ON THE DANYSZ EFFECT WITH REFERENCE TO THE TOXIN-ANTITOXIN REACTION.

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The investigation of the toxin-antitoxin reaction has been pursued during the past decade with great vigour, but the views advanced to account for the observations are, at present, highly divergent. It seems to me to be desirable, therefore, to review some recent and apparently important data, collected by Madsen and Walbum and calculated by Madsen and Arrhenius (1906) (1907), which bear directly on these interpretations.

One of the fundamental facts is that the reaction between toxin and antitoxin is practically independent of biological influences; in other words the interaction may, in many cases, be studied *in vitro*.

Ehrlich (1897) demonstrated this by experiments *in vivo* and *in vitro* for ricin and antiricin and on this basis, after numerous investigations of allied reactions, evolved his well-known "Side-Chain Hypothesis" as well as his "Spectra" for the constitution of toxins, *e.g.* of diphtheria toxin.

The striking and helpful "Side-Chain Hypothesis" is, as yet, without any worthy competitor, but Arrhenius and Madsen (1902) and Bordet (1903) and Landsteiner (1903) have advanced views that cast a new light upon the constitution of many of the best investigated toxins. Of the methods which have been acknowledged to be available in the attempt to arrive at a decision with regard to the relative value of the views advanced, that based upon the "Danysz Effect" seems to be one of the most important.

Danysz (1902) found that when ricin or diphtheria toxin was brought into contact with its corresponding antibody the degree of neutralisation depended upon the method adopted in preparing the mixtures, in the sense, that when the toxin was added to the antitoxin

in two fractions, a considerable time being allowed to elapse between the additions, the resultant mixture contained a much larger amount of free toxin than in the case when the total quantity of toxin was added at once to the antitoxin.

v. Dungern (1904) confirmed this result for diphtheria toxin and antitoxin, and attributed it to the action of a hitherto unknown substance in the toxin, viz. epitoxonoid, a view subsequently accepted by Ehrlich.

Sachs (1904) found similar relations to hold between tetanolysin, rennin and their corresponding antibodies, but not between cobra venom and its antivenene.

Craw (1905, III.) observed the "Danysz Effect" in mixtures of megatheriolysin and antilysin, and experimenting with "nearly neutral" fluids, *i.e.* such as had but a slight haemolytic effect, found the "Effect" greater with larger quantities of lysin.

Madsen and Arrhenius (1906) (1907) have given a very condensed account of their work and that of Walbum upon this theoretically very important phenomenon. They find the "Effect" greater when the antilysin is "in excess," but this difference seems to be less due to contradictory results than to our diverse definitions of a "neutral mixture" and to the nature of the materials studied. Madsen and Arrhenius ascribe the "Effect" to the production and presence of a modified antitoxin. It seems to me doubtful whether it is necessary to assume either a new constituent in the toxin or in the antitoxin; further, neither of these assumptions appears to me to be satisfactory, for the reasons given below.

In the first place the material, tetanolysin, used by Madsen and Arrhenius is unsuitable. Madsen (1899) himself showed that the haemolytic power of a 4 % solution kept at 20° C. for five hours diminished by 50 %. At 37° C. this effect is much greater amounting to 25 % in one hour, as I have found on examining various brews.

In these experiments we are, therefore, dealing with an unnecessarily complicated phenomenon—(1) the deterioration of tetanolysin, and (2) the true "Danysz Effect." It would seem then to be impossible, from experiments with tetanolysin, to arrive at a general interpretation of the "Danysz Effect," applicable, for example, to diphtheria toxin, by the simple procedure adopted by Arrhenius in his calculations. The basis upon which the equivalence of the lysin and antilysin has been estimated is still open to many of the grave doubts advanced by Nernst (1904) and Craw (1905, I.) (1905, III.).

The purely arbitrary assumption is made that one "molecule" of tetanolysin combines with 1 "molecule" of antilysin to produce 2 "molecules" of compound; the formula used in the interpretation of the experimental results was, namely,

$$\frac{1}{T_0}\left\{np\frac{1}{T}-\left(\frac{1}{T}-\frac{1}{T_0}\right)\right\}=K\left(\frac{1}{T}-\frac{1}{T_0}\right)^2,$$

where T_0 represents the original or total amount of toxin and T the amount left free, n the number of c.c. of antitoxin, p a constant indicating the ratio between units of toxin and antitoxin and K a supposed equilibrium constant.

Now, as has been pointed out by Nernst (1904), the use of two constants p and K in this equation practically reduces it to an interpolation formula, and this seems to me to be confirmed by the fact that in the calculations made on other nearly related reactions in immunity Arrhenius and Madsen have been compelled to modify the power to which the right hand member of the equation is raised.

That the equivalents between toxin and antitoxin so deduced have no relation to those enunciated by Ehrlich is obvious, from the fact that a mixture of Arrhenius and Madsen's equivalents has a toxicity equal to 23·7 % of that of the original toxin; such a mixture can only be described as "neutral" when all their assumptions are supposed to be correct. The definition of a "neutral mixture" is obviously purely arbitrary, and, in deference to the methods of Ehrlich, in my paper (1905, III.) on the toxin-antitoxin reaction, I defined a "neutral mixture" of megatheriolysin and antilysin as one which, after heating for three hours at 37° C., just failed to give a trace of haemolysis on heating for a further two hours under standard test conditions. All the mixtures which I investigated would from the point of view of Arrhenius contain excess of antitoxin. In such mixtures Madsen and Arrhenius find that the "Danysz Effect" increases when the first fraction of toxin is allowed to remain for longer periods, or "reaction times," in contact with the antitoxin, before the addition of the second fraction.

On this doubtful basis Arrhenius has calculated the Danysz Effect, and he makes further assumptions that seem to me even less tenable, as will be shown below. It was found that 0·72 c.c. of tetanus antilysin was "equivalent" to 4 c.c. of a certain brew of tetanolysin, and that an "equilibrium constant" could be deduced. The "Danysz Effect" was then determined by bringing 0·8 c.c. of the antilysin in contact with

4 c.c. of lysin but in two fractions, the first being 1 c.c. and the second 3 c.c. The first fractions in various series of experiments were allowed to stand for different lengths of time at 37° C., the second fractions were then added and the whole heated for 30 minutes at 37° C.

The toxicity of a mixture, made by adding to the antilysin 4 c.c. of lysin at once, was taken as unity and a comparison was made with the haemolytic values of the other mixtures. In the table column (1) gives the time during which the first fraction of lysin was heated with the antilysin, and column (2) the toxicity of the final mixtures. The upper half of the table (A) refers to one brew of lysin and the lower (B) to a second.

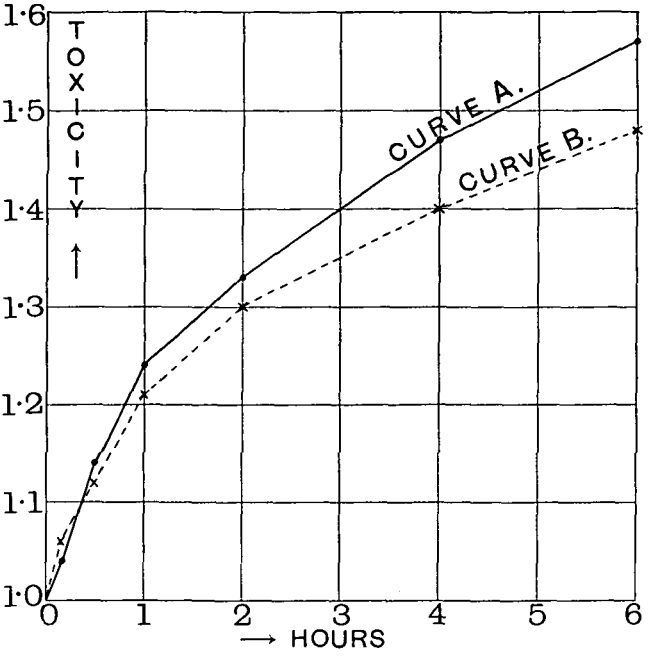
TABLE (A) AND (B).

The Danysz Effect.

	Time	Toxicity	$E_\infty - E$	K	$E_\infty{}^* - E$	$K_1{}^*$	$K_2{}^*$
A.	0·0	1·00	60	—	90	—	—
	0·17	1·04	56	0·180	86	0·115	0·0030
	0·5	1·14	46	0·230	76	0·146	0·0041
	1	1·24	36	0·222	66	0·134	0·0040
	2	1·33	27	0·173	57	0·099	0·0032
	4	1·47	13	0·168	43	0·080	0·0030
	6	1·57	3	0·217	33	0·056	0·0032
	∞	1·60	0	—	—	—	—
	∞ *	1·90	—	—	0	—	—
B.	0·0	1·00	52	—	70	—	—
	0·17	1·06	46	0·318	64	0·228	0·0080
	0·5	1·12	40	0·228	58	0·163	0·0058
	1	1·21	31	0·225	49	0·155	0·0061
	2	1·30	22	0·187	40	0·122	0·0053
	4	1·40	12	0·159	30	0·092	0·0049
	6	1·48	4	0·186	22	0·084	0·0052
	∞	1·52	0	—	—	—	
	∞ *	1·70	—	—	0	—	

It will be observed that the effect of contact-time in the first fraction is very considerable and that with increment of time the phenomenon of Danysz increases, but with longer intervals, such as four hours and six hours, the rate of increase is less than with those below an hour. This will be better realised from the curves shown in the accompanying figure, which I have plotted from Madsen and Arrhenius's data.

INFLUENCE OF CONTACT-TIME.



Curve (A) corresponds to the upper half (A) of the table and curve (B) to the lower half (B), toxicity being represented on the ordinates and time on the abscissae.

Arrhenius assumes that the "Danysz Effect" "appears to tend towards a limiting value," and that "one cannot easily imagine that the toxicity would increase with the time without limit."

These assumptions seem to me to be purely gratuitous, since from the curves which I have plotted the trend might, for lengthened periods, be as well parabolic as hyperbolic. Granting however that there may be a limiting value, is the magnitude chosen by Arrhenius in accord with the experiments quoted? It seems to me that this is not the case. In the table (A) the limiting value (∞) has been "estimated from the experiments" as 1·60, whereas from curve (A) in the figure it seems to me incredible that it would suddenly become parallel to the abscissa after say seven hours. Again in table (B) the limit (∞) is taken as 1·52, but from curve (B) no such value is warranted. It is true that these selected values agree best with Madsen and Arrhenius's theoretical views, expressed subsequently, viz., that the "Effect" obeys the law of

a monomolecular reaction, but the question is whether the data that they have furnished us with do not point to some quite other conclusion. It seems to me that this is so, for if a limit exist to curve (A) it would by graphical interpolation lie in the neighbourhood of 1·90, indicated in table (A) by ∞^*, and for curve (B) it would be nearly 1·70; using these values it becomes apparent on calculation by the method adopted by Arrhenius that the monomolecular formula does not hold.

In the third column of the table the differences between Arrhenius's limiting "Effect" E_∞ and the "Effect" E at any particular time are reproduced—the value being multiplied by 100; in the fourth column the values of K represent the constant obtained by manipulating the differences, $E_\infty - E$, as if a monomolecular reaction were being dealt with. The values of K do not appear to me as showing any remarkable constancy; further the first value in table (A), column (2) is too low and that of table (B) too high to render the curves smooth. If an intermediate value be chosen, *e.g.* 1·055 for table (A), a difference well within the experimental error, a much smoother curve is obtained and then it is found that the value of K shows a gradual diminution throughout five of the six members of series (A) and also of series (B). This in itself indicates that the monomolecular formula does not exactly represent the experiments. Now let us consider the effect of similar manipulations when we take the values of the limits, $E_\infty{}^*$, which I have provisionally interpolated. The fifth column indicates the new differences $E_\infty{}^* - E$ and the sixth gives the values of $K_1{}^*$ which should be constant if the monomolecular formula is applicable.

The uniform diminution of $K_1{}^*$ and the magnitude of the decrease indicate that the formula does not apply even approximately. Moreover, when the above-mentioned intermediate value for the first "Effect" in table (A) is used, the magnitude of $K_1{}^*$ diminishes throughout the entire series both of (A) and (B). In the equation $\frac{dE}{dt} = K(E_\infty - E)^n$, where t is the time and n a constant; supposing this equation to be applicable, I conclude that n is not equal to unity.

The question now becomes, is there any value of n which will give a constant value for K? This seems to be the case, for, retaining the values of the "Effect" given in the second column and taking n equal to 2, a relatively high degree of constancy was obtained for K in both series (A) and (B). The results are shown in the seventh column $K_2{}^*$ and indicate that the course of the "Danysz Effect" may be represented by the formula used for bimolecular reactions with close approximation.

If in table (B) the first "Effect" E be taken as 1·05 instead of 1·06, a value which gives a better fitting curve (B) and a difference below the experimental error, the constant K_2^* becomes 0·0065 instead of 0·0080 and the agreement with the bimolecular formula is therefore highly satisfactory. On the other hand, if in table (A) the above-mentioned value 1·05 be taken as the most probable for the first "Effect" the constant K_2^* becomes 0·0043, and thus there is a very slight falling off of K_2^* throughout the series (A), which indicates that the magnitude of n probably only differs from 2 by a small additional fraction and further it is certain that the value $n=3$ is, by far, too great. It does not seem to me profitable to pursue the re-calculation of the meagre data placed at our disposal, and it is futile and perhaps misleading to give, as Arrhenius does, the toxicities which have been calculated by means of a constant derived from the experimental results. If a constant be obtained, that in itself is sufficient to prove the validity of the formula; and the subsequent calculation of toxicity and comparison with observed toxicity in tabular form are liable to give rise to a false impression in the minds of workers in Immunity, who have often but slight knowledge of the methods of estimation, and lead to the belief that the theoretical views underlying the arithmetical manipulations have been substantiated. Further, as the remainder of Arrhenius's calculations depends upon estimated limiting values, E_∞, of the "Danysz Effect," which are probably open to objections of a similar nature to those advanced above, we must remain in doubt as to the actuality of the apparent correspondence between the experimental and calculated results until the original data are published.

In illustration of this I may cite Arrhenius's manipulation of the evidently valuable experimental work of Madsen and Walbum on the influence of excess of antitoxin on the "Danysz Effect."

Madsen finds that a constant quantity of toxin, viz. 4 c.c. added in two fractions of 1 c.c. and 3 c.c. to a quantity of antitoxin varying from 0·2 c.c. to 1·2 c.c. gives a "Danysz Effect" which is practically proportional to the amount of antitoxin present. Arrhenius assumes that this strict proportionality holds when the antitoxin is further diminished, and concludes that the "Effect" would disappear when the quantity of antitoxin used is less than 0·16 c.c. This view does not seem to me to harmonise with Arrhenius's own conceptions, for even with 0·16 c.c. of total antitoxin there should be a considerable proportion free which should be subject to the same laws of change ascribed by Arrhenius to excess of antitoxin. Further, when no antitoxin is added there can be

no "Effect" and when a great excess is present the "Effect" should be negligible, consequently with increasing quantities of antitoxin a gradual rise in the value of E_∞ is to be expected for very small quantities followed by an increase approximately proportional to the added antitoxin until a maximum value is reached, after which the "Effect" would gradually diminish. It does not, then, appear to me that these experiments give a method of determining the equivalents of toxin and antitoxin and consequently they do not form a "very strong support" of the view of Madsen and Arrhenius.

With regard to the application of Madsen and Walbum's experimental results to the theory of the toxin-antitoxin reaction, the increment in the "Danysz Effect" with increasing antitoxin throughout such a long range of concentrations seems to me to indicate that it cannot be due to a modified toxin, epitoxinoid, having the properties assumed by v. Dungern, for with greater quantities of antitoxin more should be left free to combine with the toxin and the "Effect" should diminish; this however may depend upon the range of antitoxin concentrations selected, as indicated above. To go to the root of the matter, Madsen and Arrhenius have advanced no theoretical justification of their treatment of the differences, $E_\infty - E$, as a monomolecular reaction, and no grounds why this relation between toxicities should be ascribed to the antitoxin. It has been seen that the formula used is merely an interpolation, without, as yet, any definite significance from the point of view of the mass law of Guldberg and Waage, and further, as such, it is probably incorrect. Moreover, the introduction of a new modification of "Antitoxin" which reacts more slowly with the toxin, but fixes it more firmly, and during which 1 "molecule" of toxin probably binds 2 "molecules" of antitoxin, renders the explanation of Madsen and Arrhenius as complicated as that of v. Dungern. This is the more to be regretted as Arrhenius and Madsen's views on Immunity have been confirmed in many respects and have the advantage of relative simplicity. It was then with some curiosity that I had recourse to the third view of the "Danysz Effect," viz. that of Bordet. Bordet (1903), Craw (1905, I.) and Bayliss (1906) have shown that a very similar effect is obtained in the staining of filter paper by anilin dyes, the paper being regarded as the antitoxin and the dye as the toxin. This "Danysz Effect" in staining is regarded as belonging to that class of phenomena called "Adsorption," the quantitative investigation and theoretical interpretation of which are at present subjects of numerous researches. It became apparent on the first few days' investigation that there

is a high probability that the relations existing between staining substances such as fuchsin, methylene blue, methyl green, erythrosin, etc., and absorbent matter such as filter paper, porcelain and ball clay are of an entirely similar nature to those described by Madsen and Arrhenius in their extensive work on "The Danysz Effect" in mixtures of toxin and antitoxin, viz. (1) increment of the time interval between the addition of the fractions of dye increases the amount of dye left free, and (2) the "Effect" is augmented by increment in the amount of absorbent material, throughout a certain range. The experimental work, which is at present in hand, on these matters will shortly be published.

These results strengthen the views advanced by Craw (1905, I.) (1905, III.) in support of the interpretation of immune reactions initiated by Bordet (1903) and Landsteiner (1903) and supported by Biltz (1904), Nernst (1904), Bayliss (1906), Freundlich (1906), and others. In my paper (1905, III.) it was shown that Arrhenius and Madsen were correct in assuming that free toxin and free antitoxin exist side by side in all mixtures of the two substances. Atoxic mixtures on filtration through gelatin became toxic, whereas the residual unfiltered fluid was antitoxic. This of course has nothing to do with the "reversibility" of the reaction as Arrhenius (1907) erroneously concludes in his *Immunochemie*, p. 18. The fact that the toxin-antitoxin combined together may be separated in part, but in part only, is shown however by other experiments in the same paper (1905, III.); this is the meaning I attached to the term "partially reversible." It will be observed that Madsen and Arrhenius (1907) have been compelled to assume this incomplete reversibility, as they now suppose that their "modified antitoxin" binds the toxin more firmly than the original antitoxin did.

Further as the toxin-antitoxin reaction especially at 20° C.—the temperature at which a considerable number of observations with tetanolysin have been made—requires an appreciable time for the completion of the union, the mechanism of the "Danysz Effect" must also operate when the whole quantity of toxin is added to the antitoxin at once; consequently the original relatively simple and now complicated views of Arrhenius and Madsen are as inapplicable as those of v. Dungern and Sachs, for the values of the equivalents of toxin and antitoxin of both of these schools of Immunity must be seriously influenced in many cases by what has been termed "false equilibrium," but really means an equilibrium which is not of the type met with in

the neutralisation of acids by bases. Reverting then to the standpoint of adsorption it seems to be admitted, even by Arrhenius, that the antitoxins are colloids, but as regards the toxins it is still a matter of doubt to what, if any, extent they are colloidal.

Arrhenius (1907) erroneously attributes to me the assumption that the toxins are colloidal and act as fine suspensions. I found (1905, III.) however that antitoxin does not appreciably diffuse through gelatin. The results arrived at by Arrhenius and Madsen (1902), which they consider show a "marked diffusibility" and of which Arrhenius (1907) seems to believe I had no knowledge, but which I personally discussed with Madsen shortly after their publication, seem to me capable of another interpretation, viz. that by the superposition of an aqueous solution of antitoxin over a gelatin column the transmission effect may not be due to diffusion but to imbibition, as I had found for megatheriolysin (1905, III.).

In my experiments the toxin, antitoxin, or mixtures, were contained in a gelatin layer superimposed on a column of gelatin, and imbibition effects thereby eliminated.

Arrhenius (1907, p. 19) appears to be unfamiliar with the mechanism of the gelatin filter. This method (Craw, (1906)) gives an indication in a few minutes of the crystalloidal or colloidal nature of a solution—under certain conditions, a confirmation of C. J. Martin's (1896) view—whereas a similar differentiation by means of dialysis or diffusion would require days or even weeks. Arrhenius has failed to grasp the meaning of my remarks on suspensions (1905, III.). The "theoretical considerations" had the object of showing that the suspension view was untenable if the union of toxin and antitoxin were purely chemical. The conclusion arrived at was that the toxin-antitoxin reaction had the greatest number of points of analogy with adsorption phenomena. This view seems to me to be materially strengthened by the experimental work of Madsen and his pupils. The calculations of Arrhenius are in my opinion of doubtful value and afford inadequate support to the purely chemical interpretation of the reaction between toxin and antitoxin.

Summary of Conclusions.

1. It is inadmissible to study the "Danysz Effect" on tetanolysin owing to its rapid deterioration.

2. The so-called "equivalents" of toxin and antitoxin deduced by Arrhenius and Madsen are arbitrary.

3. No evidence has yet been advanced that the "Danysz Effect" has a limiting value when the time of contact of the first fraction of toxin with the antitoxin is prolonged.

4. If a limiting value of the "Danysz Effect" exist that calculated by Arrhenius is probably erroneous.

5. The monomolecular formula used by Arrhenius is merely an interpolation.

6. The "Danysz Effect" is much better represented by a bimolecular formula.

7. No confirmation of the "equivalents" of toxin and antitoxin has as yet been obtained from the "Effect."

8. Expediency appears to be the only justification for assuming that the "Effect" is due to either a modified antitoxin or to a modified toxin, viz. epitoxonoid.

9. All the phenomena of the "Effect" hitherto advanced have their counterpart in the staining of paper, porcelain, etc., by anilin dyes.

REFERENCES.

ARRHENIUS (1907), *Immunochemie*, Leipzig: Akademische Verlagsgesellschaft m. b. H.

ARRHENIUS and MADSEN (1902), *Festskrift ved Invielsen af Statens Seruminstitut*, III. Copenhagen.

BAYLISS (1906), *Bio-Chemical Journal*, vol. I. p. 175.

BILTZ (1904), *Göttinger Nachrichten*, math.-phys. Kl., No. 1.

—— *Zeitschr. f. physikal. Chemie*, vol. XLVIII. p. 615.

BORDET (1903), *Ann. de l'Inst. Pasteur*, vol. XVII. p. 161.

CRAW (1905, I.), *Journ. of Hygiene*, vol. V. p. 115.

—— (1905, III.), *Roy. Soc. Proc.*, B. vol. LXXVI. p. 188.

—— *Zeitschr. f. physikal. Chemie*, vol. LII. p. 569.

—— (1906), *Roy. Soc. Proc.*, B. vol. LXXVI. p. 311.

DANYSZ (1902), *Ann. de l'Inst. Pasteur*, vol. XVI. p. 331.

v. DUNGERN (1904), *Deutsche med. Wochenschr.*, vol. XXX. pp. 275, 310.

EHRLICH (1897), *Fortschr. d. Medicin*, vol. XV. p. 41.

FREUNDLICH (1906), *Ueber die Adsorption in Lösungen*, Habilitationsschrift, Repr. Leipzig: Engelmann.

LANDSTEINER (1903), *München. med. Wochenschr.*, vol. L. p. 764.

MADSEN (1899), *Zeitschr. f. Hygiene*, p. 227.

MADSEN and ARRHENIUS (1906), *Meddelh. fråm Vet.-Akads. Nobelinstitut*, vol. I. No. 3.

—— *Communic. de l'institut sérothérapique de l'état Danois*, Extraits, vol. I.

—— (1907), *Centralbl. f. Bakteriol.*, Ref. vol. XXXIX. p. 189.

MARTIN, C. J. (1896), *Journ. of Physiol.* vol. XX. p. 364.

NERNST (1904), *Zeitschr. f. Electrochemie*, vol. X. p. 377.

SACHS (1904), *Berlin. klin. Wochenschr.*, No. 16.

—— *Centralbl. f. Bakteriol.*, vol. XXXVII. No. 2.