THE ESTIMATION *IN VITRO* OF SMALL AMOUNTS OF DIPHTHERIA ANTITOXIN BY MEANS OF A HAEMAGGLUTINATION TECHNIQUE

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The accurate estimation of diphtheria antitoxin is essential for the standardization of commercial antitoxic sera, for evaluating diphtheria prophylactics and investigating antitoxic immunity in diphtheria.

There are three classical methods for the estimation of diphtheria antitoxin. One of these, the flocculation method of Ramon, is carried out *in vitro*. It is usually employed when estimating the antitoxin content of therapeutic antitoxic sera which have been produced in the horse and contain large amounts of antitoxin. The other two methods involve the use of laboratory animals and depend on the neutralization of standard amounts of toxin by the serum under test. In the method of Ehrlich the guinea-pig is the test animal and the dose of toxin injected is at least 1 M.L.D.; in the method of Römer the power of the serum to neutralize the action of intradermally injected diphtheria toxin is determined using either the guinea-pig or the rabbit. These two methods can be used for the estimation of small quantities of diphtheria antitoxin; of the two techniques the intradermal is the more sensitive.

Although the methods of Ehrlich and of Römer, or their modifications, give excellent results, they are costly, slow and depend on the availability of highly potent and accurately standardized toxins.

Boyden (1951) reported that washed sheep erythrocytes treated with dilute solutions of tannic acid can adsorb different proteins, and that cells so treated are agglutinable by antisera specific to the adsorbed protein. Using Boyden's technique diphtheria toxin can be attached to sheep erythrocytes which have been treated with tannic acid. Investigations on such cells and their reactions with immune sera are described in this paper.

MATERIALS AND METHODS

Erythrocytes. Sheep were bled aseptically into modified Alsever's solution (Bukantz, Rein & Kent, 1946). The blood was kept in the cold room for periods up to 4 weeks. Portions were removed as required, the cells sedimented and washed in three changes of physiological saline.

Diphtheria toxin and toxoid. Samples of crude toxin and toxoid, prepared in modified Dernby's veal infusion broth and containing usually about 20 Lf per ml., and a sample of purified and concentrated toxin, prepared in casein hydrolysate medium (Mueller & Miller, 1941), containing 150 Lf per ml., were used.

Sera. A reference serum was prepared in a rabbit. The animal was given six bi-weekly subcutaneous injections of soluble formalinized toxoid, the dose being

https://doi.org/10.1017/S0022172400019732 Published online by Cambridge University Press

gradually raised from 0·5 to 2 ml. After a week's rest the rabbit was bled from the heart. The serum was separated, Seitz filtered, inactivated and stored in the cold room after the addition of merthiolate to give a concentration of 0·01 %.

Blood samples from children were taken by venepuncture. The separated sera were stored in the cold room without the addition of any antiseptic and usually without inactivation.

Treatment of cells with tannic acid. The method of Boyden (1951) was used. A 5 % suspension of washed erythrocytes was prepared in buffered (pH 7·2–7·4) saline. 0·1 g. tannic acid (May and Baker) was dissolved in 100 ml. saline just before use and a 1 in 25 dilution of the solution prepared in saline. Some of this solution was added to an equal volume of the erythrocyte suspension and the mixture placed in the water-bath at 37° C. for 15–30 min. The cells were sedimented on the centrifuge and washed once with saline.

Sensitization of tannic acid-treated cells with diphtheria toxin. 20 parts of toxic filtrate, containing 15–30 Lf per ml., or, when necessary, diluted to that strength, were added to one part of packed, washed, tannic acid-treated red cells and the cells suspended evenly either by gentle shaking or by mixing with a Pasteur pipette. After incubation in the water-bath at 37° C. for 45 min., the cells were spun down on the centrifuge, washed twice in isotonic veronal buffer (Michaelis, 1931) at pH 5·7–6·0 and finally resuspended in saline to the desired concentration for use.

The haemagglutination test. Before titration all sera were absorbed with an equal volume of washed normal sheep erythrocytes to remove naturally occurring anti-sheep red cell agglutinins. Sera which had not been inactivated by heating were absorbed in an ice-water bath; all inactivated sera were absorbed at room temperature. Absorption was allowed to take place for 15–20 min., after which time the cells were removed by centrifugation.

Serial doubling dilutions of the absorbed sera were prepared in saline containing 1 % normal rabbit serum, and aliquots of the sensitized cell suspension added to the tubes containing the serum dilutions. Negative controls were provided by tubes containing only normal or sensitized cells and the diluent and by tubes containing the first dilution of each serum and normal red cells. As a positive control a titration of the rabbit reference serum was included with each batch of titrations of unknown sera. After the contents of the tubes had been thoroughly mixed, the cells were allowed to settle at temperatures ranging from 4 to 37° C., until the sedimentation patterns at the bottoms of the tubes had become sufficiently distinct to permit the reading of the end-points of the titrations.

Because the sensitized red cells often agglutinated in normal saline, normal rabbit serum was added to the saline used for diluting the sera. However, even in 1 % serum saline the sensitized cells frequently showed slight agglutination. Sensitized cells when completely agglutinated by immune sera usually gave fine sedimentation patterns, but occasionally the film of finely agglutinated erythrocytes collapsed before settling completely, producing a coarser arrangement of the deposit.

The preliminary experiments were carried out in Wassermann tubes, using 10 drops of serum dilution and 1 drop of 5 % red cells per tube. Routine titrations

were performed in Dreyer tubes; the sera were diluted in 1 drop volumes with a pipette which was washed with water and then with saline after every two or three dilutions; 1 drop of 1 % red cells was added to every tube.

Titres were expressed in terms of the reciprocal of the final dilution in which complete agglutination of the sensitized cells occurred. In tests in which 1 drop volumes of serum dilutions were used, the increase in volume caused by the addition of the cell suspension was taken into account in calculating titres.

Intradermal antitoxin titrations. These tests were mostly carried out on white rabbits, but occasionally guinea-pigs were used. Saline dilutions of the sera were mixed in equal amounts with Schick test toxin. Schick toxin was used as the test toxin, as it is readily available and is an accurately standardized product; portions of the same batch of toxin were used for all the tests to be described. The mixtures stood on the bench for 2 hr., while combination was taking place. 0·1 ml. of each mixture was then injected into the shaved skin of the animal. Results were read 48 hr. after injection. Erythema exceeding half an inch in diameter and sometimes possessing a haemorrhagic centre was recorded as a positive result; erythema of smaller size, as a partial result; and no visible change, as a negative result.

RESULTS

(*a*) *Observations on the test system*

Effects of alterations of technique. Erythrocytes sensitized with tannic acid and diphtheria toxin as described were agglutinable by the reference serum to its full titre, and were but little susceptible to haemolysis and agglutination in saline. Failure of the sensitized cells to be agglutinated by the reference serum to its full titre was due to the following conditions: tannic acid concentration lower than 1 in 30,000; diphtheria toxin concentration outside the stated limits; pH above 6 of the fluid used for washing the cells after the treatment with toxin. If the concentration of tannic acid exceeded 1 in 10,000 or if the pH of the washing fluid was below 5·5, the sensitized cells were liable to be haemolysed or to be very agglutinable. It was particularly important to have the washing fluid adjusted to the correct pH. Veronal was a satisfactory buffer; other buffers were not tried. Variations within the stated limits of the temperature at which settling of the cells took place in the agglutination test had no effect on the titres obtained.

Attempts at sensitizing cells with diphtheria toxoid. In an experiment, the cells, after treatment with tannic acid and washing, were divided in two portions. One portion was suspended in toxin and the other in toxoid. After incubation and washing parts of both portions were tested with the reference serum for haemagglutination. The cells treated with toxin were agglutinated by the serum, but those treated with toxoid were not.

A second treatment was now carried out on the remainder of both portions of cells. Those cells already sensitized with toxin were treated with toxoid, washed and tested with the reference serum; their behaviour was not affected by the second treatment. Those cells which had been treated with toxoid were suspended in toxin, washed and tested with the reference serum; they were now agglutinable.

It was concluded that, under these experimental conditions, cells treated with tannic acid and capable of adsorbing diphtheria toxin were incapable of adsorbing diphtheria toxoid.

The effect of complement. Addition of fresh guinea-pig complement did not cause haemolysis of the sheep cells at any stage of the sensitization or testing. This result did not conform with the findings of Boyden (1951); differences between the samples of tannic acid used may have accounted for the discrepancy, since, according to Boyden, treatment with tannic acid sufficed to render the cells liable to haemolysis by complement.

Relationship between the antitoxin content of the rabbit reference serum and its power to agglutinate sheep cells sensitized with tannic acid and diphtheria toxin. Neither diphtheria antitoxin nor antibodies capable of agglutinating sensitized sheep cells could be demonstrated in the serum of the rabbit before immunization. After immunization the serum agglutinated optimally sensitized sheep red cells to a reciprocal titre of 200. Its antitoxin content determined by Ehrlich's method was between 1 and 2 units per ml.

Inhibition by diphtheria toxin and toxoid of the sensitized sheep cell haemagglutinating antibody. In reactions between erythrocytes sensitized with bacterial fractions and immune sera specific to the sensitizing agents, neutralization of the haemagglutinating antibodies has been demonstrated by mixing the bacterial fractions with the sera before the addition of the sensitized cells (Keogh, North & Warburton, 1948; Warburton, Keogh & Williams, 1949; Fisher, 1950, 1951; Boyden, 1951).

Doubling dilutions of the rabbit reference serum were prepared in bulk and dispensed in rows of Dreyer tubes in 1 drop volumes. Threefold saline dilutions of crude diphtheria toxin (22 Lf per ml.), purified diphtheria toxin (diluted to contain 15 Lf per ml.), crude formalized diphtheria toxoid (14 Lf per ml.) and of a sample of tetanus toxoid were prepared and 1 drop of each dilution was added to each tube of each row. 1 drop of saline was added to the tubes of a control row. After shaking, the tubes were allowed to stand on the bench for half an hour. Cells which had adsorbed the purified toxin were then added to all the tubes, the contents mixed, the cells allowed to settle and the test read for agglutination.

The results are shown in Table 1. All agents, with the exception of the tetanus toxoid, caused reduction of the haemagglutinating titre of the serum. In terms of Lf per ml., the antibody fixing abilities of the three inhibitory preparations were of the same order; a reduction of titre from 320 to 80, 40 and 20 was caused by the same dilutions of the toxoid and the two toxins. Proportionately more toxin or toxoid was required for the inhibition of larger amounts of haemagglutinating antibody than for the inhibition of small quantities; two minimal haemagglutinating doses were neutralized by 0·02–0·03 Lf of toxin or toxoid per ml.

The agglutination of sensitized red cells by the sera of immunized mice and horses. Sera of mice immunized with diphtheria P.T.A.P. (purified toxoid adsorbed on aluminium phosphate) gave positive haemagglutination tests with clear-cut endpoints; sera of unimmunized mice failed to agglutinate the sensitized cells. Sera of horses undergoing immunization with fluid toxoid gave positive haemagglutina-

tion tests; often, however, the end-point was difficult to determine owing to the presence of partial agglutination in several tubes at the end, or even, occasionally, in the middle of the series of dilutions. An antiglobulin test (Coombs, Mourant & Race, 1945), set up to determine whether the irregular results given by the horse sera were caused by 'incomplete' antibodies, was inconclusive due to equivocal readings in the controls containing normal horse serum.

Table 1. *Neutralization by diphtheria toxin and toxoid of the haemagglutinating antibody in rabbit reference serum*

Neutralizing agent and strength (undiluted)	Crude diphtheria toxin (22 Lf per ml.)	Purified diphtheria toxin (15 Lf per ml.)	Crude formalized diphtheria toxoid (14 Lf per ml.)	Tetanus toxoid	Saline (control)
Dilution of neutralizing agent	Haemagglutinating titre after addition of equal volume of neutralizing agent				
1/1	< 10	< 10	< 10	320	320
1/3	< 10	< 10	< 10		
1/9	< 10	10	< 10		
1/27	10	20	10		
1/81	20	20	20		
1/243	40	40	40		
1/729	80	80	80		
1/2187	320	320	320		

(*b*) *Relationship between the diphtheria content of human sera and their power to agglutinate sensitized sheep cells*

Haemagglutination tests on 175 *sera.* Sixty-five of the specimens were cord sera, obtained at the Women's Hospital, Melbourne. 110 were sera obtained from normal children at a Melbourne children's home. The ages of 18 of the children were 3 years or less; these children were not subjected to Schick testing. The remaining 92 children were between 3 and 17 years old; of these, 50 were Schick-positive and 42 Schick-negative.

The distribution of the titres is shown in Table 2. The titres ranged from less than 2, to 1024. Of the 65 cord sera, 35 showed detectable amounts of antibody. Two sera out of 18 from the children aged 3 years or less, and 8 specimens out of

Table 2. *Haemagglutinating antibody in human sera against erythrocytes sensitized with diphtheria toxin—distribution of titres*

Group	Reaction to Schick test	No. of specimens giving titre of: < 2	2	4	8	16	32	64	128	256	512	1024	Total	G.M. of titres
Cord sera	Not tested	30	1	9	6	4	5	5	3	1	—	1	65	> 4 < 8
Sera of children under 3 years of age	Not tested	16	1	—	1	—	—	—	—	—	—	—	18	< 1
Sera of children between 3 and 17 years of age	Positive	42	3	2	—	1	—	—	1	1	—	—	50	< 1
Sera of children between 3 and 17 years of age	Negative	10	4	9	6	1	4	1	1	4	1	1	42	8
	Total	98	9	20	13	6	9	6	5	6	1	2	175	> 1 < 2

G.M. = geometric mean.

50 from the older and Schick-positive children, gave antibody titres of 2 or more; in two instances the antibody titres of Schick-positive children were 128 and 256, respectively; these specimens will be referred to again. Haemagglutinating antibody could not be demonstrated in 10 of the 40 sera from Schick-negative children in the older age group.

The geometric mean titres in these groups were: cord sera, between 4 and 8; children under 3, less than 1; Schick-positive older children, less than 1; and Schick-negative older children, 8.

Relationship of age of children to haemagglutinating titre of sera. The titres of the 110 sera from normal children were grouped according to the child's age. Antibody was demonstrated in 6 out of 35 sera (17 %) from children aged 5 years or less; in 11 out of 31 sera (35 %) from children between 6 and 10 years old; in 24 out of 44 specimens (54 %) from children 11–17 years of age. The geometric mean titres of the sera containing detectable amounts of antibody were: lowest age group, between 4 and 8; middle age group, between 8 and 16; highest age group, 16 (Table 3).

Table 3. *Relationship between age of children and power of their sera to agglutinate sheep erythrocytes sensitized with tannic acid and diphtheria toxin*

Age (years)	No. of specimens			G.M. of titres of specimens with titres of 2 or more
	Titre < 2	Titre 2 or more	Total	
0–5	29	6	35	< 4 < 8
6–10	20	11	31	> 8 < 16
11–17	20	24	44	16
Total	69	41	110	

G.M. = geometric mean.

Haemagglutination tests on sera of five children suffering from diphtheria. Specimens taken just before and at varying intervals after the administration of therapeutic antitoxic serum were tested. The weights of the children were not known.

No antibody was detected in the sera taken before the injection of the antitoxin. In the other specimens, larger doses of antitoxin and shorter intervals since its administration were associated with higher titres. The average time taken for the disappearance of 50 % of the antibody was 6 days (Table 4).

The positive results in these tests were thought to be due to antibodies passively acquired by the patients.

Relationship of haemagglutinating titre to antitoxin content as determined by intradermal test. In order to investigate the correlation between haemagglutinating titre and antitoxin content, intradermal antitoxin titrations were carried out on 36 of the 110 sera from normal children. The results are shown in Table 5.

Since Schick-test toxin, the toxin used in the tests, is standardized so that 0·2 ml. is neutralized by 0·001 unit of antitoxin, serum containing not less than 0·005 unit of antitoxin per ml. will neutralize an equal volume of Schick-test toxin.

Table 4. *Sera of children suffering from diphtheria: haemagglutinating titres before and after the administration of antitoxic serum*

Case no.	Age (years)	Dosage of diphtheria antitoxin (no. of units)	Interval in days between injection of antitoxin and taking of specimen	Haemagglutinating titre of serum
1	10	4,000	14	16
			26	8
2	5	16,000	7	64
			14	32
			22	8
3	7	60,000	18	64
			39	4
4	11	90,000	7	256
			15	64
			22	32
			31	16
5	10	180,000	19	128

A specimen of blood was taken from every child immediately before the administration of the antitoxin. In all these specimens the haemagglutinating titre was less than 2.

Table 5. *Relationship between diphtheria antitoxin content and titre of haemagglutinating antibody in sera of* 36 *normal children*

Haem-agglutinating titre	Sera of Schick-positive children: Antitoxin content unit(s) per ml.	Sera of Schick-positive children: No. of specimens	Sera of Schick-negative children: Antitoxin content unit(s) per ml.	Sera of Schick-negative children: No. of specimens	Total
<2	$<0{\cdot}005$	11	$<0{\cdot}005$	2	14
			$>0{\cdot}01<0{\cdot}02$	1	
2	$<0{\cdot}005$	3	$<0{\cdot}005$	1	5
			0·04	1	
4	$<0{\cdot}005$	1			6
	0·01	1	0·01	1	
			$>0{\cdot}01<0{\cdot}02$	1	
			0·04	1	
			$>0{\cdot}08<0{\cdot}16$	1	
8			0·01	1	4
			0·02	1	
			0·025	1	
			$>0{\cdot}025<0{\cdot}125$	1	
16	$>0{\cdot}04<0{\cdot}08$	1			1
128			0·125	1	2
	$>0{\cdot}125<0{\cdot}5$	1			
256			0·625	1	3
	$>0{\cdot}625<3{\cdot}125$	1	$>0{\cdot}625<3{\cdot}125$	1	
1024			3·125	1	1
	Total	19		17	36

https://doi.org/10.1017/S0022172400019732 Published online by Cambridge University Press

The actual volume of Schick-test toxin injected in one dose was 0·05 ml. This amount contains several minimal skin reacting doses. A partial reaction, i.e. one showing erythema of less than full size, was taken as an indication that the greater part of the toxin in the test dose had been neutralized, and that the amount of antitoxin in that serum dilution closely approximated to 0·005 unit per ml.

The dilution of serum which, when mixed with 0·05 ml. of the Schick-test toxin, gave a partial reaction in the intradermal test, was therefore assumed to contain 0·005 unit of antitoxin per ml. If this occurred, only one figure is given for the antitoxin content of the serum in Table 5. When one of two successive serum dilutions gave a positive and the other a negative result, with no partial reading intervening, both readings are given.

Of 14 sera with no detectable amount of haemagglutinating antibody only 1 showed antitoxin in excess of 0·005 unit per ml.; it contained between 0·01 and 0·02 unit per ml. Five sera had *in vitro* titres of 2; one of these contained 0·04 unit of antitoxin per ml. and the others less than 0·005 unit per ml. Of 6 sera, possessing haemagglutinating titres of 4, 1 contained less than 0·005 unit of antitoxin per ml. and the others between 0·01 and 0·16 unit per ml. The antitoxin content of 4 sera with a titre of 8, was between 0·01 and 0·125 unit per ml.; of 1 serum, titre 16, between 0·04 and 0·08 unit per ml.; of 2 sera, titre 128, between 0·125 and 0·5 unit per ml.; of 3 sera, titre 256, between 0·625 and 3·125 units per ml.; and that of 1 serum, titre 1024, was 3·125 units per ml.

The haemagglutinating titres corresponded with the intradermal titres better than with the results of the Schick tests. Three sera from Schick-positive children showed *in vitro* titres of 16 or more; they all contained antitoxin in excess of 0·04 unit per ml. It was concluded that in these children the Schick-test readings were 'false positives', since, according to Vahlquist & Högstedt (1949), a positive Schick test indicates that the antitoxin content of the serum is below 0·02 unit per ml.

The results obtained on the 17 sera (Table 5) which gave definite readings in both *in vivo* and *in vitro* tests were analysed statistically as follows. Where the end-point of the intradermal antitoxin titration fell between two readings, the geometric mean of the two antitoxin unit figures was calculated. The titres and the antitoxin unit figures were then transformed into $\log_2$ units, assigning the $\log_2$ value of 1 to a haemagglutinating titre of 2, and to an antitoxin content of 0·005 unit per ml. serum.

The linear regressions between the numbers of antitoxin units per ml., and the haemagglutinating titres were then calculated with the following results:

Regression Y/X: $Y = 0{\cdot}84X + 1{\cdot}35$, $P < 0{\cdot}001$. Slope of line and S.E. of slope $b = 0{\cdot}84 \pm 0{\cdot}12$;

Regression X/Y: $X = 0{\cdot}95Y - 0{\cdot}41$, $P < 0{\cdot}001$. Slope of line and S.E. of slope $b = 1{\cdot}05 \pm 0{\cdot}13$;

where X = haemagglutinating titre, and Y = antitoxin content in units per ml., both transformed into $\log_2$ units as described.

In a test of this nature, the slope of the regression line has the value of 1 if the parameters measured by the two methods are exactly identical. The values ob-

tained above did not differ significantly from unity. These results, as well as the high significance of the regressions, indicated that the correlation between the *in vitro* and *in vivo* tests was close, and it was concluded that the haemagglutinating titre was a measure of the antitoxin content of the serum.

The value of the slope was taken as 1 for calculating antitoxin content from haemagglutinating titre. The factor required for this calculation was derived from the ratio of the geometric mean of the bench titres and the geometric mean of the antitoxin unitages. A titre of 254 thus corresponded to 1 unit of antitoxin per ml. and a titre of 2, the smallest detectable amount of haemagglutinating antibody, indicated an antitoxin content of 0·0078 unit per ml. serum. There was good agreement between the latter figure, which represented the sensitivity of the test, and the antitoxin content of the reference rabbit serum estimated from its *in vitro* titre.

Reliability of the haemagglutinating titre of a single serum specimen as a measure of its antitoxin content. This factor was assessed by applying the relationship described to the data of Table 5. Where the determined antitoxin content fell between two values, their geometric mean was used. The determined and the expected values were then compared at the corresponding *in vitro* titre level. The differences were expressed in terms of $\log_2$ units, adjusting the values, where necessary, to the nearest unit; the $\log_2$ value of 0 was used when the amount of antibody was below the detectable level in either test. The incidence of the differences is shown in Table 6.

Table 6. (Derived from Table 5.) *Antitoxin content of children's sera: differences between values determined by intradermal tests and values calculated from haemagglutinating titres*

	Amount of antibody in sera		
	Insufficient to give definite reading in *in vitro* or *in vivo* test or in both	Sufficient to give definite readings in both tests	
Differences in $\log_2$ units	No. of observations		Total
0	13	6	19
±1	4	7	11
±2	2	3	5
±3	0	1	1
Total	19	17	36

The method of calculating antitoxin content from haemagglutinating titre is described in the text.

In 13 of the 17 sera which gave definite readings in both tests, the divergence between the calculated and determined antitoxin values did not exceed 1 $\log_2$ unit; 17 of 19 sera in the other group behaved similarly. Altogether, therefore, in 30 of 36 sera (83 %) the deviation was not greater than the error of titrations using the technique of doubling dilutions. The maximum deviation, 3 $\log_2$ units, was observed on 1 specimen (3 %).

Antitoxin content of human sera. Using the relationship referred to above, the mean antitoxin titres of the groups of human sera in Table 2 were calculated. The results are:

Group	Units of antitoxin per ml. serum
Cord sera	Between 0·016 and 0·03
Sera of children under 3 years of age	Less than 0·008
Sera of Schick-positive children, aged between 3 and 17 years	Less than 0·008
Sera of Schick-negative children, aged between 3 and 17 years	0·03

Of 65 cord sera, 46 (71 %) contained 0·03 unit of antitoxin per ml. or less, 4 (6 %) contained 0·06 unit, 5 (7 %) 0·12 unit, 5 (7 %) 0·25 unit, 3 (5 %) 0·5 unit, 1 (1·5 %) 1 unit and 1 (1·5 %) 4 units per ml.

DISCUSSION

It was known from previous experience (S. Fisher, unpublished work) that normal, washed sheep erythrocytes did not adsorb diphtheria toxin. If, however, the cells were subjected to a preliminary treatment with a dilute solution of tannic acid, according to the technique of Boyden (1951), adsorption of the toxin to the cells took place readily.

Erythrocytes so treated with tannic acid and diphtheria toxin were agglutinable by the serum of a rabbit immunized with soluble diphtheria toxoid, by the sera of horses and of mice immunized with diphtheria toxoid in different forms, and by a proportion of children's sera and human cord sera, as well as by sera of children suffering from diphtheria and treated with antitoxic serum.

Tetanus toxin, with the commonly associated natural haemolysin for sheep red cells removed or inactivated, could also be adsorbed to tannic acid-treated cells, rendering the latter agglutinable by specific antisera (S. Fisher, unpublished work).

Diphtheria toxoid was not adsorbed to tannic acid-treated cells under the same conditions as the toxin. The presence of toxoid should, therefore, not affect the suitability of a toxin for sensitizing tannic acid-treated erythrocytes to haemagglutination by antitoxin. Both toxin and toxoid were capable of neutralizing the haemagglutinating antibody in the rabbit serum used as a reference standard, and their inhibitory powers were proportional to their strengths in terms of Lf units per ml.

Intradermal antitoxin titrations were carried out on a number of children's sera, selected from a group previously submitted to estimations of haemagglutinating antibody. Statistical examination of the results showed that (1) the titre of the haemagglutinating antibody was a measure of the antitoxin content; (2) the haemagglutinating titre of 2, the lowest detectable titre in the *in vitro* test, corresponded to an antitoxin content of 0·0078 unit per ml. This ratio also applied when the *in vitro* titre of the rabbit reference serum was compared with its antitoxin content as determined by the guinea-pig lethal method.

The method described in this paper for titrating diphtheria antitoxin *in vitro* enables amounts to be estimated which are almost as small as the Schick sensitivity level, stated to be between 0·002 and 0·004 unit per ml. (Vahlquist & Högstedt, 1949; Barr, Glenny & Randall, 1950). In tests on sera of a group of people, the mean result can be expected to be accurate. It cannot be expected that the same level of accuracy would be reached in single titrations; however, the correlation between the results of *in vitro* and *in vivo* tests was close in most of the individual specimens tested.

SUMMARY

1. Treatment of washed sheep erythrocytes with dilute solutions of tannic acid rendered them capable of adsorbing diphtheria toxin from solution. Diphtheria toxoid was not adsorbed under similar conditions.

2. Cells thus sensitized with diphtheria toxin were agglutinable by a proportion of children's sera and human cord sera; sera of rabbits, mice and horses, immunized with soluble or adsorbed diphtheria toxoid, also agglutinated sensitized cells, as did also the sera of children suffering from diphtheria and treated with antitoxic serum. Clear-cut results were usually obtained, but the end-point of titrations on horse sera was, in some cases, difficult to determine.

3. Using intradermal toxin neutralization tests, a highly significant linear relationship was demonstrated between the haemagglutinating titres of children's sera for the sensitized red cells, and the antitoxin content of their sera. The antitoxin content corresponding to the smallest detectable amount of haemagglutinating antibody was 0·008 unit per ml. serum.

4. The haemagglutinating titre was an accurate measure of the mean antitoxin level of a group of sera and a close measure of the antitoxin content of most specimens in individual tests.

The author is indebted to Miss May Kennedy and Mr R. W. Patterson for the supply of toxins and toxoids and for the performance of the guinea-pig lethal test; to Dr J. J. Graydon for the statistical work; to Dr Bell Brodrick for results of Schick tests; to Dr A. A. Ferris for the supply of the sera of children suffering from diphtheria; and to Dr E. V. Keogh for advice.

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(*MS. received for publication* 1. I. 52)