
Anti-ty. serum 1/3000							
B. coli Emulsion	+++	+++	+++	++	+	+	–
Distilled Water	–	–	–	–	–	–	–
Anti-ty. serum 1/9000							
B. coli Emulsion	++	++	++	+	+	+	–
Distilled Water	–	–	–	–	–	–	–
Anti-ty. serum 1/27,000							
B. coli Emulsion	++	++	+	+	+	+	–
Distilled Water	–	–	–	–	–	–	–
Acid Solution No. 5							
B. coli Emulsion	++	+	+	–	–	–	–
Distilled Water	–	–	–	–	–	–	–

0·5 c.c. of extract dilution, and anti-serum dilution or acid in each tube, and in addition either *B. coli* emulsion 0·5 c.c. or D. W. 0·5 c.c.

added as an indicator. The extract nevertheless was so potent that even when diluted 1/96 macroscopic agglutination of *B. coli* was obtained on addition of serum 1/27,000. The absence of agglutination of the *B. coli* by the serum or acid without extract is shown in the last column in each case.

Table IX shows similar results to those in Table VIII. In these experiments (1) the three alternatives distilled water, emulsion of kieselguhr or emulsion of *B. coli* were added to mixtures of (2) *B. typhosus* extract in varying dilutions, and (3) anti-typhosus serum 1/1000 or acid solution No. 5.

(1), (2) and (3) were added to the tubes in equal quantities, *i.e.* 0·5 c.c. of each.

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TABLE IX.

Agglutination of B. coli *and kieselguhr by* B. typhosus *extract with serum or acid.*

Dilutions of *B. ty.* Extract—	1/2	1/4	1/8	1/16	1/32	1/64	1/128	D. W.	1/2
Anti-ty. serum 1/1000	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·0
Distilled Water	++	+	–	–	–	–	–	–	–
Kieselguhr	+++	++	+	+	+	–	–	–	–
B. coli Emulsion	+++	++	++	+	+	–	–	–	–
Acid Solution No. 5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·0
Distilled Water	+	+	–	–	–	–	–	–	–
Kieselguhr		++	+	–	–	–	–	–	–
B. coli Emulsion	+	+++	+++	+++	–	–	–	–	–

These experiments show that the extract and serum may be present in sufficient quantities to cause agglutination, but the reaction cannot be demonstrated without the presence of some material which will make the precipitate sufficiently large to be seen. An extract can sometimes be diluted four or eight or more times beyond the minimum strength in which the agglutination is still visible when the extract is used alone, and yet a good agglutination of indifferent particles can be obtained on the addition of serum or acid.

In this way *B. coli* or kieselguhr can be used as an indicator to show the presence of an agglutination reaction, when the extract is too weak to form a visible precipitate. For this purpose a thin suspension of the indifferent particles is most suitable.

The strain of *B. coli* which was generally used for this purpose agglutinated in acid No. 10 and sometimes also in 9 and 8 after long standing, but never in 5 or 6.

After addition of *B. typhosus* extract to the *B. coli* emulsion agglutination was sometimes confined to tube 5 and no agglutination occurred in tubes 9 and 10. In this case the *B. typhosus* extract appeared to act as a "protective colloid" and each whole particle (*B. coli* + *B. typhosus* extract) behaved as though it were composed of the extract. An adsorbed coating of each bacillus with *B. typhosus* colloid being assumed as probable (see Table XI, also Table II *B. typhosus* "L").

If normal serum (horse or rabbit) in a dilution of 1/50 to 1/60 was added to extract the precipitate on addition of specific serum 1/9000 was increased, in this case the globulin of the normal serum probably acted by increasing the bulk of the precipitate, as occurs in the ordinary precipitin reaction when strong serum is used. The addition therefore of strong normal serum can make visible an agglutination reaction

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which cannot be seen when only dilute typhoid extract and dilute anti-typhoid serum are used. This increased precipitate on addition of normal serum is probably of the same kind as that described under the name of conglutination by Bordet and Streng as occurring on the addition of ox serum.

A similar increase in the precipitate was found by Dean to occur when mid-piece of guinea-pig complement was added to dilute typhoid extract and dilute anti-typhoid serum.

A low dilution of agglutinating serum may therefore be quite unnecessary in order to produce a precipitate with a bacterial extract. High dilutions of the specific serum may be used if enough indifferent substance (*e.g.* normal serum, indifferent bacteria, etc.) is present to make a sufficiently bulky precipitate.

(7) *The relation of electric charge to the agglutination of bacterial extracts.*

If acid, slightly stronger than the optimum for agglutination, is added to an emulsion of *B. typhosus*, *e.g.* regulator mixture 8 or 9, the bacilli remain dispersed for a variable length of time, often for many hours, or they may not become agglutinated at all. The bacilli dispersed in neutral or alkaline solutions always have a negative charge, that is they can be shown to move to the positive electrodes in a cataphoresis experiment.

It was expected by Michaelis and his co-workers that in a solution of optimum acidity for agglutination the charge would be nil (isoelectric point) and that in higher concentrations of acid when the bacilli are again dispersed that the charge on the bacilli would be positive, on the analogy of particles of serum protein, etc. These workers have however failed to demonstrate any change of charge on the bacilli. They showed by U-tube cataphoresis experiments that the bacilli always moved to the positive pole in solutions 5, 6, 7, the optimum acidity for agglutination, and also in stronger acid Nos. 8 and 9 as they do in weaker acids and in alkaline liquids. The writer has repeatedly confirmed these observations, using a microscope cell with platinum electrodes such as was used by Chick and Martin (1912) in their experiments on serum proteins.

Michaelis explains these results by pointing out that the bacilli are made up of numerous heterogeneous particles and suggests that they do not all become isoelectric at the same point of hydrogen-ion concentration.

It seemed possible to the writer that, since the agglutination in No. 5 was due to some substance contained in a watery extract, and

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agglutination in No. 10 to a substance which remained in the bacillary bodies after repeated washing, then by use of an extract it might be possible to obtain the substance agglutinable in No. 5 in a state of comparative purity, and in this way the agglutination optimum and the isoelectric point might be shown to be identical. On making the experiment it was found that even when a perfectly clear extract was used the agglutinated particles in tubes 5, 6, 7, 8, and 9 were all negatively charged, as in tubes 1 to 4. In tubes 8 and 9, in which agglutination did not appear, the very few particles seen moving under the microscope may not have been composed of the substance under investigation, as this may have been completely dispersed in this degree of acidity, but the negative charge observed in tubes 5, 6, and 7 was determined by watching the movement of clumped particles, which were undoubtedly composed (at least in part) of the substance which is characteristic of *B. typhosus* and is agglutinated in tubes 5 and 6.

It was found however that in emulsions and in re-emulsified deposits the substance agglutinated in tube 10 became isoelectric (no movement to either pole) about this hydrogen-ion concentration, and sometimes a positive charge could be demonstrated on bacilli in tube 10 or 11. Also in HCl N/100 the bacilli can be shown to have a positive charge, and in slightly weaker acid to be isoelectric, moving toward neither electrode.

In Table IV are recorded observations as to the charge on the bacilli in an original emulsion and in a re-emulsified deposit (Dep. 1) and also on the particles seen in a first extract (Ext. 1). It is shown that the particles in the extract remain negatively charged throughout, whilst the bacilli in the " whole emulsion " and in the deposit become isoelectric in tube 11 or 12, *i.e.* in $[H^{\cdot}]$ about $2{\cdot}2 \times 10^{-3}$.

Table X shows the agglutination with acid of emulsions of washed bacilli of a strain of *B. typhosus*, and the charge carried by the bacilli in the different tubes. Both regulator mixtures and hydrochloric acid were used. The HCl was diluted so that each tube contained two-thirds of the acid in the preceding tube. It is seen that the bacilli became isoelectric at or about the optimum for agglutination and a positive charge was demonstrated in the tube containing HCl N/100. The agglutination only occurred in the zone corresponding to tubes 9 and 10 and not at all in the zone with lower $[H^{\cdot}]$, as shown by the series of tubes with regulator mixtures.

The experiments recorded in Tables IV and X show that the optimum $[H^{\cdot}]$ for agglutination and the isoelectric points approximately coincide for the washed bacilli, but no change of charge is associated with the

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agglutination oi the substance which is agglutinated in Nos. 5 and 6.

TABLE X.

Agglutination with acid regulator mixtures and with dilute HCl of the re-emulsified washed bacilli of a strain of B. typhosus. *The electric charge on the bacilli is also recorded.*

Lactic acid regulator	12	11	10	9		7	6	5	4	D. W.	
B. ty., Dep. 2	+	+	⧻	⧻	+s	–	–	–	–	–	Agglutination.
,, ,,	0.	0	–	–	–	–	–	–	–	–	Charge.
HCl	**N/100**	**N/150**	**N/225**	**N/337**	**N/505**	**N/757**	**N/1135**	**N/1702**	**N/2553**	**D. W.**	
B. ty., Dep. 2	⧺	⧻	⧻	+s	+s	–	–	–	–	–	Agglutination.
,, ,,	+	0	0	–	–	–	–	–	–	–	Charge.

D. W. = distilled water; agglutination ⧻, ⧺, +, +s and – = complete, marked, distinct, slight and absent agglutination; charge +, 0, – = positive, isoelectric and negative charge.

As stated above, if a clear watery extract of *B. typhosus* is added to a weak watery solution of *B. coli* and the mixture put into tubes containing different regulator mixtures, agglutination occurs in tubes 5 and 6, or in 5, 6, 7, and 8, whereas when *B. coli* emulsion is present alone, the agglutination is in tube 10 or 8, 9, and 10. A very similar effect as regards agglutination may be produced by adding weak serum (*e.g.* normal horse serum) instead of *B. typhosus* extract. Agglutination then occurs in tubes 4 and 5, or in tubes 4, 5, 6, 7, and 8. If serum 1/300 is used the zone of agglutination is narrowed to tubes 4 and 5, if serum 1/1500, more tubes are involved. Thus the appearance of the tubes is nearly the same whether *B. typhosus* extract or serum 1/1500 is added. When however the electric charge on the bacilli is examined a very marked contrast is observed. In the series containing serum the bacilli move to the anode (negative charge) in distilled water and in tube 3 or weaker acid, but move to the kathode (positive charge) in tubes 8, 9, and 10. In tubes 4 and 5 no movement occurs, and in tubes 6 and 7 the bacilli are also isoelectric or they may move to the kathode according to the strength of serum employed.

Table XI shows an experiment of this kind; the influence of *B. typhosus* extract and of diluted normal serum on the agglutination and on the charge carried by *B. coli* is well shown. The agglutination optimum in the presence of serum corresponds to the isoelectric point, the dispersal in tube 3 and in distilled water corresponds to a negative charge and the dispersal in tubes 8 to 10 to a positive charge. On the other hand, though a similar appearance of the tubes, as regards agglutination and dispersal, is seen in the series of tubes which contain extract

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TABLE XI.

Agglutination of washed B. coli *by acid* (1) *with distilled water,* (2) *with extract of* B. typhosus, (3) *with normal horse serum* 1/1500. *The charge on the bacilli is also recorded.*

Regulator acid mixture	10	9	8	7	6	5	4	3	D. W.	
(1) *B. coli* + D. W.		⧺	+	+	−	−	−	−	−	Agglutination.
,, ,,		0	−	−	−	−	−	−	−	Charge.
(2) *B. coli* + *B. ty*. Ext.	−	−	⧺	⧻	⧻	⧺	−	−	−	Agglutination.
,, ,,	0	−	−	−	−	−	−	−	−	Charge.
(3) *B. coli* + Serum 1/1500	−	−	+	⧻	⧻	⧻	⧺	−	−	Agglutination.
,, ,,	+	+	+	0	0	0	0	−	−	Charge.

Agglutination ⧻, ⧺, +, − = complete, marked, distinct and no agglutination.
Charge +, 0, − = positive, no movement, and negative charge.

instead of serum, the charge on the bacilli in these tubes is negative in tubes 3 to 9, *i.e.* throughout the region of agglutination. If Michaelis' hypothesis with regard to the negative charge through such a series of tubes is correct and the substance in the extract which agglutinates in tube 5 is isoelectric in this $[H^{\cdot}]$, then the charge carried by this substance must be so weak as not to influence the movement of the particles which are seen to move to the anode in tubes 5 to 10. And since the same phenomenon of movement of all visible particles to the anode is seen throughout the series of tubes when extract of *B. typhosus* is used alone, this extract, on this hypothesis, must contain two substances, (1) agglutinable in 5 with its isoelectric point in 5 or weaker acid, (2) with isoelectric point in 9 or stronger acid.

III. Summary.

The experiments detailed above have already been discussed in the sections in which they have been recorded. The following is a brief summary of the results obtained.

(1) In many ways a clear watery extract of *B. typhosus* behaves like an ordinary emulsion of the bacilli. This is true in a general sense in respect of agglutination by serum, and the property of agglutinability by weak salts, which some emulsions of *B. typhosus* possess, is also exhibited by a watery extract of the same strain.

(2) The optimum hydrogen-ion concentration for the agglutination of an emulsion is also approximately the optimum for the agglutination of a watery extract made from the same emulsion. The washed bacilli and the original emulsion show a second optimum. Agglutination appears later at this secondary optimum which is in stronger acid. There is a zone of absent or deficient agglutination in solutions of

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intermediate strength. The lower [H˙] of the two is the characteristic optimum for *B. typhosus*, *i.e.* about $3{\cdot}6 \times 10^{-5}$. The second optimum [H˙], *i.e.* $1{\cdot}1 \times 10^{-3}$ to $2{\cdot}2 \times 10^{-3}$, is approximately the same as the optimum for the agglutination of emulsions of many different bacteria, *e.g.* some strains of *B. coli*, staphylococcus, etc., and moreover is about the optimum for the agglutination of an emulsion of *B. typhosus* after it has been heated to 100° C.

These two optima appear to indicate the presence of two acid agglutinable substances in an emulsion of *B. typhosus*, (*a*) which is characteristic of this bacillus and is present in a watery extract (this substance is rendered inagglutinable by heating to 80° C. for 30 minutes or to 100° C. for five minutes, or longer, *e.g.* one hour), and (*b*) which is present in the bodies of the bacilli after repeated washing and which is not characteristic of *B. typhosus*, but has characters shared by similar substances in other bacilli; it resists heating to 100° C. for five minutes and is apparently little, if at all, affected by heating to 80° C. for half an hour.

(3) The substance agglutinable by serum and that agglutinable by acid No. 5, *i.e.* [H˙] $3{\cdot}6 \times 10^{-5}$, are both present in a watery extract and can both be completely removed from the bacillary bodies by washing with distilled water. The serum-agglutinable substance is however less easily completely removed. This apparent difference is perhaps due to the fact that serum is a more delicate reagent for testing for the presence of agglutinable substance. If an extract is diluted it will give a precipitate in higher dilution with serum than with acid and the bacillary bodies when repeatedly washed often react with serum after the acid agglutination in solution 5 or 6 has ceased to appear. These substances agglutinable by acid and by serum are however closely associated and are both precipitated by acid No. 5 or 6.

The identity of the two substances has however not been proved and the following considerations are very strongly opposed to this assumption, (*a*) a considerable number of strains of *B. typhosus* are agglutinable by serum and not by acid; (*b*) strains have been described which are agglutinable by acid No. 5 or 6 but not by serum; (*c*) the diminished agglutinability by serum exhibited by some strains which are inagglutinable by acid is apparently due to a total diminution of the "soluble" or extractible substance; (*d*) the substance agglutinable by acid, tube 5 or 6, *i.e.* [H˙] $3{\cdot}6 \times 10^{-3}$, is rendered inagglutinable by heating to 100° C. for five minutes, whereas Porges and Prantschoff (1906) have shown that the serum agglutinable substance is still active after being boiled.

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It is not possible at present to say for certain whether the substances agglutinable by serum and acid are identical or distinct. If they are identical and there is only a single substance, its agglutination properties vary independently in different strains of *B. typhosus*.

(4) The importance for agglutination by serum of the substances in "solution" in the liquid part of a bacterial emulsion, and the impossibility of distinguishing between agglutination and the precipitation of bacterial extracts by serum, were first pointed out by Kraus and Paltauf, and by Kraus and v. Pirquet.

Their view is confirmed and extended to the agglutination by acids Nos. 5 and 6 by the result of some of the experiments described above. The facts adduced in support of the view that agglutination is essentially the formation of a coagulum in the liquid part of an emulsion are the following:

(*a*) The property of agglutination may be completely removed from *B. typhosus* by washing with water; the resulting washed bacterial bodies no longer agglutinate with specific serum or in acid solution Nos. 5 to 7.

(*b*) The washings of the bacteria or watery extracts are agglutinable by specific serum or by acid solution No. 5 or 6.

(*c*) The watery extract even in very small quantities when added to the washed bacilli, indifferent bacilli or other finely divided particles, confers on them the property of being agglutinated by specific serum or acid solution 5 or 6.

(*d*) The extract reacts with highly diluted serum, *e.g.* 1/27,000, and may be considerably diluted, *e.g.* 1/96, without entirely losing its agglutinable properties with serum or acid.

(*e*) In order to demonstrate the presence of agglutinable substance in very low concentration (especially if highly diluted serum or if acid is used) it is necessary to add some indifferent substance, *e.g. B. coli*, kieselguhr, globulin, etc. in order to increase the bulk of the precipitate.

(*f*) The extract or successive extracts appear to contain all the characteristic agglutinable substance of the original emulsion. This is shown by the delicacy of the reaction, and the small amount of extract required to agglutinate an indicator, *e.g. B. coli.* The demonstration of the reaction with highly diluted serum 1/27,000 and much diluted extract 1/96 appears to be an advance on the work of previous writers. Nicolle did not use indifferent particles with high dilutions of serum or of extract, and the only other use of an indicator under such conditions appears to be the use of conglutinin by Bordet and Gay, and Streng, and of mid-piece by Dean.

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(*g*) The microscopic demonstration of the coagulum formed in the process of agglutination has been made easy by taking a watery extract containing very few bacilli, *i.e.* ordinary emulsion from which most of the bacilli have been removed by centrifuging. After adding dilute specific serum or acid the formation of the coagulum may be watched in a microscope cell with dark ground illumination or by adding dilute fuchsin or eosin to the microscope cell.

IV. CONCLUSIONS.

(1) Emulsions of *B. typhosus* in distilled water contain two substances agglutinable by acid which have two different optima of hydrogen-ion concentration for agglutination.

(2) One of the substances, (*a*), which is contained in a clear watery extract has an optimum [H˙], of $3{\cdot}6 \times 10^{-5}$ to $7{\cdot}2 \times 10^{-5}$. The other, (*b*), is contained in the thoroughly washed bacterial bodies ; its optimum [H˙] is about $1{\cdot}1 \times 10^{-3}$.

(3) Substance (*a*) is rendered inagglutinable by being heated to 80° C. for 30 minutes or 100° C. for five minutes; substance (*b*) is unaffected by heating to 80° C. or 100° C. for similar periods.

(4) The substance in an emulsion of *B. typhosus* which is agglutinable by specific serum is closely associated with the substance whose optimum [H˙] for agglutination is $3{\cdot}6 \times 10^{-5}$. The identity of these two substances is however on the whole improbable.

(5) The agglutination of an ordinary emulsion of *B. typhosus* by specific serum or acid of [H˙] $3{\cdot}6 \times 10^{-5}$ is brought about (practically entirely) by the substance which passes out of the bodies of the bacilli into the surrounding liquid, *i.e.* the view of Kraus, v. Paltauf and Nicolle as regards serum agglutination is confirmed by experiments with dilute extract and highly dilute serum, and its application is extended to agglutination by acid.

(6) Acid of [H˙] $3{\cdot}6 \times 10^{-5}$ or high dilutions of specific serum, *e.g.* 1/27,000, will cause an agglutination reaction in much diluted clear watery extract (*e.g.* 1/96) of *B. typhosus*, but the addition of indifferent particles is necessary to produce a visible agglutination or precipitate.

(7) There is no direct evidence of change of electric charge associated with the agglutination by acid of the substance in an emulsion or extract whose optimum for agglutination is $3{\cdot}6 \times 10^{-5}$.

The observations of Michaelis and Beniasch on the cataphoresis of emulsions have been confirmed microscopically and found to apply to extracts also.

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(8) Washed bacillary bodies of *B. typhosus* become isoelectric in [H·] $1 \cdot 1 \times 10^{-3}$ or $2 \cdot 2 \times 10^{-3}$, *i.e.* they do not move towards either electrode in this [H·], and move to the kathode in stronger [H·].

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