TETANUS ANTITOXIN TITRATION BY HAEMAGGLUTINATION

By A. J. FULTHORPE

The Wellcome Research Laboratories (Biological Division), Beckenham, Kent

A method of preparing erythrocyte suspensions sensitized with protein antigens which were agglutinable by their respective antiprotein antibodies was described by Boyden (1951). This technique was applied by Fisher (1952) to the titration of diphtheria antitoxin in the sera of groups of children. Borduas & Grabar (1953) investigated the behaviour of albumin and anti-albumin systems and found that the method was extremely sensitive, but complicated by selective absorption of certain antigens. They also noted that soluble antigen-antibody complexes in the region of antigen excess were capable of producing agglutination of sensitized cells. Stavitsky (1954) made a considerable investigation of various protein-antiprotein systems by the haemagglutination method and concluded that it had the great advantage of extreme sensitivity. He ascribed the occasional lack of reproducibility to variation in different batches of erythrocytes, and the occasional lack of specificity to unabsorbed sheep haemagglutinins which could be detected by adding sensitizing antigen to agglutinating serum. He found good correlation between the agglutinin titre and the diphtheria antitoxin content of rabbit sera over a wide range, but poor correlation with human diphtheria antitoxin below 1·0 unit and above 10 units. Landy, Trapani, Formal & Klugler (1955) also compared the haemagglutination technique with the rabbit intracutaneous method of assay of diphtheria antitoxin. They observed that the quantity of sensitizing antigen capable of giving maximal sensitivity varied widely with different toxoids. In general, they found good correlation between haemagglutinin titre and antitoxin content by *in vivo* titration, with ninety-six human sera.

An investigation has been made into the titration of tetanus antitoxins both by direct haemagglutination and by haemagglutination inhibition tests.

MATERIALS AND METHODS

Erythrocytes

Sheep cells were used exclusively. Washed sheep cells were sensitized by Stavitsky's method (1954), but 1 % normal horse serum was used in the final suspension as a stabilizer. A satisfactory preparation required about 100 Lf of partially purified toxoid per 10 ml. of $2\frac{1}{2}$ % suspension of tannic acid cells. Freshly prepared suspensions were used on the day of preparation only.

Preserved sensitized suspensions

After the final washing, sensitized cells were resuspended in a minimum volume of 0·85 % saline and poured into a solution of isotonic 0·05 M sodium borate-

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

succinic acid buffer saline at pH 7·5 containing 1 % of normal horse serum and 20 % formalin. This mixture was left in contact with the cells for 3 days with frequent shaking to break up the cell clumps. The supernatant was withdrawn and replaced with 1 % normal horse serum saline, every 2 days for a week, after which the concentration of free formalin was negligible. Such cells remain apparently unchanged and with a constant sensitivity for up to 4 months.

Controls

All the sera used were tested with fresh unsensitized sheep cells for the presence of natural anti-sheep haemagglutinins. No horse serum was found with natural agglutinin in excess of 1/20. All horse sera were titrated for tetanus antitoxin at a far higher dilution than this, and absorption of natural agglutinins was therefore considered unnecessary. In addition to fresh unsensitized cell controls, the sera were tested with preserved unsensitized tanned cells. The sheep species agglutinogen was not affected by formalinization, and these cells gave the same agglutinin titre as fresh suspensions with anti-sheep sera. All toxins and toxoids were also tested against the sensitized cells, and some were found to agglutinate cells non-specifically in low dilution. This non-specific effect usually occurred only at a dilution of 1/10 or lower and could be removed or reduced by dialysing the material. The usual diluent controls were also carried out.

Antigen diluents

In haemagglutination inhibition tests it was necessary to use some stabilizer to prevent loss in potency of tetanus toxin in high dilution. A diluent containing equal parts 0·85 % saline and digest broth (papain digest of horse muscle, – pH 7·0–7·2) was satisfactory.

Performance of direct haemagglutination test

The sera under test were diluted either in 0·85 % saline containing 1 % normal horse serum (freshly prepared cells) or in 0·85 % saline alone (preserved cells). For convenience, dilutions of 1/2, 1/5, 1/10, 1/20 and so on, were used: 1·0 ml. amounts of the dilutions were dispensed in $\frac{3}{8} \times 3$ in. round-bottomed tubes, 0·1 ml. of 1·25 % suspension was added to each, the tubes were inverted to mix the contents and the test left overnight at room temperature.

Performance of the haemagglutination inhibition test

A test dose of toxin equivalent to one unit of antitoxin (L+ dose) was dispensed severally in a number of tubes and volumes of serum differing from each other by 20 % over a convenient range added. Repeat tests were made at 20 % differences on interlocking points. Usually five such test mixtures were used in each series. The tubes were inverted to mix, allowed to stand for 1 hr. at room temperature, and 0·1 ml. of sensitized cells added. The arithmetic mean of the values obtained in tests on one serum was taken as the antitoxin value of that serum. Similar tests were performed with toxoid using the Lf dose (equivalent to one unit of antitoxin by flocculation).

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Reading haemagglutination tests

The agglutination pattern consisted of (*a*) a complete smooth carpet of cells spread evenly over the bottom of the tube (+ + +), (*b*) a smooth pattern as before but with the faintest ring of more concentrated cells commencing at the periphery (+ + ±), and (*c*) a faint carpet of cells with rings of unagglutinated cells of decreasing diameter (+ + to −). It was found that the most reproducible end-point was the + + ± agglutination pattern. With increasing quantities of antitoxin the + + + agglutination often collapsed to form a ragged clump in the bottom of the tube, and still higher concentrates often resulted in the development at some point of a true 'prozone' or inhibition of agglutination. The 'prozone' effect was readily reproducible while the ragged appearance of a collapsed pattern varied from day to day.

Performance of in vivo *tests*

Test mixtures containing the L+ dose of toxin were made in the same way and injected into albino mice weighing 20–25 g. The mice were observed twice daily, and the end-point of the titration was assessed on a basis of death on the fourth day, or definite symptoms of tetanus on the third day. If an animal was dead on the second or third day and the next mouse in the series was alive, the end-point was arrived at by interpolation. Five estimations were usually made and the value allotted was the arithmetic mean of individual results.

Many sera were also tested in mice at the 0·1 L+ level of test and the dilution ratio of sera calculated in order to demonstrate the degree of avidity of the antitoxin under test.

RESULTS

Direct agglutinin titres with freshly prepared unpreserved suspensions

Sheep cells treated with tannic acid and (*a*) tetanus toxin, (*b*) tetanus toxoid, were used to estimate the direct agglutinin titre of a group of horse sera of different types. The means of the agglutinin titres of several tests were taken and the antitoxin value estimated in relation to that of the working standard (no. 8480, 1700 units/ml.). This standard had a titre of $1/1{\cdot}7 \times 10^6$ with toxoid-treated cells and $1/7 \times 10^5$ with toxin-treated cells. The titres were improved in subsequent tests by using greater quantities of sensitizing antigen. It will be seen, however, (Table 1) that although the agglutinin titres of a number of sera with the two sensitized cell preparations differ by more than 100%, the difference is fairly consistent, and when the figures are converted to antitoxin units, the differences between the two tests are, in general, reasonable for direct agglutination tests on a 100% range. The calculated antitoxin values of four sera related to that of the working standard are unreal. Of these, two horse antitoxins in group A (nos. 7897 and 8740) have calculated values only a small fraction of the true antitoxin value. These sera were obtained from hyperimmune horses, immunized many times with tetanus toxin and toxoid only. Except for no. 8825 the sera in group B produced titres in fair agreement with the *in vivo* value; these were derived from horses

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

hyperimmunized many times with tetanus antigens, but previously hyperimmunized with other Clostridial toxins such as *Clostridium welchii* type B; this group included the working standard no. 8480. Group C consisted of sera from three horses bled after two prophylactic doses of 200 Lf of tetanus toxoid. All were non-avid (dilution ratios* 0·42–0·53), and two of the three sera gave values by the direct test in reasonable agreement with the *in vivo* value. The third serum no. 9598, however, gave a discrepant result.

Table 1. *Comparison of the antitoxin values (units/ml.) estimated at the L+ level of test of a group of hyperimmune horse sera with values calculated from direct haemagglutination tests with freshly prepared unpreserved sheep cells sensitized with tetanus toxoid (TD 341 C) and toxin (XW 1322)*

Sera tested		TD 341 C cells: Reciprocals of agglutinin titres	TD 341 C cells: Calculated antitoxin values (units/ml.)	XW 1322 cells: Reciprocals of agglutinin titres	XW 1322 cells: Calculated antitoxin values (units/ml.)	*In vivo* values at L+ level of test (units/ml.)
A	LX 381/A	15×10^3	15	5×10^3	12	46
	7897	10^5	100	5×10^4	120	1350
	8740	3×10^5	300	10^5	240	1600
B	8480*	$1{\cdot}7\times10^6$	1700	7×10^5	1700	1700
	9018	4×10^5	400	15×10^4	370	360
	8348	4×10^5	400	15×10^4	370	380
	8825†	2×10^6	2000	4×10^5	1000	650
C	9611	8×10^4	80	15×10^3	37	77
	9615	5×10^4	50	25×10^3	61	75
	9598	12×10^3	12	4×10^3	10	30

Standard error of the mean of the observations on the working standard were: with TD 341 C cells ± 10 %; with XW 1322 cells ± 9 %.

* Working standard.

† This serum has been found to contain a high proportion of non-specific antibody by other tests. That is antibody other than specific tetanus antitoxin.

A: Sera from hyperimmune horses after many courses on tetanus toxoids and toxins only.

B: Sera from hyperimmune horses after many courses on tetanus antigens preceded by several courses on other antigens such as *Cl. welchii*.

C: Sera from horses after only two doses of 200 Lf of tetanus toxoid (non-avid).

Antitoxin values by haemagglutination inhibition tests with freshly prepared unpreserved suspensions

When it was found that the estimation of antitoxin values by direct haemagglutination gave results, some of which differed widely from the *in vivo* values, a series of tests were set up in which a test dose of toxin or toxoid was used as the agglutinin inhibiting agent. The volume of these inhibitory toxins or toxoids used

* Dilution ratio is a measure of *avidity*, that is, firmness of combination of toxin and antitoxin *in vivo*. It is the volume of serum that would be expected by calculation from the L+ level to neutralize a 0·01 L+ dose of toxin divided by the actual volume of serum found necessary by experiment.

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

contained the L+ or Lf dose equivalent to one unit of standard antitoxin. The apparent antitoxin values of the various sera were calculated from the volume necessary to give a convenient end-point (+ + ± agglutination) (Table 2). The cells (freshly prepared daily) were sensitized with the same toxoid and toxin as were used for the direct agglutinin tests in Table 1.

Table 2. *Estimation of the antitoxin values (units/ml.) of a group of horse sera tested by haemagglutination inhibition using the L+ or Lf dose of toxin or toxoid as the inhibitory agent. Agglutinable suspensions were freshly prepared unpreserved sheep cells sensitized with (a) toxoid TD341C, and (b) toxin XW1322*

	Agglutinin inhibiting antigen ...	(a) Toxoid sensitized cells: Toxoid TD341C	Toxin XW1322	Toxins XW1322 AW1999 (mixed)	Toxin AW1781	(b) Toxin-sensitized cells Toxin XW1322	*In vivo* value at the L+level of test
	Sera						
A	LX381/A	40	50	48	45	50	46
	7897	1040	1130	1020	1200	1000	1350
	8740	1350	1300	1320	1200	1600	1600
B	8480*	1700†	1700‡	1700§	1700	1700	1700
	9018	616	900	360	450	500	350
	8348	500	424	400	420	450	380
	8825	730	2825	630	630	600	650
C	9611	85	175	90	73	80	77
	9615	92	113	84	70	75	75
	9598	32	45	26	—	24	30

* Working standard. The standard error of the tests carried out on this serum in the six columns above expressed as a percentage of the mean were: 6·7, 2·4, 2·0, 4·0, and 3·8% (*in vivo* test).

† Apparent antitoxin values corrected to *in vivo* value of working standard −25%.

‡ Apparent antitoxin values corrected to *in vivo* value of working standard +10%.

§ Apparent antitoxin values corrected to *in vivo* value of working standard +20%.

A, B, C, see Table 1.

TD341C: filtrate toxoid concentrated by acid precipitation only.

XW1322: filtrate toxin grown on semi-synthetic medium (Muller-Miller).

AW1999: freak autolysate toxin containing only 1 M.L.D./ml. of specific toxin.

AW1781: routine autolysate toxin—a blend of several batches.

It is apparent from the figures in Table 2 that the wide discrepancies found with the sera of group A by direct agglutination in the previous tests have been greatly reduced, although the values compared with the *in vivo* results for sera nos. 7897 and 8740 are in general still too low. The sera in group B gave the best results when the agglutinin-inhibiting agent used was either AW1781, a blend of crude untreated autolysate toxins (col. 5) or XW1322 and AW1999 mixed in a ratio of 1:9. XW1322 was prepared on semi-synthetic medium and AW1999 was a 'freak' autolysate toxin containing very little specific toxin. These tests were made using cells sensitized with toxoid TD341C, prepared from toxin grown on semi-synthetic medium and concentrated by acid precipitation only. It may be assumed that toxins or toxoids prepared from culture filtrates of *Cl. tetani* will contain

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

many antigens other than the specific toxin or toxoid, and that most of these will be protein substances which can be attached to sensitized red cells in the same way. AW 1999 and AW 1781 would be expected to contain non-specific protein material derived from the autolysis of the organism in addition to the specific toxin. These results suggest that when a routine unpurified antigen is used to sensitize cells, an agglutinin-inhibiting agent containing much non-specific antigen is required to inhibit non-specific antibody at a level below that at which the true toxin-antitoxin reaction is being titrated. When XW 1322 was used by itself as an inhibiting agent with TD 341 C toxoid sensitized cells, some very discrepant results were obtained (Col. 3). This suggests that XW 1322 is lacking in some non-specific antigen necessary to block non-specific antibody in some of the sera. This suggestion is reinforced by the results obtained from tests in which the cells were sensitized with XW 1322 and the inhibiting agent was the same toxin (Col. 6). These were in fair agreement with the results of *in vivo* tests. The results obtained by using TD 341 C as an inhibiting agent with TD 341 C sensitized cells are, however, unsatisfactory, suggesting that although the proportion of non-specific blocking material is greater than that present in XW 1322 it is not enough to prevent the non-specific antibody in some sera from reacting with the cells. Thus, the results shown by one series of tests when blocking and sensitizing antigens were the same (XW 1322, col. 6) were superior to another series in which a different antigen was used for both these purposes (TD 341 C, col. 2). It may be that XW 1322, used as a sensitizing antigen, contains proportionately too little non-specific material to react to non-specific antibody. This suggests that tetanus toxoids prepared on the same type of medium may contain different proportions of specific and non-specific antigens.

Effect of suspension sensitivity on the apparent antitoxin value of sera by haemagglutination inhibition tests (preserved cells)

The lack of reproducibility of tests carried out with some batches of freshly prepared cells was a constant source of difficulty. This problem made it necessary on occasion to reject a whole series of tests owing to lack of sensitization of the cells. It was, moreover, found that some cells could not be used on more than one occasion, as the titre was lower on subsequent days, and not always proportionately so with different sera.

The effect of sensitivity was determined by direct and by haemagglutination inhibition tests using two agglutinable suspensions of widely different sensitivity (Table 3). The cells were sensitized with a partly purified toxoid of 1430 Lf/mg. P.N., the more sensitive cells (V_2) being treated with five times greater concentration of sensitizing toxoid than the less sensitive cells (V_3). The direct agglutinin titres against the working standard were $V_2 = 1/20 \times 10^6$ and $V_3 = 1/3 \times 10^6$. The direct agglutinin titres of the sera were determined and the antitoxin values calculated from the known antitoxin value of the working standard. In the inhibition tests the L+ dose of a crude autolysate toxin AW 1781 was used.

It is evident from Table 3 that there are numbers of very large differences between the *in vivo* values and those calculated from direct agglutination titres, the more serious being those from hyperimmune horses, nos. 8737, 7897/B and

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

9195. While this may suggest that an unsuitable standard has been chosen, it is evident that if any of these three sera had been used as a standard, the discrepancies would merely transfer themselves to most other sera. It will be noted that with the exception of the guinea-pig serum and possibly, to a slight extent, the rabbit serum, all the sera were avid, as was the working standard. This suggests that these differences are not related to the avidity of the antitoxins.

Table 3. *The apparent antitoxin values and corrected antitoxin values by haemagglutination inhibition and the calculated antitoxin values by direct agglutination of a group of sera of different animal species, tested with two agglutinable suspensions of widely different sensitivity. Inhibition tests carried out with an L+ dose of autolysate toxin AW 1781 as the inhibiting agent*

Sera	Apparent antitoxin values at the L+ level of test		Corrected antitoxin values at the L+ level of test		Calculated antitoxin values by direct haemagglutination		*In vivo* value at L+ level of test	Dilution ratio of anti-toxins
	V_2	V_3	V_2	V_3	V_2	V_3		
8480*	1850	1200	1700	1700	1700	1700	1700	1·06
Human	57	42	52	59	85	71	45	1·0
Guinea-pig	9·0	4·7	8·3	6·6	2·6	1·7	5·0	0·44
Dog	24	12	22	17	8·5	8·6	19	0·84
Rabbit	30	16·5	28	23	43	20	22	1·1
Cow	10	8	9·2	11·2	8·5	9·7	8·0	1·2
Horse 65	1350	1100	1240	1540	1275	1140	1200	0·92
Horse 8737	180	120	167	168	64	43	180	1·1
Horse 7897/B	475	360	437	504	50	34	425	1·1
Horse 9195	650	200	598	280	64	43	450	1·0

* Working standard.

Sensitivity of agglutinable suspensions by direct agglutination with 8480 serum:

$V_2 = 1/20 \times 10^6$: sensitized with partly purified toxoid TD 343D of 1430 Lf/mg. P.N. 250 Lf per 10 ml. of 2½ % tannic acid cells.

$V_3 = 1/3 \times 10^6$: sensitized with partly purified toxoid TD 343D of 1430 Lf/mg. PN. 50 Lf per 10 ml. of 2½ % tannic acid treated cells.

In the haemagglutination inhibition tests in Table 3 the difference in the apparent antitoxin value of the working standard no. 8480 (uncorrected value) using two different suspensions was of the order of 40 %, whereas the sensitivity of the two suspensions used, differed by about seven times. Those sera which had wide discrepancies between *in vivo* values and *in vitro* values by direct agglutination gave values by agglutinin inhibition which were much more reasonable.

From the fact that the apparent (uncorrected) antitoxin values by agglutination inhibition may be higher or lower than the *in vivo* values (Table 3, columns 2 and 3), it is evident that the L_A dose must vary according to the sensitivity of the suspension used. The L_A dose of a toxin or toxoid is that volume of antigen which when mixed with one unit of standard antitoxin will give a + + ± end-point in the haemagglutination inhibition test.

Estimation of the test dose (L_A dose) of an agglutinin-inhibiting toxin or toxoid

In order to estimate the volume of inhibiting antigen necessary to give the accepted end-point with one unit of standard antitoxin (L_A dose), volumes of antigen differing by 20 % were mixed with a volume of serum containing one unit

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

of antitoxin (by *in vivo* titration) and the test repeated at intervals (Table 4). It can be seen from this that the test dose varies with the sensitivity of the suspension used, and that the end-point with this antitoxin was relatively sharp. In other experiments when an antitoxin used to estimate the L_A dose contained much non-specific antibody, and the sensitizing antigen on the agglutinable cells contained non-specific antigen, this non-specific antibody in the serum reacted to produce agglutination at a point which did not correspond with the true toxin-antitoxin point unless it were blocked by non-specific antigen in the agglutinin-inhibiting toxin or toxoid. The apparent potency was thus greatly in excess of the L+ or Lf value of the toxin or toxoid under test. However, if cells were sensitized with a toxoid of moderate purity containing little non-specific antigen, an intermediate stage could be observed in the test dose (L_A dose) titration with an antitoxin containing much non-specific antibody. The $++\pm$ end-point was followed by a long range of partial agglutination. This is shown in Table 5. When the quantity of sensitizing antigen was reduced, the minor antigens on the surface of the sensitized cells ceased to produce this interference, and a correct end-point is easy to estimate (Table 5, suspension V_3).

Estimation of the L_A dose of different toxins and toxoids against two hyperimmune horse antitoxins using sheep cells sensitized with toxoids of different purity

It was evident from the results of L_A dose estimations that considerable differences in the antigenic composition of toxins and toxoids could be demonstrated by estimating the relative volumes necessary to inhibit agglutination of sensitized cells by sera of well-immunized animals. If sheep cells were sensitized with toxoids containing specific and non-specific antigens in different proportions, the difference in L_A dose of different inhibiting antigens would indicate the relative proportion of specific and non-specific antigens in the preparations. The L_A doses of several batches of toxin and toxoid were determined against two different sera, using cells sensitized with toxoids of varying purity. The two sera were (i) the working standard no. 8480, from a horse hyperimmunized over a long period with tetanus toxins and toxoids, and (ii) a similar serum no. 8825 thought to contain a high proportion of non-specific antibodies (see Table 2, col. 3). The sensitivity (agglutinin titres) of the four suspensions M.B.K. and T_5 differed, so that figures for the L_A dose given in Table 6 were adjusted for suspensions K and T_5 to conform with the sensitivity of suspensions M and B. This was carried out as a result of the observations on the correlation between sensitivity and test dose (Tables 4 and 5).

Table 6 (A) shows results for the working standard no. 8480. It can be seen that whether the inhibiting agents were crude autolysate toxins (AW 1781, XW 1322 + AW 1999), toxin produced on semi-synthetic medium (XW 1322 alone), autolysate toxoid MW 1470 or filtrate toxoid acid precipitated (TD 341 C), the L_A dose was not significantly different when cells sensitized with toxoids of widely different purity in terms of Lf/mg. P.N were used. When, however, this serum was inhibited with toxoids TD 343 D (1430 Lf/mg. P.N.) and T_5 (2500 Lf/mg. P.N.) the inhibitory effect was dependent upon the degree of purity of the toxoids used to sensitize the

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Table 4. *The effect of altering the sensitivity of toxoid sensitized sheep cells on the estimation of the L_A dose. The L_A dose is that volume of toxin or toxoid which when mixed with one unit of standard antitoxin will give a suitable end-point* (+ + ± *agglutination*) *in the haemagglutination inhibition test*

Volumes of toxin added to one unit of antitoxin (ml.)	Agglutinable suspensions (formalin preserved)											
	V_2				K				V_3			
0·0165	.	.	.	.	.	.	.	.	.	+ + +	.	.
	.	.	.	.	.	.	.	.	+ + +	.	+ + +	.
0·020	.	.	.	.	.	.	.	.	.	+ + +	.	+ + +
	.	.	.	.	.	.	.	.	+ + +	.	+ + +	.
0·024	.	.	.	.	.	.	.	.	.	+ + ±	.	+ + +
	.	.	.	.	+ + +	.	+ + +	.	+ + ±	.	+ + ±	.
0·030	.	+ + +	.	+ + +	.	+ + +	.	+ + +	.	±	.	±
	+ + +	.	+ + +	.	+ + +	.	+ + +	.	+	.	±	.
0·036	.	+ + +	.	+ + +	.	+ + +	.	+ + ±	.	−	.	−
	+ + +	.	+ + +	.	+ + ±	.	+ + ±	.	−	.	−	.
0·045	.	+ + ±	.	+ + ±	.	+ + ±	.	+	.	−	.	−
	+ + ±	.	±	.	±	.	±	.	−	.	−	.
0·055	.	±	.	−	.	±	.	−	.	.	.	.
	−	.	−	.	−	.	−	.	.	.	.	.
0·065	.	−	.	−	.	−	.	−	.	.	.	.
	−	.	−	.	−	.	−	.	.	.	.	.
Estimated test dose (L_A)	0·046 ml.				0·040 ml.				0·026 ml.			
Sensitivity of suspensions, i.e. direct agglutinin titre with standard antitoxin	$1/20 \times 10^6$				$1/10 \times 10^6$				$1/3 \times 10^6$			

Suspensions sensitized with moderately purified tetanus toxoid TD 343 D 1430 Lf/mg. P.N.

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Table 5. *The influence of minor antigens and antibodies on the estimation of the L_A dose against an antitoxin containing much non-specific antibody*

Volumes of toxin added to one unit of antitoxin (ml.)	Agglutinable suspensions (formalin preserved)											
	V_2				K				V_3			
0·018	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	+ + +	.	+ + +
0·022	.	.	.	.	.	.	.	.	+ + +	.	+ + +	.
	.	.	.	.	.	.	.	.	.	+ + +	.	+ + +
0·027	.	.	.	.	+ + +	.	+ + +	.	+ + ±	.	+ + ±	.
	.	.	.	.	.	+ + +	.	+ + +	.	±	.	±
0·033	+ + +	.	+ + +	.	+ + +	.	+ + +	.	−	.	−	.
	.	+ + +	.	+ + +	.	+ + +	.	+ + +	.	−	.	−
0·04	+ + +	.	+ + +	.	+ + ±	.	+ + ±	.	−	.	−	.
	.	+ + ±	.	+ + ±	.	+ +	.	+ +	.	.	.	.
0·05	+ + ±	.	+ + ±	.	+ +	.	+ +	.	.	.	.	.
	.	+ + ±	.	+ + ±	.	+	.	+	.	.	.	.
0·06	+ + ±	.	+ + ±	.	+	.	+ +	.	.	.	.	.
	.	+ + ±	.	+ + ±	.	+	.	+	.	.	.	.
0·07	+ + ±	.	+ +	.	+	.	+	.	.	.	.	.
	.	+ +	.	+	.	+	.	+	.	.	.	.
0·09	+ +	.	+ +	.	+	.	+	.	.	.	.	.
	.	+ +	.	+	.	.	.	.	.	.	.	.
	+ +	.	+ +	.	.	.	.	.	.	.	.	.
Estimated test dose (L_A)	0·045–0·065				0·040				0·027			
Sensitivity of suspensions against standard	$1/20 \times 10^6$				$1/10 \times 10^6$				$1/3 \times 10^6$			

Suspensions sensitized with moderately purified tetanus toxoid TD 343 D 1430 Lf/mg. P.N.

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Table 6. *Comparison of the test dose by haemagglutination inhibition (L_A dose) of several toxins and toxoids with their L+ and Lf dose equivalent to one unit of hyperimmune horse sera (A) 8480 and (B) 8825. The L_A dose estimated using agglutinable suspensions prepared with toxoids of different purity (preserved cells)*

Agglutinin-inhibiting toxins and toxoids tested	L+ dose (mice)	Lf dose	L_A dose *M*	*B*	*K* (corrected)	T_5 (corrected)
A. Toxins						
XW 1322	0·036		0·033	0·03	0·033	0·03
XW 1322 1 vol. + AW 1999 9 vol.	0·36	—	0·33	0·33	0·33	0·30
AW 1781	0·0165	—	0·015	0·0135	0·0135	0·011
A. Toxoids						
MW 1470	—	0·014	0·012	0·011	0·012	—
TD 341 C	—	0·011	0·0135	0·012	0·015	0·012
TD 343 D	—	0·0008	0·004	0·0016	0·0011	0·0010
T5	—	0·0004	0·03	0·002	0·0007	0·0006
B. Toxins						
XW 1322	0·035	—	0·065	0·04	0·03	0·03
XW 1322 1 vol. + AW 1999 9 vol.	0·36	—	0·4	0·38	0·33	0·30
AW 1781	0·0165	—	0·015	0·015	0·0135	0·012
B. Toxoids						
MW 1470	—	0·014	0·012	0·012	0·012	0·011
TD 341 C	—	0·011	0·0165	0·018	0·0135	0·0135
TD 343 D	—	0·0008	0·011	0·0055	0·0011	0·0010
T5	—	0·0004	0·08	0·0010	0·0008	0·0006

Cell sensitizing agents:
M: crude autolysate toxoid MW 1470 (738 Lf mg./P.N.).
B: toxoid treated by acid precipitation only TD 341 C (845 Lf/mg. P.N.).
K: toxoid purified to moderate extent TD 343 D (1430 Lf/mg. P.N.).
T_5: toxoid purified and supplied by Prof. Raynaud T_5 (2500 Lf/mg. P.N.).
Agglutinin-inhibiting toxins and toxoids:
XW 1322: laboratory test toxin grown on semi-synthetic medium (Muller–Miller).
AW 1999: a freak toxin which after autolysis of organism at the usual time contained only 1 M.L.D./ml. of specific toxin.
AW 1781: a routine batch of autolysate toxin.
MW 1470: a routine batch of autolysate toxin, toxoided in the usual way with formalin.
Also TD 341 C, TD 343 D and T_5 as above.
The L_A dose with *K* and T_5 cells corrected for sensitivity.

cells, and a much larger volume of inhibitory toxoid was required to reach an end-point with *M* cells than with T_5 cells. This demonstrates the reaction produced by non-specific antigens on the surface of *M* and *B* cells with non-specific antibody in serum no. 8480, which was incompletely blocked owing to the absence of sufficient of the non-specific antigens in toxoid TD 343 D and T_5. Serum no. 8825 (Table 6 *B*), thought to contain much non-specific antibody, behaved similarly, but in this case the blocking capacity of XW 1322 was also inadequate and had to be reinforced with AW 1999, an antigen containing chiefly non-specific material. With serum no. 8825, however, the inhibiting effect of autolysate toxin AW 1781 and autolysate toxoid MW 1470 was satisfactory, indicating that these materials

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Table 7. *Comparison of* in vivo *and* in vitro *tests by haemagglutination for assay of tetanus antitoxin*

	In vivo value at the L+ level of test (units/ml.)	Antitoxin values calculated from direct agglutinin titres (units/ml.)	Antitoxin values by haemagglutination inhibition tests at the L+ level of test (units/ml.)
		Hyperimmune sera	
8480*	1700	1700	1700
9018	360	400	360
8348	380	400	400
9096	220	220	220
65	1200	1275	1240
7897	1350	100	1020
7897/B	425	50	437
8740	1600	300	1320
7602	1100	128	1200
8737	180	64	167
LX 381/A	46	15	48
9195	450	64	600
8510	95	170	110
8825	650	2000	630
		Pepsin-refined sera	
H	5500	1700	6000
M	1725	290	1840
L	650	125	830
		Horses after 2 doses of toxoid only	
300	80	128	120 (non-avid)
302	100	34	165 (non-avid)
307	100	128	117
312	42	26	97 (non-avid)
		Sera of other species after short courses of immunization	
Human	45	85	52
Guinea-pig	5·0	2·6	8·3 (non-avid)
Dog	19	8·5	22
Rabbit	22	43	28
Cow	8·0	8·5	9·2

* Working standard.

contained a high proportion of non-specific antigens and were consequently very suitable for haemagglutination inhibition tests on sera of hyperimmunized animals.

Influence of the level of test on the antitoxin values of horse sera by haemagglutination inhibition test

Numbers of horse sera were tested by haemagglutination inhibition using the 0·01 L+ dose of an autolysate toxin as the inhibiting agent. It was found that the apparent antitoxin value of the working standard was much higher than the *in vivo* values; that is to say the 0·01 L_A dose was much greater than the 0·01 L+

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

dose. When the values obtained for the other sera were converted to the *in vivo* value of the working standard, there were numbers of large discrepancies, and the antitoxin values of those sera giving low values by direct agglutination were also low by the agglutination inhibition test at the 0·01 L+ level of test. This observation also applied to pepsin-refined antitoxins.

A toxoid of low purity (845 Lf/mg. P.N.) was used as an agglutinin-inhibiting agent against the working standard antitoxin to find the end-point by haemagglutination inhibition with multiples and fractions of one unit of antitoxin. It was found that as the dilution of the components of the reacting mixture was increased, a relatively greatly increased volume of inhibiting toxoid was required to obtain the correct + + ± end-point.

Estimations at the $L_A/1000$ level were difficult to read, the agglutination pattern being very fragile and liable to collapse under slight vibration.

Comparison of in vivo *and* in vitro *tests by haemagglutination*

The results of all tests carried out by haemagglutination inhibition at the L+ level of test, and those by direct agglutination are shown in Table 7.

The hyperimmune horse sera fall into three groups. The first contains sera which give antitoxin values in reasonable agreement by *in vivo* estimation and by both direct-agglutination and agglutination-inhibition tests. The second group contains sera which show reasonable agreement between the *in vivo* antitoxin values and those obtained by haemagglutination inhibition, but for which the calculated values by direct agglutination are widely different, and are in fact much lower in relation to the working standard. The third group consists of two horses in which the calculated antitoxin values from the direct agglutinin titres are higher than the *in vivo* value.

The immunization histories of these groups were as follows:

8480	3 courses on *Cl. botulinum* type A	7 courses on tetanus
9018	4 courses on scarlet fever	3 courses on tetanus
8348	21 courses on *Cl. welchii* type B	3 courses on tetanus
9096	4 courses on *Cl. oedematiens*	2 courses on tetanus
65		1 course on tetanus
7897	20 courses on tetanus	
7897/B	42 courses on tetanus (same horse 2 years later)	
8740	6 courses on tetanus	
7602	55 courses on tetanus	
LX381/A	3 courses on tetanus	
8737	2 courses on *Cl. welchii* type D	10 courses on tetanus
9195	3 courses on diphtheria	3 courses on tetanus
8510	19 courses on *Cl. oedematiens*	1 course on tetanus
8825	5 courses on *Cl. septicum*	17 courses on tetanus

In general the horses in the first group had had fewer courses of immunization on tetanus than those in the second, and these courses had been preceded by immunization with other antigens. The second group contains 5 sera from horses

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

immunized with tetanus antigens only, and includes nos. 7897 and 7602, which had been immunized many times with tetanus toxins. These two sera show the greatest discrepancies between the *in vivo* value and that calculated from the direct agglutinin titres. The remaining two sera in the second group (nos. 8737 and 9195), however, do not quite fit this picture; like those in the first group the horses had previously undergone immunization with other types of antigen. The two sera in the third group give calculated values by direct agglutination above the *in vivo* value, and the result for no. 8825 is probably due to non-specific antibody of high titre. This may also be the case with 8510.

In spite of the lack of complete correlation with immunization history the general picture suggests that on prolonged hyperimmunization there is some change in the quality of the antitoxin produced which is not connected with avidity but which renders it proportionately less effective as a direct agglutinin.

The pepsin-refined material H.M. and L consisted of blends of sera from many horses on hyperimmunization courses 'refined' by the pepsin process. The results obtained by calculation from the direct agglutinin titres may be the effect of refining, but are more likely to be due to the effects of repeated courses of immunization.

The results with the remainder of the sera tested do not show any particular trends in the direct agglutination tests. The antitoxin values obtained by haemagglutination inhibition at the L+ level of test with these widely different types of sera were not unreasonable.

The fact that reasonable values can be obtained by haemagglutination inhibition when the direct agglutinin titre of a serum compared with the working standard is as little as 1/10 of its expected value, requires explanation. In agglutination-inhibition tests the *indicator effect* is produced by excess antibody, while in *in vivo* tests the *indicator effect* is produced by excess toxin. It is well known that *in vivo* the sharpness of end point is affected by the number of *indicator doses* (in lethal tests, *lethal doses*) in the test dose of toxin. Provided that there is an adequate number of indicator doses in the test dose, results should be satisfactory. The actual number of indicator doses required in a test dose differs in different toxin-antitoxin systems. In haemagglutination-inhibition tests with the working standard serum and reasonably sensitive suspensions (titre with working standard $1/10^7$), one unit of antitoxin in 1·0 ml. of diluent can be diluted approximately 1/6000 and give agglutination; that is, one unit of this antitoxin contains 6000 *indicator doses*. Provided that the combining power of the antitoxin with toxin is unaffected, a reduction in the agglutinative capacity of the serum to give only 500 *indicator doses* per unit would not affect the end point in agglutination inhibition tests at the L+ level of test. This can be compared with the use of two test toxins in *in vivo* tests of avid antitoxin, in which one toxin contains much toxoid and therefore few lethal doses per L+. If the number of *indicator doses* in the test dose of the toxin with a high toxoid content is adequate, the antitoxin values of avid antitoxins is not affected.

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Factors involved in the development of prozones

It had been appreciated for some time that strong concentrations of antitoxin-inhibited agglutination of tetanus toxoid sensitized cells. When the working standard was titrated over a wide range against three different agglutinable suspensions, all sensitized with the same tetanus toxoid but in different concentrations, the change in sensitivity of the cells affected both the final titre and the length of the range of agglutination inhibition in the region of antibody excess (Table 8).

Table 8. *The effect produced on the agglutination and prozone ranges of alteration in the sensitivity of agglutinable suspensions used for testing simple dilutions of tetanus antitoxin* (8480) (1700 *units/ml.*)

Reciprocals of antitoxin dilutions	Agglutinable suspensions (unpreserved cells)		
	V_2	V_3	V_4
Undiluted	–	–	–
10	–	–	–
20	–	–	–
50	–	–	–
100	–	–	–
200	–	–	–
500	+	–	–
10^3	+++	+	–
10^4	+++	+++	±
10^5	+++	+++	++±
10^6	+++	+++	+
2×10^6	+++	++±	–
5×10^6	+++	±	–
10^7	+++	–	–
2×10^7	++±	–	–
5×10^7	–	–	–

V_2 Cells sensitized with 240 Lf of tetanus toxoid per 10 ml. of $2\frac{1}{2}$ % sheep cells.
V_3 Cells sensitized with 48 Lf of tetanus toxoid per 10 ml. of $2\frac{1}{2}$ % sheep cells.
V_4 Cells sensitized with 12 Lf of tetanus toxoid per 10 ml. of $2\frac{1}{2}$ % sheep cells.

When the same suspensions were used to test toxin-antitoxin mixtures very similar effects were found and there was an apparent fall in titre and an increase in the prozone range as the concentration of sensitizing antigen was reduced.

Several of the tetanus antitoxins used in earlier experiments were tested directly over a wide range of dilutions using a suspension of good sensitivity, and the prozone range and agglutination range observed.

The sera from horses immunized longest, or with tetanus antigens only, gave the longest prozones and proportionately the shortest agglutination range. The prozone range was shortest and the agglutination range proportionately longest where immunization had been mixed or of short duration. The behaviour of pepsin-refined sera was that which could be expected from blends of hyper-immunized sera from horses on repeated courses of tetanus antigens.

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

Haemagglutination-inhibition tests were also carried out on the same sera over a wide range with three suspensions of different sensitivity. It was found that the same principles applied as in the direct agglutination tests, sera from horses after prolonged immunization giving proportionately the greatest prozone range and the smallest agglutination range. Moreover, this phenomenon was accentuated if agglutinable suspensions of very low sensitivity were used. Sera from horses after two doses of 200 Lf of toxoid only did not produce prozones at all even with the least sensitive suspensions, and a serum from a horse hyperimmunized for a few weeks only, gave results intermediate between the long immunization group and the two horses which received only two doses of toxoid. This adds weight to the suggestion that there is some undefined qualitative difference in agglutinating capacity of sera from horses at different stages of immunization.

The development of zones of agglutination

The presence of multiple zones in the Ramon flocculation test for tetanus antitoxin with toxin or toxoid has always been a troublesome feature of this test. Investigation with different types of horse sera disclosed that not only could apparent antitoxin titres be raised very high by the presence of uninhibited non-specific antibodies, but that true zones of agglutination existed, when the agglutinin-inhibiting agent was a partly purified toxoid.

Wide ranges of volumes of purified tetanus toxoid were mixed with one unit of antitoxin and tested with sheep cells sensitized with toxoid of different purity, and the volumes of toxoid necessary to inhibit agglutination found (Table 9).

It can be seen from this table that the additional zone only develops when the system is tested with cells sensitized with the crude types of toxoid. Cells sensitized with purified toxoid (K and T_5) either have insufficient non-specific antigen present on the surface of the cell to react with antibody, or the relative proportion of antibody to antigen is so great that positive inhibition occurs. It can also be seen that with cells M and B the end-point of the first zone (presumed to be a specific toxoid-antitoxin reaction) ends at 0·0015 and 0·0012 ml. as compared with 0·0009 ml. for K and T_5. It is probable that there is some overlap and this represents additional antigen-antibody reactions at about the neutral point for the specific reaction.

The presence of a gap in the agglutination range in the titration with B cells is probably due to a 'prozone' to the second zone of agglutination, since the quantity of non-specific antigen on the surface of B cells is relatively small compared with the uninhibited non-specific antibody at that point.

A number of other horse antitoxins were tested to show the presence or absence of zones of agglutination. It was found with some sera that the quantities of inhibiting toxoid necessary to reach an end-point with the different types of sensitized cells were not greatly different, and that there were no additional zones over the range tested. These sera were from horses hyperimmunized against tetanus only. Other sera required a much greater volume of inhibiting toxoid when the mixtures were tested with M cells than with the other suspensions, but there were no separate zones of agglutination. A third group of sera required much more

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

inhibiting toxoid to reach an end-point with M cells than with other suspensions, and there existed with most of them separate additional zones of + + ± to + + + agglutination with M or B cells or with both.

Table 9. *The existence of additional zones of agglutination in haemagglutination-inhibition tests with one unit of tetanus antitoxin and a wide range of inhibitory toxoid of a fair degree of purity* (1430 *Lf/mg. P.N.*)

Volumes of toxoid mixed with antitoxin (ml.)	Agglutinable suspensions (preserved cells)			
	M	*B*	*K*	*T*
0·0007	+ + +	+ + +	+ + +	+ + +
0·0009	+ + +	+ + +	+ + ±	+ + ±
0·0011	+ + +	+ + +	+	+
0·00135	+ + +	+	−	−
0·00165	+ +	+	−	−
0·002	+ +	±	−	−
0·0024	+ + +	+	−	−
0·0030	+ + +	+ + ±	−	−
0·0036	+ + +	+ + ±	−	−
0·0045	+ + +	+ + +	−	−
0·0055	+ + +	+ + ±	−	−
0·0065	+ + +	+ + ±	−	−
0·008	+ + +	+ + ±	−	−
0·010	+ + +	+	−	−
0·012	+ + ±	±	−	−
0·015	±	−	−	−
0·018	−	−	−	−

M Sheep cells sensitized with crude autolysate toxoid (738 Lf/mg. P.N.).
B Sheep cells sensitized with toxoid treated with charcoal (845 Lf/mg. P.N.).
K Sheep cells sensitized with toxoid moderately purified (1430 Lf/mg. P.N.).
T Sheep cells sensitized with toxoid purified by Raynaud's method (2500 Lf/mg. P.N.).
Agglutinin-inhibiting toxoid TD 343 D 1200 Lf/ml. (0·00083 ml. = 1 Lf).

Effects of washing cells from the region of antibody excess

It was found that sensitized cells, tested against a wide range of toxoid-antitoxin mixture so that there were prozones and additional agglutination zones, could be freed of excess antibody by washing three times with 0·85 % saline. When this was done it was found that the cells resuspended in 0·85 % saline from the region of antibody excess (prozone) agglutinated normally. When two zones of agglutination existed, separated by a zone of incomplete agglutination, this latter disappeared and agglutination became complete over the whole range.

Flocculation tests and agglutination reactions

Flocculation tests were done to find the range over which visible reactions occurred with a number of sera already tested by haemagglutination. It was found that in general sera from horses after prolonged immunization flocculated slowly and over a much narrower range than sera from horses early in immunization, which flocculated rapidly and over a very wide range. Hyperimmune sera showed marked inhibition in the region of antibody excess.

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

DISCUSSION

In the course of the investigation it became evident that titrations of hyperimmune sera could not be carried out satisfactorily by direct agglutination tests and that reasonable correlation with the *in vivo* values could only be obtained by haemagglutination inhibition test with toxin or toxoid-antitoxin mixtures.

It had originally been assumed that the presence of several antibodies to the complex mixture of antigens in the tetanus culture filtrates, which were used for immunization of the horses, would be likely to cause discrepancies in the tests. It was also expected that these could be avoided by the use of sheep cells sensitized with purified toxoids. The discovery, however, that certain hyperimmune horse sera had such poor agglutinating power that the antitoxin values calculated from the direct agglutination titres were unreasonably low, was not anticipated.

It has been known for some time that antitoxin is dispersed in different fractions of the globulin range in horse sera, and that the proportion of antibody found in each fraction varied in animals with different immunization histories (Kekwick & Record, 1941). Moreover, some horse antibodies have been shown to be of much greater molecular weight and electrophoretic mobility than others (Heidelberger & Pedersen, 1937), although Fell, Stern & Coghill (1940) were unable to demonstrate such heavy antibodies in tetanus antitoxin.

It is possible that certain types of antibody exist in a polymerized state, which might explain the phenomenon of low agglutinability. Such polymers could have the same toxin-neutralizing capacity as unpolymerized antibody and yet because of physical characteristics associated with size and shape, be unable to give proportionate globulin cover to sensitized cells. Dilution of sera containing polymerized antibody would produce a smaller number of agglutinin particles per unit of toxin-neutralizing power than unpolymerized material of the same toxin-neutralizing power, and would thus give a lower agglutinin titre.

Many of the observations on zones of agglutination and the formation of prozones with horse antitoxins in these agglutination tests, and in haemagglutination-inhibition tests, have characteristics associated with precipitin and flocculation reactions. In precipitin reactions, the zone of antibody excess with many horse antibodies such as diphtheria antitoxin (Pappenheimer & Robinson, 1937; Pappenheimer, Lundgren & Williams, 1940), and horse anti-egg albumin and anti-rabbit serum albumin (Treffers, Heidelberger & Freund, 1947) is a zone of inhibition. This has been found to apply also to haemagglutination reactions by direct agglutination with simple dilutions of antitoxin and with toxin-antitoxin mixtures. The most marked prozones occurred with sera of proportionately poor agglutinating power (nos. 7897, 7602 & 8740). Qualitative changes in antibody have been shown to affect prozone formation in precipitin systems. Shibley (1929) produced marked prozone activity in sera modified by heating, and Bawden & Kleczkowski (1942) showed that this was due to the formation of complexes of albumin and antibody globulin. Further evidence that these agglutinating systems behave as though they were precipitin reactions at a cell surface is that

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

the extent of a prozone is increased as the quantity of antigen used for sensitizing the suspensions is reduced, thus maintaining the ratio of antigen to antibody at the end of the zone of inhibition in the region of antibody excess (Table 8). Jenkins (1946) showed that prozones could be produced in non-prozone producing salmonella suspensions by degrading the surface antigens with pyridine, thus increasing the ratio of antibody to antigen. Moreover, when the surface of human red cells was treated with different concentrations of non-agglutinating *Rh* antibodies and the cells exposed to agglutination with rabbit anti-human globulin antiserum, the length of the prozone varied inversely as the amount of sensitizing antigen (Van Loghem, Kresner, Coombs & Roberts, 1950). Thus prozone is a simple expression of antibody excess and it can be abolished by washing unagglutinated cells in the zone of inhibition and resuspending them in saline. It would appear that the qualitative differences in antibody behaviour observed in these haemagglutination reactions, however, markedly affect the results of antibody excess. Horse sera obtained early in immunization do not tend to produce prozone, they flocculate rapidly and over a wide range, and have a good agglutinating power. Conversely horse sera obtained after prolonged immunization produce marked prozones, flocculate more slowly over a narrower range and are of poor agglutinating capacity.

SUMMARY

The behaviour of sheep cells sensitized with tetanus toxins and toxoids in the presence of antitoxin has been investigated.

Sensitized sheep cells were agglutinated in the presence of tetanus antitoxin. The agglutinin titres of such direct agglutinin systems were roughly proportional to the antitoxin content of the sera with some marked exceptions. The discrepancies were associated with the presence of non-specific agglutinins (antibodies other than specific antitoxin) and with the immunization history of the horses providing the sera.

When sensitized cells were used as indicators of the presence of free antitoxin in toxin or toxoid-antitoxin mixtures, the correlation with *in vivo* tests was quite good at the L+ level of test, but not so reliable at the 0·01 L+ level of test.

Sensitized cells preserved with formalin were found to be more reliable and the results with such materials were more reproducible than those obtained with freshly prepared sensitized cells.

Cells sensitized with different concentrations of antigen gave different titres by direct agglutination with simple dilution of antitoxin; this difference was very much less in haemagglutination inhibition tests.

The L_A dose (the volume of toxin or toxoid which when mixed with one unit of antitoxin gave a $++\pm$ end-point) varied according to the sensitivity of the suspension used and the purity of the components of the reacting mixtures.

Prozones (zones of agglutination inhibition in the region of antibody excess) were found to be associated with the immunization history of the horses from which the sera were obtained. In general the more thorough the immunization the longer the prozones found. The length of prozones was proportionately increased

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press

with decrease in the concentration of sensitizing antigen. Possible reasons for the behaviour of certain antitoxins in these tests have been discussed.

I am indebted to Prof. M. Raynaud of the Pasteur Institute, Garches, for the supply of tetanus toxoids of high purity.

I am very grateful to Mr Charles Newman for considerable technical assistance.

REFERENCES

Bawden, F. C. & Kleczkowski, A. (1942). *Brit. J. exp. Path.* **22**, 208.

Borduas, A. G. & Grabar, P. (1953). *Ann. Inst. Pasteur*, **84**, 903.

Boyden, S. V. (1951). *J. exp. Med.* **93**, 107.

Fell, N., Stern, K. G. & Coghill, R. D. (1940). *J. Immunol.* **39**, 223.

Fisher, S. (1952). *J. Hyg., Camb.*, **50**, 445.

Heidelberger, M. & Pedersen, K. O. (1937). *J. exp. Med.* **65**, 393.

Jenkins, C. E. (1946). *Brit. J. exp. Path.* **27**, 127.

Kekwick, R. A. & Record, B. R. (1941). *Brit. J. exp. Path.* **22**, 29.

Landy, M., Trapani, R., Formal, Rosamond & Klugler, I. (1955). *Amer. J. Hyg.* **61**, 143.

Pappenheimer, A. M. & Robinson, E. S. (1937). *J. Immunol*, **32**. 291.

Pappenheimer, A. M., Lundgren, H. P. & Williams, J. W. (1940). *J. exp. Med.* **71**, 247.

Shibley, G. S. (1929). *J. exp. Med.* **50**, 825.

Stavitsky, A. B. (1954). *J. Immunol.* **72**, 360, 368.

Treffers, H. P., Heidelberger, M. & Freund, J. (1947). *J. exp. Med.* **86**, 83.

Van Loghem, J. J., Kresner, M., Coombs, R. R. A., & Roberts, G. F. (1950). *Lancet*, ii, 729.

(*MS. received for publication* 4. I. 57)

https://doi.org/10.1017/S0022172400037293 Published online by Cambridge University Press