On the Physical Chemistry of the Toxin-Antitoxin Reaction: with Special Reference to the Neutralisation of Lysin by Antilysin.

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Ehrlich (1898) (1903) came to the conclusion that the toxin secreted by *B. diphtheriæ* is neutralised by diphtheria antitoxin much as an acid is by a base. The course of the neutralisation seems to him to indicate the presence of several toxic substances and atoxic forms of the same substances in the toxic fluid which are successively neutralised by the gradual addition of antitoxin. After complete neutralisation of the various toxins, a substance—toxone—remains which has the property of causing diphtheritic paralysis, and also of neutralising antitoxin. Similar constitutions have been ascribed by Ehrlich and his pupils to other toxic fluids, and to the hæmolytic filtrates or lysins obtained from certain bacterial cultures.

On the other hand, Arrhenius and Madsen (1902) (1904) concluded that the toxin-antitoxin reaction is quite analogous to the action of an acid on an alcohol, and that the chemical laws of mass action, which hold for the latter, apply equally well to the former. The chief reaction is considered to be a reversible one between two substances only, toxin and antitoxin, and when the system has reached equilibrium, a fraction of the toxin and also of the antitoxin remain free. The toxone effect they ascribe to a trace of free toxin. The numerical relations deduced are approximately in agreement with the experimental observations they have made on equilibria obtaining between toxins and antitoxins, and likewise between lysins and antilysins. Nernst (1904) has, however, pointed out that the laws of mass action are not applicable to these reactions.

Bordet (1903) expressed the view that the fixation of toxin by antitoxin is similar to the fixation of a dye by a tissue, and the author has shown that this conception is consonant with the chemical and physical properties of antibodies in general (1905).

The two substances most thoroughly investigated by Arrhenius and Madsen, viz., diphtheria toxin and tetanus lysin, do not admit of exact determination. The estimation of the free diphtheria toxin is rendered uncertain by reliance on animal experiments, and tetano-lysin is itself a most unstable body. Todd (1902), however, discovered a relatively stable lysin in the filtrate

from cultures of *B. megatherium*, and succeeded in producing a very strong antilysin for the same. He demonstrated that, in constitution, this lysin resembled tetano-lysin and diphtheria toxin. I, therefore, determined to test in how far the various views were applicable to the relations existing between Megatherium lysin and antilysin. Dr. G. Dean kindly placed the antilysin prepared by Dr. Todd at my disposal. I here beg to express my deep sense of indebtedness to Dr. G. Dean for his kindly criticism and many suggestions, and to Dr. C. J. Martin for his counsel in the filtration experiments.

On the hæmolytic index employed.—That the hæmolytic effect is always proportional to the amount of lysin added seems to be more than doubtful, consequently the calculated concentrations of free lysin have not been given.

However, when the hæmolytic powers of two solutions of the same lysin in the presence of a considerable concentration of antilysin are not widely different, the powers are approximately proportional to the amounts of free lysin present. The hæmolytic power of a fluid was determined by adding 1 c.c. to 2 c.c. of a 2·5-per-cent. suspension of washed guinea-pig corpuscles in 0·8-per-cent. saline, and heating the mixture to 37° C. for three hours.

The contents were subsequently allowed to stand in the ice-chest until the corpuscles had sedimented sufficiently to allow of 1 c.c. of the supernatant fluid being removed. The intensity of colour of the fluid was then compared with that of the tinted scale of a von Fleischl's hæmoglobinometer. By using a disc of paper stained with potassium chromate, instead of the usual white illumination, the contrast in colour between hæmoglobin and scale is much diminished, and daylight may be used for the comparison. Only that portion of the scale between the numbers 30 and 70 was used, and when the fluid was strongly coloured, dilutions were made so as to bring the final tint within these limits. The scale was standardised by various dilutions of the fluid obtained when 1 c.c. of lysin completely hæmolysed 2 c.c. of 2·5-per-cent. corpuscles in saline. The intensity of tint of this fluid has been represented by the index 100, and the hæmolytic indices given below refer to this tint as a standard. The experimental error in determining the tint was found to be less than 1 per cent. of the standard tint.

In the following a "partially neutralised" mixture of lysin and antilysin is a fluid giving a marked hæmolysis under standard conditions; a "neutral" mixture gives no hæmolysis in three hours, but would hæmolyse slightly in four hours; an "over-neutralised" mixture contains a greater proportion of antilysin than a neutral mixture.

On the presence of Free Lysin and Free Antilysin in mixtures which have attained equilibrium. Method: Filtration through Gelatine.

The method employed was that of C. J. Martin (1896), and applied by Martin and Cherry (1898), in their investigation of the relations existing between diphtheria toxin and antitoxin. The fluids examined were forced by a constant air-pressure of 100 atmospheres through Pasteur-Chamberland filters filled with solid gelatine, and each filtrate was removed in successive fractions of about 4 c.c. The hæmolytic powers of these fractions of filtrates, of the residual fluid left in the filter case, and of the original fluid introduced, were then determined in the manner described above.

Lysin.—On filtering a weak lysin solution through a filter prepared from 15-per-cent. gelatine, the filtrate gave little or no hæmolytic effect, but on diminishing the concentration of gelatine to 9 per cent., and using a stronger lysin, the filtrates obtained were strongly hæmolytic. The first few fractions had practically no action on blood corpuscles, and the succeeding fractions increased at first rapidly in hæmolytic power and then more slowly until a fairly constant value was reached, which approximated to that of the original lysin introduced. The gelatine, also, proved to be intensely hæmolytic, whereas the original gelatine had no effect on red-blood corpuscles. Candles prepared from 15-per-cent., 11-per-cent., 9-per-cent., and 7·5-per-cent. gelatine were also used, and the residual fluids showed in all cases a greater hæmolytic power than the original lysin, the difference being more marked with the higher percentages of gelatine. The gelatine filter is evidently more permeable to water than to the lysin. Similar concentration relations were found to hold for the filtration of crystalloids and inferior colloids, but the filter showed considerable differences in permeability to various crystalloidal substances.*

Antilysin.—On filtering a 5-per-cent. solution of the antilysin in saline no trace of anti-hæmolytic action could be detected in the filtrates. In this respect the antilysin behaves like a typical colloid, *e.g.*, colloidal ferric hydrate was found even in concentrated solutions to be almost entirely retained by the gelatine on filtering under *constant* pressure. It is important to observe that if the pressure be *suddenly diminished* the concentrated contents of the gelatine, whether crystalloidal or colloidal, are swept into the filtrate. The filter showed considerable permeability to typical colloids, especially those which stained the gelatine, but on the whole retained this class of substances to a greater extent than crystalloids.

* *Cf.* also E. W. Reid, 1901.

Mixtures.—The mixtures of lysin and antilysin were brought to a state of equilibrium by heating at 37° C. before filtering. They embraced weakly hæmolytic fluids (Nos. 1, 2, and 3, Table I), neutral fluids, *i.e.*, mixtures which did not hæmolyse in the standard time (No. 4), and fluids containing a large excess of antilysin (Nos. 5 and 6).

Nos. 1, 2, and 3 were exactly of the same constitution, viz., equal volumes of a lysin of constant value and a 5-per-cent. solution of antilysin in saline. No. 1 was heated 1 hour at 37° C., and kept at 10° C. for 1 hour. Nos. 2 and 3 were heated for 3 hours at 37° C., and allowed to stand 18 hours at 10° C.*

No. 4 consisted of equal volumes of a 1-per-cent. solution of a lysin which had been precipitated by ammonium sulphate, and of a 5-per-cent. solution of antilysin in saline. No. 5 contained one volume of the 1-per-cent. lysin to two volumes of 5-per-cent. antilysin, and No. 6, one volume of 1-per-cent. lysin to four volumes of 5-per-cent. antilysin. Nos. 4, 5, and 6 were heated for 3 hours at 37° C., cooled 1 hour at 10° C. and filtered.

Examination of the Filtrates for the Presence of Lysin.—The last fractions of the filtrate from No. 1 indicated a trace of hæmolysis, the corresponding fractions from Nos. 2 and 3 were unquestionably hæmolytic.

The filtrates from Nos. 4, 5, and 6, as well as the original fluids introduced, did not produce hæmolysis.

Examination of the Gelatine for the Presence of Lysin.—After filtration the *gelatine* of the filters 1 to 6 was melted out at 37° C., and *in all cases was found to be intensely hæmolytic*, whereas the original gelatine had no hæmolytic effect in the standard time.

Examination of the Residual Fluids for the Presence of Lysin.—The residual fluids were in all cases decidedly hæmolytic, as can be seen in Table I. *This increment in hæmolytic power was to be expected for Nos.* 1, 2, *and* 3 *from the results given above for the filtration of lysin alone*, and is simply a concentration effect that might be brought about by removing the water in other ways, *e.g.*, by evaporation under diminished pressure. The result, however, appears to be most remarkable when it is considered that No. 4 is a *neutral mixture*, and Nos. 5 and 6 *highly over-neutralised.*

Control filtrations of saline showed no hæmolytic power in either filtrate, residue, original fluid, or gelatine, so that the hæmolysis obtained above was certainly not due to impurities introduced by the apparatus.

* The experiment with No. 3 was performed 30 days after the experiments with Nos. 1 and 2 and the agreement in the hæmolytic values obtained testifies to the constancy of the lysin.

Table I.

Fluids mixed	Fluid lysin K_{XII}. Antilysin 5 per cent. in saline.						Precipitated lysin 1 per cent. in saline. Antilysin 5 per cent. in saline.							
No. of experiment	1		2		3		4		4A		5		6	
Volume ratio of lysin to antilysin	1 : 1		1 : 1		1 : 1		1 : 1		—		1 : 2		1 : 4	
Temperature and time of contact	37° 1 hr. 10° 1 hr.		37° 3 hrs. 10° 18 hrs.		37° 3 hrs. 12° 18 hrs.		37° 3 hrs. 10° 1 hr.		37° 2½ hrs. 10° 1 hr.		37° 3 hrs. 10° 1 hr.		37° 3 hrs. 10° 1 hr.	
	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.	Vol. in c.c.	Hæm. index.
Last fraction of filtrate	5	1·0	4	7·7	16	4·5	4	0	4	0	4	0	4	0
Total filtrate	75	0·5	63	6·3	72	2·8	54	0	62	0	48	0	35	0
Residue	40	38·6	35	41·3	37	38·2	33	31·2	33	27·8	42	21·6	15	20·1
Gelatine	—	100	—	100	—	100	—	100	—	100	—	100	—	100
Original mixture	120	16·8	105	24·3	112	16·8	98	0	100	0	100	0	62·5	0

The hæmolytic power of the residues and the gelatine could be demonstrated to be due to free megatherium lysin, for on the addition of sufficient antilysin *the hæmolytic power was in all cases entirely neutralised.* For example, 1 c.c. of residue No. 1 of index (38·6) with 1 c.c. of 5-per-cent. antilysin, added directly to the test blood, gave a hæmolytic index of (8·4), the same mixture heated for one hour at 37° C. before adding to the test blood, gave as index (1·8). Again, 0·5 c.c. of gelatine from No. 1 caused complete hæmolysis (index 100), whereas, after being heated for 1 hour at 37° C. with 1 c.c. of 5-per-cent. antilysin the value was reduced to (25). Free lysin, then, exists in the residues, and since the filtration was completed in less than two hours at a temperature of 10° C., at which temperature the velocity of reaction is extremely low, there can have been no appreciable liberation or dissociation of lysin during the filtration. It follows, therefore, that *free lysin exists in neutral and highly over-neutralised mixtures of lysin with antilysin, and that the free lysin is partially removed during filtration.*

The Existence of Free Antilysin in Partially neutralised and Neutral Mixtures.

The residual fluids from Experiments 1 to 6 showed slightly smaller hæmolytic indices after standing over night at 10° C., and on heating them to 37° C. for 1 hour their hæmolytic powers further markedly decreased. This is clearly shown by the following experiment:—

1 c.c. of residue No. 1 (index 38·6) together with 1 c.c. of saline, when added directly to the test blood, gave a hæmolytic index of (35), whereas the same mixture heated for one hour at 37° C. before being added to the test blood, gave an index of only (23·4). This behaviour indicates that in Nos. 5 and 6, the filtration which causes an increase in the concentration of any free lysin or antilysin by the withdrawal of water, occasions a further reaction which has a low velocity at 10° C., but is considerably more rapid at 37° C. As this phenomenon is common to all the residues it follows that if the above interpretation be correct, free antilysin is present in all cases, and presumably must have been present in the original mixtures. Nos. 1, 2, and 3 were, however, hæmolytic mixtures, and No. 4 neutral, which points to the conclusion that *free antilysin exists in partially neutralised and neutral mixtures* as well as in over-neutralised mixtures.

This conclusion is strengthened by the high neutralising power possessed by the original mixture and by the residue of No. 3, when fresh lysin was added, as shown by the following experiments:—

To one series of tubes containing 1 c.c. of the original hæmolytic mixture

in equilibrium, gradually increasing quantities of lysin were added. The same quantities of lysin were added to a second series containing 1 c.c. of the residue of No. 3, and to a third series containing 1 c.c. of saline. The test blood was added to each and, after mixing, the tubes were placed at 37° C. for 3 hours. The hæmolytic indices obtained are shown in Table II.

Table II.

Lysin K_{XII} added, in c.c.	Hæmolytic indices.				
	Saline.	Original mixture, No. 3.	Residual fluid, No. 3.	Original mixture increments.	Residual fluid increments.
0	0	25	48·2	—	—
0·0001	0	25	48·2	0	0
0·001	0	25	48·2	0	0
0·01	2·3	25	48·2	0	0
0·1	100	33·2	55·0	8·2	6·8
0·2	100	41·4	59·1	16·4	10·9
0·3	100	50·9	63·2	25·9	15·0
0·4	100	59·1	67·2	34·1	19·0

Obviously the original mixture has neutralised a very considerable proportion of the added lysin, for 0·1 c.c. of the lysin causes complete hæmolysis in saline (index 100), whereas 0·3 c.c. in the mixture only produces half the effect (index 50·9). The residue, although itself more hæmolytic than the original mixture, shows a higher neutralising value than the latter, as may be seen from the ratio of the increments, Table II. If the hæmolytic indices of mixtures containing a high concentration of antilysin such as those investigated above, are approximate measures of the free lysin present, then, for both original and residue, the amount of added lysin left free is roughly proportional to the lysin added. The amount of added lysin left free by the original is approximately double that left free by the residue, which agrees with the conclusion that the free antilysin in the latter is present in greater concentration than in the former.

On the Reversibility of the Reaction.—The reversibility of the reaction will be demonstrated if, on removing the free lysin, the compound of lysin with antilysin dissociates, producing more free lysin; on the other hand, if no lysin be set free, the reaction is irreversible. In the case of the reaction

being reversible, the velocity of dissociation will probably increase rapidly with rise of temperature. A temperature of 37° C. was employed as more likely to show reversibility if such exists.

The methods employed were: (*a*) filtration through gelatine; (*b*) diffusion through gelatine.

(a) *Filtration through Gelatine.*—The practically constant concentration of free lysin in the residues of Experiments Nos. 5 and 6, Table I, might well be due to reversibility. To test this the residue of No. 4 (index 31·25) was diluted with saline to the volume of the original mixture, and heated for $2\frac{1}{2}$ hours at 37° C. On again filtering through gelatine until the new residue had the same volume as the first residue, the hæmolytic index was found to be (27·8).* On repeating this treatment the hæmolytic index was found within the experimental error unchanged. The gelatine obtained from the filters used was strongly hæmolytic (index 100). If the free lysin of the first residue had alone been available, viz., $33 \times 31{\cdot}25 = 1031$ "hæmolytic units," then as filtration removes 300 or more "units," the second residue could not have had a higher index than $\frac{1031-300}{32{\cdot}5}$ $= (22{\cdot}5)$ and the third residue (13). It would appear then that a portion of the apparently combined lysin has become free on heating at 37° C.

I conclude, therefore, that *in a neutral mixture the reaction is at* 37° *C. at least partially reversible.*

(b) *Experiments on the Diffusion through Gelatine of Lysin and Antilysin.*—Dr. G. Dean kindly suggested to me a suspension of red blood corpuscles in gelatine as a convenient indication of the diffusion of lysin. In applying this method I found it was necessary to allow the lysin to diffuse from a solid gelatine layer into the solid gelatine suspension, for when normal saline alone is placed above a 2·5-per-cent. suspension of corpuscles in gelatine (9 per cent.), hæmolysis occurs. This proceeds from the surface downwards at a rate which is not altered when a strong fluid lysin is used instead of the saline. To avoid this effect, which is probably an imbibition phenomenon, the precipitated lysin was dissolved in 9-per-cent. gelatine at about 37°, and poured on to the strongly cooled columns of 9-per-cent. gelatine containing corpuscles. A rate of hæmolysis was then obtained which decreased with decreasing concentrations of lysin, and was entirely absent when saline in gelatine was used. The course of the diffusion is evidenced by a zone of transparent gelatine coloured with the freed hæmoglobin and a second zone in which partial hæmolysis has taken place, which

* *Cf.* Table I, No. 4A.

latter has a fairly sharp boundary against the unhæmolysed and turbid suspension. The experiments were carried out at about 18° C. The gelatine suspensions were contained in tubes of 1 cm. diameter, and were 10 cm. in length. The test volume of supernatant fluid was 1 c.c.

Lysin.—The rate at which hæmolysis proceeded from the surface decreased when the concentration of the lysin decreased from 1 to 0·0625 per cent., but lower concentrations down to 0·0078 per cent. gave almost the same effect as 0·0625 per cent. *For low concentrations, then, the rate of the hæmolytic effect is not dependent on the mass of the lysin.* Controls with saline gave no trace of hæmolysis.*

Antilysin.—A layer of the gelatine blood suspension 2 mm. thick was placed on a gelatine column containing 10 per cent. antilysin, and after standing for 40 hours at 18° C., a gelatine layer containing 0·5 per cent. lysin was superimposed. The blood was completely hæmolysed in 40 hours, whereas in a control with gelatine saline no hæmolysis took place. When, however, antilysin was mixed with the gelatine suspension, so that the concentration was 2·5 per cent., it was found that a gelatine layer containing 0·5 per cent. lysin failed to give a trace of hæmolysis in 40 hours. It follows, therefore, that the antilysin in the first experiment could not have been present in a concentration of 2·5 per cent. even in the layer immediately in contact with the 10-per-cent. antilysin in gelatine.

Again, as 0·5 per cent. lysin hæmolyses 2 mm. in 40 hours under normal conditions, no retardation has taken place, and it is probable that *megatherium antilysin does not diffuse appreciably through gelatine.*

Mixtures.—*A neutral mixture*, containing 0·5 per cent. precipitated lysin and 3·73 per cent. antilysin in saline gelatine (Table IV, No. 2), *and an over-neutralised mixture*, No. 1, containing 0·5 per cent. lysin and 5 per cent. antilysin, after being brought to equilibrium, *showed the presence of at least a trace of free lysin.* The hæmolytic effects were equal and of the magnitude for which

Table III.—Diffusion of Megatherium Lysin through Gelatine Columns, showing Hæmolysis, in Millimetres.

Percentage of lysin	1	0·5	0·25	0·125	0·0625	0·0317	0·0159	0·0078	Saline controls.
40 hrs.	2·6	2·0	1·7	1·4	1·0	1·0	1·0	1·0	0·0
70 „	4·5	3·5	2·2	1·5	1·0	1·0	1·0	1·0	0·0

* Compare Table III.

Table IV.—Diffusion of Lysin (0·5 Per Cent.) with Antilysin after attaining Equilibrium, through Gelatine Columns, showing Hæmolysis, in Millimetres.

Percentage of antilysin	No. 1. 5	No. 2. 3·73	No. 3. 2·5	No. 4. 1·25	No. 5. 0·625	No. 6. 0·317	Saline controls.
40 hrs.	tr.	tr.	tr.	tr.	1·4	2·0	0
70 „	1·0	1·0	1·0	1·0	1·5	3·5	0

no proportionality had been found to exist between mass of lysin and effect. It is remarkable that although in 70 hours this effect was evident, yet in less than 40 hours the corresponding free lysin had a greater hæmolytic effect. This seems most easily explained on Ehrlich's view that *lysins are complex and that antilysin will neutralise the most active constituents first.* The same effects were obtained from mixtures of lysin and antilysin in the same relative proportions, but present in $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$th of the concentration obtaining in the above experiment. Controls with saline showed no effect. The partially neutralised mixture containing 0·5 per cent. lysin and 2·5 per cent. antilysin No. 3, gave the same effect as the neutral mixture and likewise a mixture of 0·5 per cent. lysin and 1·25 per cent. antilysin, No. 4, but when the antilysin concentration was reduced to 0·625 per cent. (No. 5), the effect indicated a concentration equal to 0·125 per cent. free lysin. With 0·3175 per cent. antilysin (No. 6), and less the effect indicated 0·5 per cent. lysin free.

These results indicate that *the addition of antilysin up to* 1/10*th the amount required to entirely prevent hæmolysis* at 37° in the standard time (neutral mixture) *does not appreciably neutralise the lysin.* That the combination of lysin with antilysin is easily reversible at 18° C., does not seem probable, for on the addition of double the amount of antilysin the free lysin decreases to less than 1/4th of the total amount added. This then apparently conforms well with Ehrlich's view that no neutralisation of toxin takes place when small quantities of antitoxin are added.

On the Nature of the Equilibria: Fractional Addition of Lysin to Antilysin.

Danysz (1902) found that when a certain quantity of diphtheria toxin was added in fractions to antitoxin, less toxin was neutralised than in the case in which the whole quantity was added in one portion. This result has been confirmed by von Dungern (1904). Dr. C. Todd first drew my

attention to the fact that false equilibria were attained on the fractional addition of megatherium lysin to antilysin (experiments unpublished).

On further investigation I found, however, that the nature of the equilibria obtained in partially neutralised and in neutral mixtures is not the same. This may be seen from the following experiments :—

One c.c. of 0·625 per cent. antilysin was placed in each of two series of tubes. To one series 1 c.c. of various dilutions of lysin was added, and the mixture heated for two hours at 37° C. To the other series 0·5 c.c. of the same dilution of lysin was added, and the mixtures heated for one hour at 37° C. Another similar addition of 0·5 c.c. was then made, and the mixtures heated one hour at 37° C. Table V shows that the mixtures

Table V.

Addition at once.			Fractional addition.				
2 hrs. at 37°.			Part I, 1 hr. at 37°.		Part II, 1 hr. at 37°.		
Added to 1 c.c., 0·625 per cent. antilysin.					Added to Part I.		
Lysin in c.c.	Saline in c.c.	Hæm. index.	Lysin in c.c.	Saline in c.c.	Lysin in c.c.	Saline in c.c.	Hæm. index.
0·1	0·9	18·3	—	—	—	—	—
0·2	0·8	20·8	0·1	0·4	0·1	0·4	18·3
0·3	0·7	24·6	—	—	—	—	—
0·4	0·6	31·7	0·2	0·3	0·2	0·3	32·1
0·5	0·5	39·2	—	—	—	—	—
0·6	0·4	48·3	0·3	0·2	0·3	0·2	60·0
0·7	0·3	71·2	—	—	—	—	—
0·8	0·2	91·7	0·4	0·1	0·4	0·1	100
0·9	0·1	98	—	—	—	—	—
1·0	0·0	100	0·5	0·0	0·5	0·0	100

which were made in this intermittent way have higher hæmolytic indices than the mixtures which were made at one operation. The above was only found to be true when the resulting mixtures were strongly hæmolytic. When, however, the mixtures contained considerably less free lysin, the hæmolytic indices were the same in both series. From this and similar experiments, in which the volume of the antilysin was not appreciably modified by the addition of the lysin in concentrated form, it follows that false equilibria are attained in the lysin-antilysin reaction when the final concentration of the free lysin is high. In more nearly neutral mixtures

an apparently true equilibrium results. From Table V the irreversibility in nearly neutral solutions gives a slight effect in the opposite direction to the effect obtained by Danysz and von Dungern.

On the Mass Action of Lysin and Antilysin.

If the equation, toxin + antitoxin = 2 (toxin, antitoxin), proposed by Arrhenius and Madsen, holds for lysin and antilysin, then, when the same relative concentrations of these bodies are brought together, the equilibria attained will be the same, *i.e.*, the relative concentrations of the substances will be independent of the volume of the mixture. Thus, 10 c.c. of a mixture of lysin and antilysin in equilibrium will contain as much free lysin as 1 c.c. of a mixture 10 times as strong in total lysin and antilysin.

In Table VI (*a* 1, 2) and (*a* 5, 6) are such mixtures which were brought to equilibrium at 37°, the test volume (3) of the first mixture was 1 c.c., and of the other 0·1 c.c., added with 0·9 c.c. saline (7). The hæmolytic indices (4) (8) are not equal, being respectively (50) and (60).

Table VI.—Hæmolytic Indices of Multiple Mixtures of Lysin and Antilysin in Equilibrium.

	1.	2.	3.	4.	5.	6.	7.	8.
	Lysin conc. per c.c.	Antilysin conc. per c.c.	Test volume in c.c.	Hæm. index.	Lysin conc. per c.c.	Antilysin conc. per c.c.	Test volume in 1 c.c.	Hæm. index.
a	0·005	0·01	1·0	50	0·05	0·1	0·1	60
b	0·0025	0·005	1·0	26	0·025	0·05	0·1	50
c	0·0005	0·001	1·0	0	0·05	0·1	0·01	42·5
d	0·00025	0·0005	1·0	0	0·025	0·05	0·01	41

Two similarly related mixtures of half the strength (*b* 1, 2) and (*b* 5, 6) showed a more marked difference, viz. (26) and (50). When the strength of the mixtures were widely different as 1—100, the difference is extremely marked, *e.g.*, (*c* 1, 2) and (*c* 5, 6) gave the indices (0) and (42·5) when 1 c.c. and 0·01 c.c. with 0·99 c.c. saline were used as the test volume respectively. Two similarly related mixtures of half the strength (*d* 1, 2) and (*d* 5, 6) showed a similar effect and indices (0) and (41).

It must then be concluded that Arrhenius and Madsen's equation does not apply to the lysin-antilysin reaction so far as megatherium lysin is concerned.

The Validity of the Application of Chemical Mass Action Equations to the Lysin-Antilysin Action.

Nernst (1904) has insisted that the views of Arrhenius and Madsen on the mass action of toxin and antitoxin can have no true theoretical foundation. If the reaction consist in a chemical change, it seems to me that the chemical law of mass action would be applicable in the manner given below. If, on the other hand, the observed mass action differs widely from the deduced, we can conclude that the toxin-antitoxin reaction is not a purely chemical one.

The anti-body, *e.g.*, antilysin, is a typical colloid, and its active chemical mass will, like that of a solid suspension, be constant. Similarly, the chemically active mass of the compound between antilysin and lysin is constant, as this also occurs in the form of a fine suspension. The active mass of the lysin will vary with its concentration, as this substance is not present in the form of a typical colloid. It has properties such as diffusibility, and the power of passing a gelatine filter similar in magnitude to those found for crystalloids and inferior colloids.

These conditions are not unlike those studied by Walker and Appleyard (1896) in the case of a solid suspension of diphenylamine in picric acid solutions. If this analogy is a true one, the chemical equation expressing the combination of lysin with antilysin should be similar to that given by these authors for the combination of picric acid with diphenylamine, viz., lysin, water + antilysin = compound + water.

The lysin is removed by the solid suspension of antilysin, and a solid suspension of compound (lysin, antilysin) is produced with the liberation of the water which held the lysin in solution. The velocity of combination will be K_1CK' where K_1 is the velocity constant, C the concentration of the lysin, and K' the constant active mass of the antilysin. When the reaction is reversible, *i.e.*, possibly at high concentrations of antilysin, the velocity of dissociation will be $K_2K''K'''$ where K_2 is the velocity constant, K'' the constant active mass of the compound, and K''' the constant active mass of the water. For equilibrium $K_1CK' = K_2K''K'''$, or

$$C = \frac{K_2K''K'''}{K_1K'} = \text{constant.}$$

From which it follows that at any definite temperature the concentration of the lysin must be a constant. The compound (lysin, antilysin) and free antilysin can only exist side by side in the aqueous medium when the latter has a certain fixed absolute concentration. The relative concentrations of

free antilysin and compound have no influence on the equilibrium. Thus Walker and Appleyard found that the concentration of aqueous picric acid in contact with varying amounts of diphenylamin is constant, but that the latter was stained more deeply when present in smaller quantities. The relations observed between lysin and antilysin are, however, totally different, for in this case the amount combined varies continuously with the concentration of the lysin.

I therefore conclude that *the removal of lysin from a solution by antilysin is not capable of interpretation as a purely chemical change, and the law of chemical mass action does not apply when the lysin is present in excess.*

From the filtration experiments with neutral and overneutralised mixtures it, however, seems possible that when excess of antilysin is present, the chemical law of mass action holds, for the concentration of free lysin was found to be practically constant when the concentration of antilysin was largely increased.

The application of Adsorption and Surface Tension hypotheses to the Lysin-Antilysin Action.

Walker and Appleyard also investigated the phenomena of the fixation of picric acid by silk, to which the relations of lysin to antilysin, which have been described above, present a much closer analogy. They found that the concentration of the dye bath varies continuously with the depth to which the silk is dyed, and that the concentration of picric acid (C_2) in the silk was proportional to the concentration of free picric acid (C_1) raised to a constant power n, or $C_2 = KC_1^n$. The adsorption of substances, *e.g.*, of iodine from solutions by charcoal (Schmidt, 1894); of iodine from water by starch (Küster, 1894), etc., have in general been found to obey similar relations. It is, then, not improbable that some such adsorption formula may be found to hold for the fixation of lysins, etc., by their respective antibodies, as I suggested in a recent paper on the phenomena of agglutination (1905). I am at present engaged in further investigating the phenomena of lysin-antilysin relations from this point of view, and, so far, the results have been encouraging.

Summary of Conclusions.

1. Megatherium lysin passed through a gelatine filter, and is diffusible through gelatine.

2. Megatherium antilysin does not pass through a gelatine filter, and is not appreciably diffusible through gelatine.

3. The filtration and diffusion of mixtures show that free lysin is present in neutral mixtures and in mixtures containing excess of antilysin.

4. Free antilysin exists in neutral mixtures, and in mixtures containing excess of lysin.

5. The reaction is at least partially reversible when excess of antilysin is present.

6. False equilibria are produced with greater facility when the lysin is in excess.

7. The neutralisation equation of Arrhenius and Madsen does not hold for multiple mixtures.

8. The removal of lysin from a solution by antilysin is not capable of interpretation as a purely chemical change, but is more analogous to certain adsorption phenomena.

REFERENCES.

Arrhenius and Madsen (1902). 'Festskrift ved Indvielsen af Statens Seruminstitut,' III.
Arrhenius and Madsen (1904). 'Bulletin de l'academie royale des sciences de Danemark,' p. 271.
Bordet (1903). 'Annales de l'Institut Pasteur,' vol. 17, p. 161.
Craw (1905). 'Journal of Hygiene,' vol. 5, p. 115.
Danysz (1902). 'Annales de l'Institut Pasteur,' vol. 16, p. 331.
Von Dungern (1904). 'Deutsche Med. Wochenschrift,' vol. 30, pp. 275, 310.
Ehrlich (1898). 'Deutsche Med. Wochenschrift,' vol. 24, p. 597.
Ehrlich (1903). 'Berliner Klin. Wochenschrift,' vol. 40, pp. 793, 825, 848.
Küster (1894). 'Liebig's Annalen der Chemie,' vol. 283, p. 360.
Martin, C. J. (1896). 'Journ. of Physiol.,' vol. 20, p. 364.
Martin and Cherry (1898). 'Roy. Soc. Proc.,' vol. 63, p. 420.
Nernst (1904). 'Zeitschrift für Elektrochemie,' vol. 10, p. 377.
Reid, E. W. (1901—2). 'Journal of Physiology,' vol. 27, p. 161.
Schmidt (1894). 'Zeitschrift für Physikalische Chemie,' vol. 15, p. 56.
Todd (1902). 'Trans. of the Pathological Society of London,' vol. 53, Part II, p. 196.
Walker and Appleyard (1896). 'Journal of the Chemical Society,' vol. 69, p. 1334.