# ON THE MECHANISM OF AGGLUTINATION.

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In view of the large number of hypotheses advanced to account for the phenomenon of agglutination of bacterial cells, it was suggested to me by Dr Allan Macfadyen, that I should investigate the agglutination of typhoid bacilli from a physico-chemical standpoint. I here beg to express my indebtedness to Dr Allan Macfadyen and Dr George Dean for much kindly aid and criticism.

In a preliminary notice to the Pathological Society (13. II. 1904), I indicated that my results supported the view first advanced by Bordet, that the phenomenon of agglutination may be divided into two stages:

1. Fixation of agglutinin by bacilli.
2. Aggregating influence of salts and other substances.

Further, I suggested that the fixation obeyed the general laws of staining and that the aggregation was a surface-tension effect. In this investigation the first question was whether agglutination was a strictly specific phenomenon or one of a general character. Agglutination is not entirely specific. According to Köhler (1900) typhoid bacilli are agglutinated not only by typhoid immune serum but also to a smaller extent by the sera obtained from *B. coli*, pneumococcus and meningococcus infections. Lubowski and Steinberg (1904) obtained agglutination by immune sera from proteus bacilli and staphylococcus, and Rodet (1904) found that the normal serum of the rabbit, etc. if not too much diluted agglutinated typhoid bacilli. Stern (1903) has shown that the serum produced by a bacillus generally agglutinates allied bacilli but is specific in the sense that the bacillus itself is most easily agglutinated. In addition to the specific agglutination caused by immune sera, a non-specific agglutination can, according to Malvoz (1897), be brought about by saffranin and other dyes, and Gilardoni (1903) states that, in general, any reagent which precipitates proteids will cause agglutination.

Since the most diverse fluids thus lead to the same end the reaction cannot be regarded as a definite chemical change, and further investigation is required to ascertain whether the physical part of the reaction (or mechanism) is the same in all cases.

The chief views regarding the essential nature of agglutination which have been put forward are those of Pfeiffer, Gruber, Paltauf, and Bordet.

(1) Pfeiffer (1896) and Emmerich and Löw (1899) regarded agglutination as a vital paralysis of the bacilli due to the action of a bacteriolytic enzyme. It has, however, been shown by Joos (1901) that the agglutinating substance in typhoid immune serum is in the absence of salts absorbed without diminishing the motility of the bacilli, and further, that after the agglutination is completed by the addition of an electrolyte, *e.g.* sodium chloride, reproduction does not cease. Moreover, the enzymic character of the agglutinating substance, or agglutinin, is rendered doubtful by the fact that the bacteriolytic enzymes are destroyed by temperatures at which the agglutinins remain unaffected.

(2) Gruber (1899), Dineur (1898), and Nicolle (1898), supposed that a glutinous substance, "glabrificin," was absorbed from the serum by the bacilli, thereby causing small adhesive prominences on the membranes, or rendering the flagella, or the membranes themselves, adhesive on contact.

These hypotheses, which ascribe agglutination to the adherence of bacilli after contact, are insufficient, however, to account for the movement of dispersed non-motile bacilli towards a common centre.

(3) Paltauf (1897) and Duclaux considered that a specific precipitate is produced in the medium which during flocculation mechanically carries the bacilli with it.

Wassermann (1903) and Kirstein (1904) have shown that the precipitable substance in a culture filtrate and the agglutinable substance in the bacilli are very closely related, and that the former may be regarded as agglutinable substance ejected by the bacilli into the medium.

Beljaeff (1904), however, finds that the agglutinating power of a typhoid immune serum is not parallel to its power of precipitating a typhoid filtrate, and Norris (30. v. 1904) considers that many facts point to at least some of the substances concerned in specific agglutination and specific precipitation being distinct.

That agglutination can be brought about by a visible precipitate

is certain, and that extremely fine precipitates are sometimes formed round the isolated organisms during agglutination, has been shown by Löwit (1904), but agglutination of *B. typhosus* has been observed by Hinterberger where no precipitate was demonstrable, and it remains an open question whether precipitation in the medium is a necessary part of the mechanism of agglutination. As regards precipitation within the bacilli it will be seen from the following (part 2) that this is highly probable.

(4) Bordet (1899) separated the mechanism of agglutination into two stages as mentioned above, (1) fixation, and (2) aggregation. The fixation of agglutinin by the bacilli he considers to be analogous to the fixation of a dye by a tissue, and that once the agglutinin is fixed, the bacilli obey the laws of inert particles, and that aggregation is caused by changes in the molecular attraction between the organisms and the surrounding medium.

Since staining is usually regarded as a special case of adsorption, *i.e.* the condensation or concentration of gases or dissolved matter on substances with highly developed surfaces, one might expect that the fixation of agglutinin would obey the general laws of adsorption. If the second part of the hypothesis is correct, since the relations between inert particles and a surrounding medium are directly governed by the surface tension of the particles against the medium, one would expect the bacilli to be subject to corresponding surface tension effects.

It being, therefore, possible to control both parts of Bordet's view by direct experiments on adsorption and surface tension, an investigation on these lines was instituted.

### *The Fixation of Agglutinin.*

According to Arrhenius (1903) agglutinin is partitioned between bacilli and medium much as iodine is between water and carbon-disulphide. He further deduces that the molecular weight of agglutinin dissolved in the medium is one and a half times that of the agglutinin dissolved in the bacilli.

The following objections may be raised to the views advanced by Arrhenius. Specific agglutinins and the agglutinable substances, precipitins and precipitable substances, toxins and antitoxins, belong to the same class of substances. (Bordet, 1903.) This class also includes lysins and antilysins, immune bodies and complements with their antibodies. All these substances belong to the division of

colloidal solutions of compounds of high molecular weight. (Müller, 1903.)

Toxins, lysins, and agglutinins are possessed of some degree of diffusibility, but in general the substances with which they react have a very low rate of diffusion of the order of magnitude of that of globulin and other typical colloids. (Billitzer, 1903.) A recent determination of the molecular weight of a well-purified typical organic colloid, viz., Glycogen, by the Nernst-Abegg cryoscopic method according to Gatin-Gruzewska (1904), gave no depression of the freezing-point of the aqueous medium. Glycogen is then either a difficultly soluble substance with a molecular weight over 140,000, or it is incapable of forming a true solution and its molecular weight *indefinite*. This glycogen migrated in an electric potential gradient like other colloids, to the anode. Sabanejev had previously found for glycogen the molecular weight 1620, probably owing to the presence of crystalloidal impurities.

E. W. Reid (1904) using Starling's (1899) method found that by washing salted out or crystallised proteids, solutions of proteids are obtained which give no osmotic pressure against a membrane of gelatine.

The view that colloidal solutions of high molecular organic compounds are not in homogeneous solution is supported by their dispersion of polarised light. Raehlmann (1904), Römer and Siebert (1904), using the Siedentopf-Zsigmondsky microscope, have observed in serum and other albumin, globulin, and glycogen solutions a similar granular appearance to that exhibited by colloidal gold, silver, and platinum. Taken in conjunction with the low diffusibility and absence of osmotic pressure this granular structure seems to indicate that typical colloidal proteids are almost entirely in suspension, and that the pseudo-solutions are really heterogeneous systems. The conditions under which the laws of Boyle, Gay-Lussac, and Avogadro apply being absent, it is probable that for proteids of this class the application of the gas laws in the manner made by Arrhenius is not a true interpretation of the mechanism of their reactions and equilibria with other substances. Nernst (1904) has recently pointed out that for reactions in heterogeneous systems, in so far as they take place entirely or partly on the interfaces of the different phases, the velocity of reaction depends entirely or partly on velocities of diffusion, which in general have nothing to do with the order of a reaction, that is, with the number of reacting molecules. Since at least one of the substances in the reactions with which we are concerned must be considered as in the typical colloidal state

van't Hoff's laws cannot be applied to the reactions of specific substances with their antibodies.

On the other hand, typical colloidal solutions being heterogeneous systems with highly developed interfaces, the velocities of their reactions with other substances, and the equilibria they attain, should be governed by the laws which hold for the absorption of substances from solutions by suspensions, porous substances, and highly developed surfaces in general, classed generally as adsorption phenomena.

That there are quantitative laws of adsorption has been fully recognized by Ostwald (1891). A dilute solution of hydrochloric acid digested with animal charcoal slowly reaches equilibrium, which does not change when portions of the solution or of the charcoal are removed. The equilibrium is then only dependent upon the ratio of the concentration of hydrochloric acid in the solution to that in the charcoal. On the addition of water the charcoal gives up a portion of its hydrochloric acid within a few minutes and a new equilibrium is established. A given mass of charcoal can be obtained in this manner so uniform in concentration as regards hydrochloric acid that the experiments agree within fractions of a percentage. With various substances, the amounts absorbed by charcoal, when plotted as ordinates against the amounts of water as abscissae in right-angled coordinates, gave hyperbolic curves. The laws governing the absorption of the most varied substances are the same, yet, in many cases the charcoal undoubtedly reacts chemically on the substance absorbed. It is important to observe that the state of equilibrium is easily and rapidly attained when the solid body is treated with a concentrated solution of the substance to be absorbed and subsequently by addition of water brought to the desired dilution. On the contrary, if the solid substance be brought into contact with the already diluted solution days may elapse before equilibrium is attained.

A further important feature of adsorption equilibria is that the general formula

$$C_1 = KC_2^n$$

is capable of wide application, where $C_1$ is the concentration of the free substance in the aqueous medium, $C_2$ the concentration of the bound substance in the adsorbing material, $K$ and $n$ constants dependent on the chemical nature of the substances brought into contact. Thus when a solution of iodine is mixed with animal charcoal the relation is

$$C_1 = 0{\cdot}0014\, C_2^4.$$

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Schmidt (1904) found that acetic, succinic, and oxalic acids with charcoal, similarly give definite and characteristic constants $K$ and $n$. It may be well to observe that the values of $n$ and $K$ do not give any definite idea of the magnitude of the adsorbed molecule or grain as compared with that in the aqueous medium but rather of the relative intensity and capacity of adsorption of charcoal and water for the substances partitioned.

It has been observed that colloids are adsorbed by charcoal, which leads to the consideration of the more immediately interesting case in which an inferior colloid or a crystalloid is adsorbed by a typical colloid.

For this we can assume within certain limiting concentrations that

$$C_1 = KC_2^n$$

where $C_1$ is the concentration of inferior colloid that is retained in the aqueous medium as a consequence of the solvent-like attractive or adhesive intensity of the latter, $C_2$ is the concentration of inferior colloid that is bound or taken up by the typical colloidal medium in virtue of its adhesive intensity.

From Eisenberg and Volk's (1902) experimental investigation of the equilibria which obtain between agglutinin and typhoid bacilli, Arrhenius has deduced the relation

$$\frac{C}{A} = KB^{\frac{2}{3}}$$

where $\frac{C}{A}$ represents the concentration of agglutinin in the bacilli, $B$ the concentration of free agglutinin in the medium, and $K$ is a constant. This equation is, it seems to me, a special case of the adsorption formula where the power $\frac{2}{3}$ has only significance in indicating the relative intensity of adsorptive power of the medium and the bacilli for agglutinin. The validity of the special formula given is however questionable, for in calculating the agglutinating value of a serum an assumption has been made by Eisenberg and Volk which is very open to criticism. They take it for granted that a serum which gives a concentration of agglutinin per c.c. equal to one 24 hours' unit when diluted to 20,000 times its original volume, will contain in 1 c.c. of the original serum 20,000 units of agglutinin. In other words, they assume that the number of agglutinin units present in a fluid is independent of its volume, no association, dissociation of the units, or, in other words, no change in the binding capacity of the agglutinin units takes place. When we consider that flocculation by sera and other chemicals reaches

a maximum at certain concentrations, *i.e.* that there are optimum concentrations of sera, etc., above and below which the flocculation is diminished, it seems to me, that without further experimental investigation, we cannot assume that the fixation of agglutinin is unaffected by this phenomenon or that the binding capacity of the diluted unit is equal to that of the same quantity of agglutinin when in the concentrated state. The experimental error being the same for the estimation of free agglutinin in dilute and concentrated solutions, the deviations from the calculated values for dilute solutions shown by Eisenberg and Volk's figures prove that Arrhenius's formula, as it stands, does not embrace the entire range of dilutions. In Table III (Arrhenius, 1903, p. 417), assuming that in more dilute solutions we have a different relation, we obtain a better constant by using the formula

$\frac{C}{A} = KB^{\frac{3}{4}}$ for the more concentrated solutions, viz.,

$K = 13{\cdot}6,\ 12{\cdot}3,\ 12{\cdot}5,\ 12{\cdot}54,$

whereas

$\frac{C}{A} = KB^{\frac{2}{3}}$ gives $K = 23{\cdot}2,\ 24{\cdot}8,\ 26{\cdot}3,\ 28{\cdot}2.$

Again, rejecting according to the principle adopted by Arrhenius the last value of Table XIII we obtain for $\frac{C}{A} = KB^{\frac{1}{2}}$, $K = 23, 17,\ 23{\cdot}2,$ $24{\cdot}5$, which seem to be as good as $\frac{C}{A} = KB^{\frac{2}{3}}$, $K = 12{\cdot}7,\ 11{\cdot}8,\ 8{\cdot}4,\ 10{\cdot}6.$ But a change in the power to which $B$ is raised would give from Arrhenius's standpoint a radical change in the molecular relations of agglutinin, for then the molecular weight of free agglutinin would be to that of bound agglutinin as 4 is to 3 or 2 to 1, entirely different dissociations to that found in other cases. From the adsorption point of view the variation of $K$ and $n$ from one serum to another is to be expected. From the hypothetical gas-law standpoint, however, $K$ and above all $n$ must be constant. Moreover, as Arrhenius himself points out, if multiples of a certain concentration of agglutinable substance and agglutinin be brought together the power $\frac{2}{3}$ is incompatible with Eisenberg and Volk's experimental series in which they find the ratio between agglutinin bound and free to be unchanged on bringing multiple concentrations together. It seems to me that this last result of Eisenberg and Volk would be explicable on the partition formula $\frac{C}{A} = KB$, and this would lead to the conclusion that $n$ was dependent

upon the conditions of the experiment. The apparently constant active mass of the agglutinable substance within the bacilli cannot be ascribed to the non-participation of the cell contents in the reaction, for, as we have seen, Wassermann (1903) has demonstrated that agglutinin reacts with precipitable substance and gives rise to a precipitum, and we have no reason to assume that this reaction does not occur within the bacilli when agglutinin is absorbed; on the contrary, the second phase of agglutination is most easily accounted for by the assumption that the cell contents experience a decided change in structure, either physical or chemical, on the absorption of agglutinin. From a physico-chemical point of view it does not then seem probable theoretically that the gas laws apply to the fixation of agglutinin by agglutinable substance, nor experimentally that there is foundation for the application. As far as the equilibria are concerned, a superficial analogy is, however, to be expected, such as the phenomena of adsorption would lead us to expect. With respect to the more general problem of the velocity of reaction of substances which may be classed with agglutinin, and agglutinable substance such as toxin and antitoxin, the agreement between the experimentally observed relations and those calculated by Arrhenius is also merely superficial. Similar agreement between observations on the velocity of reaction in heterogeneous systems and calculations on the assumption of homogeneity has been observed by Hantsch (1904), who recognised fully the untenability of the assumption, for the absorption of ammonia gas by solid organic acids and of hydrogen chloride by solid amine bases.

With a view to the elucidation of the class of phenomena to which the fixation of agglutinin may be attributed the following experiments were carried out:

The unit concentration of agglutinin was arbitrarily fixed as that dilution of a serum 1 c.c. of which on addition to 1 c.c. of a standard suspension of typhoid bacilli in a tube 6 mm. in diameter showed clumps just visible with the aid of a hand-lens at the end of 2½ hours, at room temperature. The same lens was used throughout the series compared. In thus reducing the time at which the reading took place I hoped to eliminate part of the error due to the growth of a living culture in a very weakly agglutinating fluid. In Eisenberg and Volk's experiments this error may have influenced their results, for they allowed their test mixtures to remain 24 hours before reading. The absolute quantity of agglutinin represented by a unit depends upon the time given to the test mixture to flocculate, a 2½ hours' unit contains more agglutinin

than a 24 hours' unit, hence when an agglutinating value is ascribed to a fluid the time value of the unit must be given.

The standard suspension was prepared from an agar culture of a good agglutinating strain, grown at 37° C. for 18 hours. Each agar tube was rinsed out with 15 c.c. of 0·82 % sodium chloride. To secure uniformity the suspensions, obtained from a dozen tubes, were mixed and filtered through glass-wool to remove agar particles.

The typhoid immune serum first employed was obtained from Horse No. 1 on December 5th, 1903, and gave agglutination at a dilution of 1 in 40,000, equivalent to an agglutinating value of 40,000 diluted $2\frac{1}{2}$ hour units.

Since the use of killed cultures might reduce the experimental error, a few comparative series, to test the relative agglutinability of the above suspension and of the standard suspension after it had been heated to 58° C., for 30 minutes, were carried out and gave as a mean the following result. With the unheated standard suspension :—

4 Series.

| Serum concentration | 1/20 | 1/200 | 1/2,000 | 1/20,000 | 1/40,000 |
|---|---|---|---|---|---|
| Time of agglutination | 8 | 19 | 29 | 40 | 150 minutes |

With the heated standard suspension :—

4 Series.

| Serum concentration | 1/2,000 | 1/8,000 | 1/20,000 |
|---|---|---|---|
| Time of agglutination | 10 | 80 | 150 minutes |

The serum in the higher dilutions, corresponding to the $2\frac{1}{2}$ hour units, showing from this only half the agglutinating power on the heated bacilli of that which it has for the unheated, I decided to use the unheated bacilli, as they form a more delicate indicator of diluted free agglutinin in a solution. Again, since heating probably alters the nature of the absorbing substance and since from the above it seems probable that the laws which hold for the fixation of agglutinin by the heated agglutinable substance would be different to those governing the fixation in the unheated substance, the unheated standard suspension was also used for the experiments on equilibrium. A further investigation to ascertain whether the motility of the bacilli was connected with their agglutinability gave entirely negative results. Two non-motile cultures obtained by 18 transfers on agar at 42° C., gave values for their agglutinability which were, within the experimental error, identical with those given above for the unheated standard suspension. On staining

by Loeffler's method the flagella were observed in this non-motile culture to be still attached to the cell bodies. That the contents of the cell play an important, if not the chief, part in the fixation of the agglutinin seems to be indicated by the fact that one sample of typhoid residue obtained by grinding the organisms at the temperature of liquid air and washing repeatedly with distilled water only gave agglutination with the above immune serum in the concentration of 1/20; a second sample did not agglutinate even with the undiluted serum. This result is in entire harmony with Bordet's view.

With regard to the minutiae of the experimental method it is important that after mixing bacilli and agglutinating fluid the mixture be not disturbed. To trace the possible effect of shaking, the heated culture, which with serum in a concentration of 1 in 2,000 agglutinated in 10 minutes, was shaken up for 1 minute, the bacilli reagglutinated in 1 hour; on repeating the agitation a third agglutination took place in 3 hours. This result is also interesting in showing that the second phase of agglutination, *i.e.*, the aggregation, is quite distinct from the fixation of agglutinin which is completed in a very short time.

The equilibria existing between various concentrations of serum and a constant concentration of bacilli were then determined:—10 c.c. of various dilutions of serum were added to equal volumes of suspension, thoroughly mixed and allowed to stand 3 hours at 17° C., the whole was then centrifugalised, the supernatant fluid decanted and the dilution in which it just caused agglutination in $2\frac{1}{2}$ hours determined. The results obtained expressed in the manner adopted by Eisenberg and Volk and the value of $K$ calculated by Arrhenius's formula were as follows:—

*Serum of Horse No.* 1 = 40,000 $2\frac{1}{2}$ *hour units.*

Mean values obtained from 6 Series.

| Concentration of serum | Units of agglutinin added | Units of agglutinin absorbed | Units of agglutinin free | $K$ |
|---|---|---|---|---|
| 1/20 | 2,000 | 1,300 | 700 | 16·5 |
| 1/30 | 1,333 | 1,133 | 200 | 38·9 |
| 1/40 | 1,000 | 840 | 160 | 28·5 |
| 1/50 | 800 | 768 | 32 | 76·2 |
| 1/60 | 666 | 646 | 20 | 87·6 |
| 1/200 | 200 | 200 | 0 | — |

With the exception of the 1/30 dilution the value of $K$ calculated according to the formula of Arrhenius shows a constant increase.

Taking Eisenberg and Volk's (1902) agglutinating unit, viz.—the dilution of agglutinating fluid 1 c.c. of which on addition to 1 c.c. of

the above-mentioned bacterial suspension showed a definite sediment after 24 hours—and using another serum, less regular results were obtained. 15 c.c. of suspensions were mixed with 15 c.c. of serum,

$$S, \frac{S}{2}, \frac{S}{4}, \frac{S}{8}, \frac{S}{16}, \frac{S}{32}, \frac{S}{64}, \frac{S}{128}, \frac{S}{256}, \text{ and } \frac{S}{512}.$$

The highest concentration of serum was obtained by adding 30 c.c. serum to one agar tube of culture. The values obtained were as follows:—

*Serum of Horse "Tom" = 44,000 24 hour units.*

Mean values obtained from 4 Series.

| Concentration of serum | Units of agglutinin added | Units of agglutinin bound | Units of agglutinin free |
|---|---|---|---|
| 1/1 | 44,000 | 24,000 | 20,000 |
| 1/2 | 22,000 | 8,000 | 14,000 |
| 1/4 | 11,000 | 3,000 | 8,000 |
| 1/8 | 5,500 | 500 | 5,000 |
| 1/16 | 2,750 | 750 | 2,000 |
| 1/32 | 1,375 | 675 | 700 |
| 1/64 | 688 | 488 | 200 |
| 1/128 | 344 | 329 | 15 |
| 1/256 | 172 | 170 | 2 |
| 1/512 | 86 | 86 | 0 |
| 1/1,024 | 43 | 43 | 0 |

The mixtures were heated for 2 hours at 37° C., and then allowed to stand 24 hours. No constant could be found by Arrhenius's formula for these figures.

Owing to the wide deviations obtained from Arrhenius's formula the question arose whether equilibrium between bound and free agglutinin could be easily and certainly attained or whether we had here only an apparent or so-called "false" equilibrium. The serum of the horse "Tom" ($S$) was brought together with a suspension of typhoid bacilli ($T$) of a concentration equal to one agar tube of culture grown for 18 hours at 37° C., in 5 c.c. of saline. The concentrations $\frac{S}{3}$ and $\frac{T}{3}$ were obtained by a dilution with saline. The final volume was 30 c.c., and the final concentration of serum and bacilli in all cases was the same. The following mixtures were made:—

2 Series.

I. 15 c.c. $\frac{S}{3}$+15 c.c. $\frac{T}{3}$.

II. 15 c.c. $\frac{S}{3}$+ 5 c.c. $\frac{T}{3}$, after 18 hours+10 c.c. $\frac{T}{3}$.

III. 15 c.c. $\frac{S}{3}$+ 5 c.c. $T$, after 18 hours+10 c.c. saline.

IV. 15 c.c. $\frac{T}{3}$+ 5 c.c. $\frac{S}{3}$, after 18 hours+10 c.c. $\frac{S}{3}$.

V. 15 c.c. $\frac{T}{3}$+ 5 c.c. $S$, after 18 hours+10 c.c. saline.

VI. 5 c.c. $T$+ 5 c.c. $S$, after 18 hours+20 c.c. saline.

VII. 5 c.c. $T$+ 5 c.c. $S$, after 2 hours+20 c.c. saline.

VIII. 15 c.c. $\frac{T}{3}$+15 c.c. $\frac{S}{3}$, mixed at the time of addition of the second portion in the other cases.

After allowing to stand for 24 hours further, the supernatant fluids had practically the same agglutinating values, the variations being at the most 200 units in 8,000 free units of agglutinin measured by bringing 1 c.c. of the fluid together with 1 c.c. of $\frac{T}{3}$ and heating to 37° C., for 2 hours.

Given a considerable time then we attain a fairly constant equilibrium with a concentrated serum. This equilibrium was found, however, to show the characteristics of "false" equilibria, when the serum was more diluted. *The successive addition of the bacillary suspension to the serum removed more agglutinin from the solution than when the addition was made at one time.* 10 c.c. of the diluted serum of Horse No. 1 were added to 10 c.c. of the standard suspension at once, and in a second case at the rate of 5 drops every 5 minutes. The concentrations of serum used were $\frac{S}{10}$ and $\frac{S}{200}$. The equilibria seemed to be, within the experimental error, uninfluenced by the rate of addition. When, however, 10 c.c. of the suspension were gradually added to an equal volume of the $\frac{S}{10}$ concentration of serum, the free agglutinin corresponded to 95 units ($2\frac{1}{2}$ hr.) when the addition was made at one time, whereas for the addition in parts 60 units ($2\frac{1}{2}$ hr.) were left free.

The serum of the horse "Tom" of concentration $\frac{S}{10}$ was then mixed with the standard suspension in the following manner:—

2 Series.

I. 15 c.c. $\frac{S}{10}$ + 15 c.c. suspension, centrifugalised after 3 hours.

II. 15 c.c. $\frac{S}{10}$ + gradually increasing fractions of suspension.

III. 15 c.c. suspension + gradually increasing fractions of $\frac{S}{10}$.

IV. 15 c.c. $\frac{S}{10}$ + 15 c.c. suspension, centrifugalised after 30 minutes.

In II and III the entire addition of the second 15 c.c. was spread over 2½ hours and the mixtures centrifugalised after a further 30 minutes. The supernatant fluids of I, III and IV showed 2,600 units of free agglutinin in a 2 hours' test at 37° C., whereas II showed 2,400 units; I, III and IV gave 3,200 six-hour units and II 2,800. Since I and IV gave the same result the agglutinin equilibrium is set up in less than 30 minutes at room temperature. No. II confirms the view that we are dealing with a "false" equilibrium.

It cannot then be assumed that the gas laws apply to the partition of agglutinin between bacilli and medium, nor that the formula $\frac{C}{A} = KB^{\frac{1}{n}}$ expresses the relation, and in the application of the general formula $\frac{C}{A} = KB^n$ $K$ and $n$ will probably be dependent upon the conditions of the experiment, such as the manner in which serum and bacilli are brought together, the temperature, the strain of bacilli used, their age, and the constituents of the serum other than agglutinin.

Since these results agreed well with the conception of the fixation of agglutinin advanced by Bordet, I carried out a staining experiment which lends further probability to the view that the mechanisms of both have much in common. In these experiments the staining substance (fuchsin) corresponds to the agglutinin, and the bacillary substance is represented by absorbent paper.

A few drops of saturated alcoholic fuchsin were added to a litre of water. 100 c.c. of water and 100 c.c. of this solution were placed in a porcelain tray *A*, 100 c.c. of water and 33·3 c.c. of the solution in a tray *B*. A piece of thick absorbent paper of superficial area nearly equal to that of the bottom of the tray was placed in *A* and a similar

piece in $B$; the paper weighed 4·4 grammes in each case. After an interval of 24 hours a similar experiment $A^1$ was started, and at the same time 66·7 c.c. of fuchsin solution were added to $B$. The three systems were then allowed to stand a further two hours and the intensity of colour of the supernatant fluids compared. $A$ was slightly less coloured than $A^1$, but $B$ was strongly coloured. On diluting 5 c.c. of $B$ until it had the same intensity as $A^1$ 7·5 c.c. of water had to be added. $B$, therefore, contained 2·5 times the concentration of free fuchsin in $A^1$.

Similarly the addition of the absorbent paper in parts removed more fuchsin from the solution than in the case where the whole amount of paper was added at once.

Von Dungern (1904), and Sachs (1904), have found similar false equilibria to exist in the relations between toxin and antitoxin. Indirectly then we have further support for the view that the gas laws are not applicable to specific substances and their antibodies, and the view that we are here dealing with adsorption phenomena appears to be confirmed.

## Summary.

1. The hypotheses of Pfeiffer, Emmerich and Löw, attributing agglutination to a vital paralysis due to the action of a bacteriolytic enzyme, and those of Gruber, Dineur, and Nicolle, which ascribe the action to the glutinous nature of the membranes or cilia, are insufficient to account for the observed phenomena.

2. The views of Paltauf and Duclaux that a specific precipitate is formed in the medium which mechanically carries the bacilli together are sufficient, but probably do not account fully for the agglutination of washed bacilli.

3. Arrhenius's assumption that the gas laws are applicable to the partition of agglutinin between bacilli and medium is improbable since the conditions under which these laws can be applied are absent.

4. The formula given by Arrhenius for the partition of agglutinin seems to be a special case of a general formula holding for the absorption of substances from solution by substances with highly developed surfaces, *e.g.*, the adsorption of iodine from solution by charcoal. A superficial analogy between the gas partition law and the adsorption partition law for equilibria is to be expected, likewise an equally superficial analogy between reactions in true solutions and colloidal solutions, *e.g.*, of agglutinin, for the velocity of reaction.

5. The special formula given by Arrhenius does not apply to the entire range of agglutinin solutions and the change which must be made in the constants is incompatible with the application of the gas laws, but agrees with the view that the fixation of agglutinin is due to adsorption.

6. The fixation of agglutinin from two different typhoid immune sera by living typhoid bacilli did not correspond to the partition law deduced by Arrhenius from the experiments of Eisenberg and Volk.

7. The rate of addition of bacillary suspension to agglutinating serum is a factor determining the amount of agglutinin fixed by the bacilli. By adding the suspension in parts more agglutinin is removed than in the case where the whole amount of suspension is added at once. This points to the equilibria belonging to the class met with in absorption.

8. Similar adsorption equilibria are obtained by experiments on staining.

9. The cell contents probably play an important part in agglutination as the washed membranes are but slightly agglutinated.

10. Motile cultures of *B. typhosus* grown at 37° C., and non-motile cultures grown at 42° C., agglutinated equally well, the agglutinable substance probably being unchanged.

11. The law governing the fixation in *B. typhosus*, heated to 58° C. for 30 minutes, is probably different to that holding for living cultures owing to modification of the agglutinable substance.

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