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A Reproducible Method for the Removal of Non-Specific Flocculating Antibodies from Tetanus Antitoxin Serum

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SUMMARY

The culture filtrate of a toxinogenic strain of *Cl. tetani* grown in medium supplemented with excess glutamate was proved by the Ouchterlony's immunodiffusion test to consist mostly of antigenic components other than toxin. On the basis of this finding, an attempt was made to remove all non-specific flocculating antibodies from pepsinized horse tetanus antitoxin serum by absorption with concentrated culture filtrate. The removal of the antibodies was effected by a stepwise absorption procedure and was found to be nearly complete after two or three absorption steps. The final absorbed serum either gave almost a single precipitation line in an Ouchterlony plate or a single flocculation zone in test tubes with crude tetanus toxin or toxoid preparations. The yield of the absorbed serum was more than 90 per cent of the original unabsorbed serum in terms of antitoxin units. Evidence was obtained for the existence of a linear relationship between the L + /10 doses of various toxin preparations and their Lf values titrated with absorbed serum by Ramon's method. It was shown that the excess antigenic components remaining in the absorbed serum could be partially removed by ammonium sulfate fractionation and almost completely removed by zone-electrophoresis. Removal of the excess antigen from the serum resulted in a shortening of the flocculation time (Kf).

INTRODUCTION

Unlike the case with diphtheria, when tetanus toxin or toxoid is titrated with an ordinary antitoxin serum by Ramon's method, it is frequently very difficult to distinguish a specific flocculation with toxin and antitoxin from those with other antigenic components and their antibodies. As reported previously by many workers (Ramon, 1937, Prevot, 1938, Glenny and Stevens, 1938, Goldie *et al.*, 1942, Moloney *et al.*, 1944), this has become a major obstacle in *in vitro* titration of tetanus toxin or toxoid. Among the methods to be considered in approaching this problem, the best, of course, would be to obtain tetanus antitoxin serum in which specific antitoxin is the sole precipitating antibody. Thus, the flocculation occurring on mixing this serum with any tetanus toxin or toxoid would be due

to a specific toxin and antitoxin reaction. In practice, there appear to be two possible ways to prepare such serum; first, by immunizing animals with highly purified tetanus toxoid and toxin preparations and second, as has been attempted by several workers (Moloney *et al.*, 1944, Hirabayashi *et al.*, 1953 and Murata *et al.*, 1953), by absorbing an ordinary horse tetanus antitoxin serum with an appropriate tetanus antigen preparation which has been proved to contain all antigenic components other than the specific toxin. Evidently, the former way depends on the purity of the toxin or toxoid and the latter, entirely on the antigenic constitution of the material used for the absorption. Recently, the authors have found (1964) that excess supplementation of sodium glutamate to medium markedly suppresses toxin production by a toxinogenic strain of *Cl. tetani* and the culture filtrate of this medium, which contains negligible amounts of tetanus toxin, has the same antigenic composition as that of crude toxin preparations. On the basis of this finding, attempts have been made to employ this filtrate as antigen for absorption and to remove all detectable non-specific flocculating antibodies from horse tetanus antitoxin serum. The present paper describes the absorption procedure adapted for this filtrate and data on some immunochemical analyses of the absorbed serum. Results of experiments to remove excess antigenic components remaining in the serum after the absorption are also presented.

MATERIALS AND METHODS

1. *Tetanus toxin*

A substrain (Biken) of the Harvard A 47 strain was grown in P-11 medium (Kubota *et al.*, 1960) at 35°C for 6 days. At the end of this period, the whole culture was centrifuged at 8,000 rpm for 30 minutes and the supernatant was then filtered. The filtrate was stocked in the cold after thorough mixing with a small amount of toluene and usually employed as the toxin. The toxicity of the toxin was expressed as L +/10/ml using mice of Japanese stock (corresponding to the dd line) of 12g average weight

2. *Tetanus toxoid*

Toxoid was prepared by detoxifying the toxin with 0.4 per cent formalin at 35°C for 2 weeks.

3. *Antigen for absorption (Antigen-ab)*

A substrain (Biken) of the Harvard A 47 strain was grown at 35°C for 6 days in P-II medium supplemented with 1 per cent sodium glutamate. The culture filtrate obtained after centrifugation and filtration was concentrated with a solution of Carbowax 20,000 (polyethylene glycol) to 1/10th of the original volume of the filtrate. The concentrate, which had a toxin content of less than 100 in terms of L + /10/ml was usually used as the antigen for absorbing the tetanus antitoxin serum. For the flocculation and immunodiffusion tests, the original unconcentrated culture filtrate (*Filtrate-al*) was usually employed as control antigen.

4. *Tetanus antitoxin serum subjected to absorption (Unabsorbed serum)*

Horse tetanus antitoxin serum (Batch No. 128, 700 antitoxin units/ml) which was pepsinized and partially purified by ammonium sulfate fractionation was subjected to absorption with the material described above. Titration of antitoxin in the serum before and after the absorption was carried out according to the method described in the Minimum Requirements for Biologic Products of Japan

(1963), using standard toxin (TA-No.2) and antitoxin (5 international units/ml) provided by the National Institute of Health of Japan.

5. *Flocculation test*

The flocculation test was usually carried out by Ramon's method. Thus, a constant volume (1.0 ml) of antigen was added to varying volumes of antitoxin serum and physiological saline was then added to make up a constant volume. Incubation of the mixture of antigen and antitoxin was ususally carried out at 42°C in a water bath. The preliminary test for calculating the necessary volume of the *Antigen-ab* to be added to the serum for absorption was done by adding a constant volume of the serum to varying volumes of the antigen.

6. *Immunodiffusion test*

The double diffusion precipitation method in agar described by Ouchterlony (1948) was employed with a slight modification. The results were usually read after standing the agar plate at 30°C for one week.

RESULTS

1. *Antigenic properties of the culture filtrate derived from sodium glutamate supplemented medium*

Before employing *Antigen-ab* as an antigen for absorption, the unconcentrated culture filtrate from sodium glutamate supplemented medium was compared with a crude tetanus toxin preparation in terms of its precipitating activity with *Unabsorbed serum* (Batch No. 128) by both Ramon's flocculation and Ouchterlony's imm-

Table I. Flocculation of Toxin and Antigen-ab with Unabsorbed Serum

Unabsorbed serum	10u	20	30	40	50	60	70	80
Antigen-ab	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		F_1 (5')				F'_1 (10')		
*Toxicity of Supernatant	—	—	—	—	—	—	—	—
Toxin	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		F_1 (10')				F_1' (15')		
*Toxicity of Supernatant	$+_4$	—	—	—	—	—	—	—

F_1 or F_1' : Initial flocculation tube (Kf)

* Toxicity was examined by injecting 0.4 ml of the supernatants into mice

\+ death, number indicates survival days.

— no toxicity

Toxin: 160 L + /10/ml

unodiffusion method. The content of toxin in the culture filtrate was less than 10 L + /10/ml and the toxin was 160 L + /10/ml.

In Ramon's flocculation method, both the culture filtrate and toxin exhibited double flocculation zones and the initial flocculation took place, in both cases, in the tube containing 20 and 60 units of antitoxin, respectively. The supernatants of these initial tubes were found to be antitoxic by *in vivo* tests both in the case of the culture filtrate and of the toxin, while that of the tube containing 1 ml of toxin and 10 units of antitoxin was slightly toxic. This result is shown in Table 1.

A similar experiment was done with these antigens after they had been heated at 63°C for 15 minutes to test the heat stability of their precipitating activity. As may be seen in Table 2, no flocculation occurred in the tubes containing around 60 units of antitoxin but it took place in the tubes containing around 20 units of antitoxin. This clearly indicates that the former flocculation is due to heat labile antigens and the latter to heat stable antigens.

Table 2. Flocculation of Heated Toxin and Antigen-ab with Unabsorbed Serum

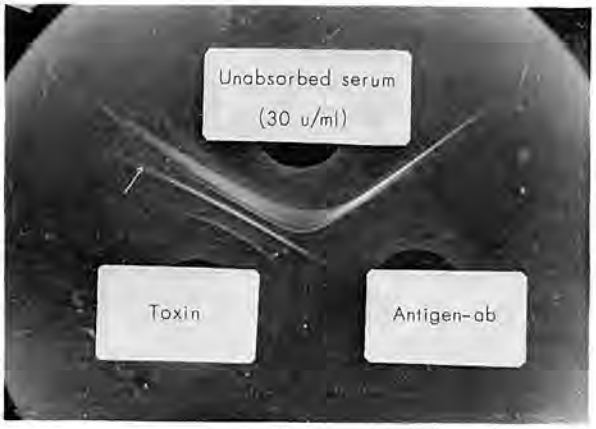
Unabsorbed serum	0u	20	30	40	50	60	70	80
Antigen-ab Heated	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
						F_1' (10) heat stable		
Toxin Heated	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
						F_1' (10) heat stable		

F_1 : Initial flocculation tube (Kf)
Toxin aud Antigen-ab were heated at 63°C for 15 minutes

The result of Ouchterlony's immunodiffusion test is illustrated in Photograph 1.

As may be seen in this photograph, there were many precipitation lines between the *Unabsorbed serum* (30 units/ml) and toxin or culture filtrate wells and, except for one line, all the lines of toxin fused completely with those of culture filtrate. Between the culture filtrate and the serum wells, no line corresponding to the exceptional precipitation line described above was detectable. This indicates that the content of the antigen relevant to the line in the culture filtrate is much lower than that of the toxin and further suggests that it may be specific for toxin.

To see whether this particular precipitation line was due to a true toxin and antitoxin reaction, an experiment was made as follows; a small aliquot of agar was taken from both the serum and toxin sides nearest this precipitation line and the toxicities of these aliquot were examined by injecting them subcutaneously into mice. It was found that mice injected with agar from the toxin side all showed typical tetanus intoxication and died, while mice receiving agar from the serum side did not show any symptoms in mice. This indicates that the above precipi-



Photograph 1
Agar Gel Diffusion Pattern Demonstrating the Antigenic Relationship between Toxin and Antigen for Absorption
The arrow indicates the line of the toxin-antitoxin reaction.
Toxin: 160 L + /10/ml
Antigen-ab; Original culture filtrate from glutamate supplemented medium

tation line may represent a true toxin-antitoxin precipitation reaction.

2. *Absorption procedure*

The absorption of *Unabsorbed serum* (Batch 128, 700 units/ml) with *Antigen-ab* described previously was carried out stepwise two or three times and the changes in the antibody composition of the serum were examined after each absorption step by both Ramon's flocculation and Ouchterlony's immunodiffusion method. Appropriate volumes of *Antigen-ab* to be added to *Unabsorbed serum* for the absorption were calculated from the result of a preliminary precipitation reaction using a constant volume of the serum and varying amounts of antigen. Table 3 gives the result

Table 3. Preliminary Precipitation Test

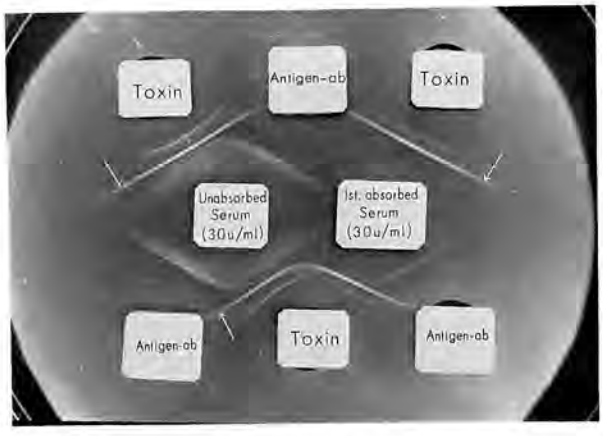
Tube No	1	2	3	4	5	6	7	8	9
Unabsarbed Serum (40u/ml)	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Antigen-ab*	0.06ml	0.09	0.14	0.20	0.30	0.45	0.68	1.0	1.5
P.B.S.	0.94ml	0.91	0.86	0.80	0.70	0.55	0.32	0	0
Precipitation	−	±	++ ↑ heat stable	+	+	++ ↑ heat labile	±	−	−

* Antigen-ab : ×10 concentrated (<100 L +/10 /ml)
− ±, +, ++ : degree of precipitation.

of a preliminary precipitation test showing that maximal precipitation took place in tubes 3 and 5. A similar experiment using heat treated antigens showed that the former precipitation was due to heat stable antigens and the latter to heat labile ones.

a) *First absorption*

Based on the volumes of *Antigen-ab* and *Unabsorbed serum* in tube 3, 280 ml of *Antigen-ab* was mixed with 114.3 ml of *Unabsorbed serum.* The mixture was incubated at 37°C for one hour and then in the cold for 2 days. The mixture was then centrifuged in the cold and the resulting supernatant was employed as the *1st absorbed serum.* In the *1st absorbed serum,* 190 units/ml of antitoxin remained, the yield thus being 93.6 per cent. Comparison of the absorbed serum with *Unabsorbed serum* by immunodiffusion in an Ouchterlony plate indicated that the antibodies precipitating with heat stable antigens were removed from the *Unabsorbed serum* by the 1st absorption. This is shown in Photograph 2.



Photograph 2
Agar Gel Diffusion Pattern Demonstrating the Effect of the 1st Absorption
The arrows indicate the lines of the toxin-antitoxin reaction
Toxin: 160 L + /10/ml
Antigen-ab: Original culture filtrate from glutamate supplemented medium

This photograph shows that there were four less precipitation lines between the toxin and *1st absorbed serum* than between the toxin and *Unabsorbed serum* wells. In a separate experiment, these four lines were not found to disappear after heating the toxin or *Antigen-ab* at 63°C for 15 minutes, indicating that heat stable antigens are responsible for the formation of the four precipitation lines.

Table 4 illustrates the result of Ramon's flocculation test with the *1st absorbed serum* and toxin or *Antigen-ab.*

It will be seen that initial flocculation takes place only in the tube containing

Table 4. Flocculation of Toxin and Antigen-ab with 1st Absorbed Serum.

1st absorbed serum	1.0u	20	30	40	50	60	70	80
Antigen-ab	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
flocculation		F_1 (5') heat labile						
Toxicity of ※ supernatant		—	—	—	—	—	—	—
Toxin	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
flocculation		F_1 (10') heat labile.						
Toxicity of ※ supernatant		$+_4$	—	—	—	—	—	—

F_1 : Initial flocculation tube (Kf)
※ Toxicity was examined by injecting 0.4 ml of the supernatants into mice
+ death, number indicates survival days.
— no toxicity
Toxin : 160 L +/10/ml

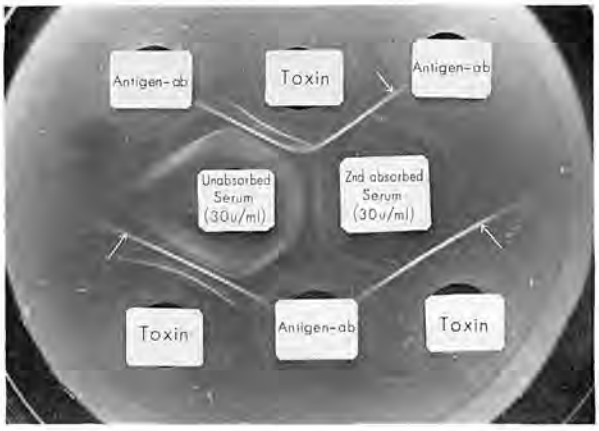
20 units of antitoxin and not in the 6th tube in which antother flocculation would occur in the case with *Unabsorbed serum*, as shown in Table 1. The results of both Ramon's flocculation and Ouchterlony's immunodiffusion analysis clearly indicate that antibodies against heat stable antigens can be effectively removed from *Unabsorbed serum* by the 1st absorption.

b) *Second absorption*

To remove the remaining antibodies other than antitoxin, the *1st absorbed serum* was again absorbed with *Antigen-ab*. In this case, 70 ml of *Antigen-ab* was thoroughly mixed with 100 ml of *1st aborbed serum* because of the relative volumes of *Antigen-ab* and *Unabsorbed serum* in tube 6 in Table 2. The mixture was incubated at 37°C for one hour and then in the cold for 2 days. It was then centrifuged in the cold and the supernatant thus obtained was employed as the *2nd absorbed serum*. The yeild of this serum was about 92 per cent of the original *Unabsorbed serum* in terms of antitoxin units.

The effect of the 2nd absorption on the removal of antibodies other than antitoxin from the *1st absorbed serum* was examined, as described above, by both Ramon's flocculation and Ouchterlony's immunodiffusion anlysis. The results are given in Table 5 and Photograph 3, respectively.

As may be seen in the photograph, the *2nd absorbed serum* (30 units/ml) gave a strong precipitation line with toxin and no line corresponding to this was detectable between the serum and *Antigen-ab* wells. Careful inspection of the agar plate, however, showed that there was a very faint line between the serum and *Antigen-ab* wells and that this line fused with a faint line formed between the toxin and serum



Photograph 3
Ager Gel Diffusion Pattern Demonstrating the Effect of the 2nd Absorption
The arrows indicate the lines of the toxin-antitoxin reaction
Toxin: 160 L + /10/ml
Antigen-ab: Original culture filtrate from glutamate supplemented medium

wells which was closely adjacent to the strong precipitation line. The toxicities of the agar on both sides of the strong precipitation line were examined, as described above. It was found that only the mice injected with the agar from the toxin side died as a result of typical tetanus intoxication.

Table 5. Flocculation of Toxin and Antigen-ab with 2nd Absorbed Serum

2nd absorbed serum	6u	8	10	12	14	16	18	20
Antigen-ab	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Flocculation	undetectable							
Toxicity of supernatants	—	—	—	—	—	—	—	—
Toxin	1.0ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Flocculation			F_1 (115)					
Toxicity of supernatants	$+_1$	$+_2$	$+_4$	—	—	—	—	—

F_1 : Initial flocculation tube
Toxicity was examined by injecting 0.4ml of the supernatants into mice
+ death, numbers indicate survival days.
— no toxicity
Toxin : 160 L +/10/ml

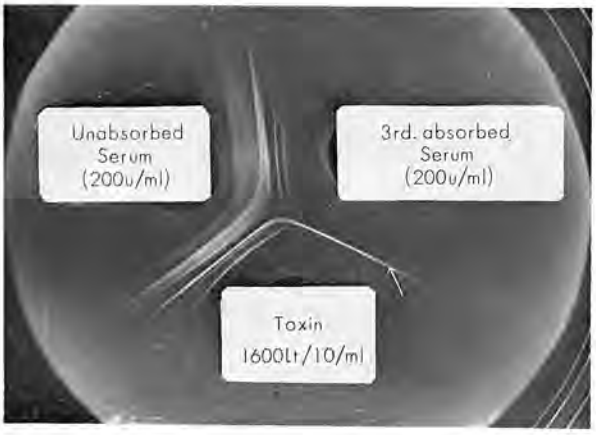
Ramon's flocculation test demonstrated that the *2nd absorbed serum* gave a single flocculation zone with toxin while no flocculation took place with *Antigen-ab*

using the proportions of antigen and serum described in Table 5. As is seen in this table, the initial flocculation was observed in the tube containing 10 units of antitoxin and 160 L + /10 of toxin. Animal tests showed that the supernatant of this tube was still slightly toxic indicating the occurrence of flocculation near an antigen excess zone. However, the toxin content of the supernatant was so small (about $1/10^5$ of the toxin added to the tube) that the unneutralized toxin may be either a contamination obtained during the procedure or be dissociated from the toxin-antitoxin complexes.

The results described above clearly suggest that nearly all the antibodies other than antitoxin in *Unabsorbed serum* are removed so satisfactorily after the 2nd absorption that the floccul tion of a crude toxin or toxoid with the *2nd absorbed serum* represents only the true toxin-antitoxin precipitation reaction. Actually, as will be described in a later section, a quantitative relationship has been found between the *in vivo* titers (L + /10 or Lb) of various toxin or toxoid preparations and their Lf values titrated with the *2nd absorbed serum.*

c) *Third absorption*

Although the *2nd absorbed serum* described above has been shown to give perfectly reliable antitoxin serum for routine *in vitro* titration of toxin or toxoid, it in fact still contains traces of antibodies other than antitoxin which can be detected by the immunodiffusion method. To remove these antibodies, the serum was absorbed again with *Antigen-ab*. Before this, the *2nd absorbed serum* was concentrated by precipitation at 50 per cent saturation of ammonium sulfate. The antitoxin titer of the resulting concentrated solution was 420 units per ml. Then, based on the



Photograph 4
Agar Gel Diffusion Pattern Demonstrating the Antibody Composition of the 3rd Absorbed Serum
The arrow indicates the line of the toxin-antitoxin reaction
Toxin: Concentrated with Carbowax 20,000

equivalent proportion of *Antigen-ab* to the *2nd absorbed serum* (concentrated with ammonium sulfate, 420 units) obtained from a preliminary flocculation test, 50 ml of the *Antigen-ab* were added to an equal volume of the concentrated *2nd absorbed serum* with thorough mixing. The mixture was incubated at 37°C for one hour and then in the cold for 2 days. The supernatant obtained from the mixture by centrifugation was finally employed as the *3rd absorbed serum*. There was no appreciable loss of antitoxin by this absorption procedure. Photograph 4 illustrates the result of the 3rd absorption demonstrated in an Ouchterlony's agar plate.

As may be seen in the photograph, the *3rd absorbed serum* (200 units/ml) gave only one strong precipitation line with concentrated toxin (1600 L + /10/ml). Between the *Unabsorbed serum* and *3rd absorbed serum* wells, there appeared many precipitation lines and these lines fused completely with those between the *Unabsorbed serum* and toxin wells indicating the presence of excess antigenic components in the *3rd absorbed serum* introduced by both the 2nd and 3rd absorption procedure. Incidentally, the *3rd absorbed serum* gave no detectable precipitation line even with concentrated *Antigen-ab*. These results clearly indicate that the removal of traces of non-specific antibodies can be accomplished by the 3rd absorption.

Removal of antibodies other than antitoxin from *Unabsobed serum* by stepwise absorption was further confirmed by carrying out a quantitative precipitation test with the *3rd absorbed serum* and concentrated crude toxin. The serum employed in this test was fractionated and concentrated with 30-45 per cent saturation of ammonium sulfate. The antitoxin content of the serum was 200 units per ml. The crude toxin used contained 0.875 mg protein N per ml and 700 Lf per ml determined with the *3rd absorbed serum*.

The quantitative precipitation test was done as described by Heidelberger and Kendall (1935) with a slight modification. Thus, to a series of 12 centrifuge tubes

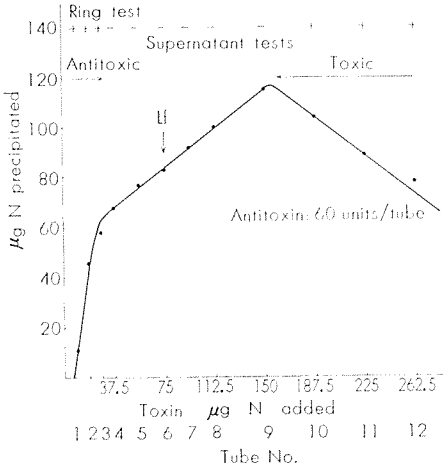


Fig. 1. Quantitative Precipitation of a Crude Tetanus Toxin with 3rd Absorbed Serum

were added increasing amounts (from 9.4 to 262.5 μg N) of toxin in a final volume of 0.3 ml and a constant volume (0.3 ml) of the *3rd absorbed serum* fraction was then added to each tube with thorough mixing. The mixtures were placed in a water bath at 37°C for 1 hour and then in the cold for 3 days. Then they were centrifuged and the resulting supernatants were used for assay of the antigen or antibody remaining unprecipitated. The precipitates were washed three times with 2 ml of cold saline and then their nitrogen contents were determined by a modification of the Kjeldahl Nessler method (Yokoi and Akashi, 1955).

Figure 1 summarizes the results obtained. It may be seen from this figure that, with between 37.5 and 150 μgs of total protein nitrogen added, the precipitable protein shows a linear increase and no detectable amounts of precipitating antigen or antibody remain in the supernatants in the linear region. This clearly indicates that this region is the zone of equivalence. Examination of the toxin and antitoxin in all the supernatants by *in vivo* tests also shows that this is the case. The supernatant of tube 9 corresponding to the end of the equivalence zone was found to be slightly toxic. However, the toxin content was less than $1/10^4$ of the toxin added and therefore the toxicity may possibly be accounted for by a trace of toxin contaminating or dissociated from toxin-antitoxin complexes during this procedure. The arrow in Figure 1 indicates the tube in which the initial flocculation of the toxin used would occur by Ramon's method with the amount of antitoxin (60 units) employed in the quantitative precipitation test. It will be seen that the flocculation point (tube 6) is in the region of equivalence indicating that the flocculation is due to a true reaction of toxin and antitoxin. From the slope of the linear precipitation curve, it can be calculated that about 41 per cent of the protein added was specifically precipitated by the antitoxin serum.

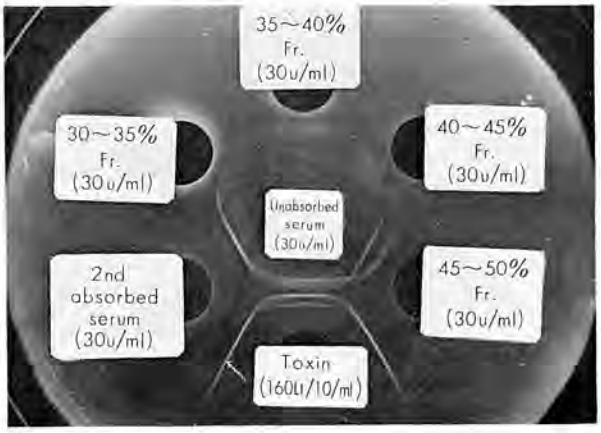
3. *Removal of the excess antigen from the absorbed serum*

Considering the absorption procedure described above, it is readily understandable that a considerable amount of the heat stable antigens in *Antigen-ab* should remain as excess antigens in the *2nd* or *3rd absorbed serum* and in fact this is so, as seen in Photograph 4. Moreover, in our experience, the presence of such excess antigens in the serum appears greatly to delay the time (Kf) of flocculation, particularly when antigen of low toxin content is used. Therefore, attempts were made to remove the excess antigens from the absorbed serum by the following three methods.

1) *Ammonium sulfate fractionation*

To the *2nd absorbed serum* were added appropriate volumes of saturated ammonium sulfate solution (pH 7.0) and the precipitates obtained by centrifugation were dissolved in M/75 phosphate buffered saline (pH 7.2). These solutions were dialyzed against M/75 phosphate buffered saline and antitoxin titers of the dialyzed materials were assayed by *in vivo* tests. Then these materials were each subjected to both Ouchterlony's immunodiffusion and Ramon's flocculation test. The results are shown in Tables 6, 7 and Photograph 5.

This photograph shows that removal of excess antigens from the serum was par-



Photograph 5
Agar Gel Diffusion Pattern Demonstrating the Effect of Ammonium Sulfate Fractionation of the Removal of Excess Antigens
The arrow indicates the line of the toxin-antitoxin reaction

tially effected by ammonium sulfate treatment. Thus, the precipitation lines between *Unabsorbed serum* and the 30-35, 35-40 and 40-45 % fractions became much fainter than those between the *Unabsorbed* and original *2nd absorbee serum* wells. Furthermore, the former lines were considerably nearer to the *Unabsorbed serum* well than the latter lines. This indicates that the contents of excess antigens in these serum fractions are much less than that in the unfractionated serum.

Table 6. Ammonium Sulfate Fractionation of Antitoxin

Fraction. % saturation of $(NH_4)_2SO_4$	Before fractionation units/ml × vol (ml)	After fractionation units/ml × vol (ml)	Yield % *
30—35 %	110×50	70×5	6.4
35—40 %		220×5.3	21.3
40—45 %		300×5.1	24.8
45—50 %		210×5.2	19.8
			72.3

* The yields are expressed as percentages of the original 2nd absorbed serum in terms of antitoxin units.

Tables 6 and 7, respectively, show the yields of the various serum fractions and their flocculations with a crude toxin preparation. Table 7, shows that the time (Kf) for flocculation of the serum is much shortened by fractionation with low concentrations of ammonium sulfate.

2) *Column-chromatography on Sephadex G-200*

The *2nd absorbed serum* was concentrated by precipitation at 50 per cent saturation of ammonium sulfate. Then 1 ml was applied to a column (1.3 × 95 cm)

Table 7. Flocculation of Toxin and Various Ammonium Sulfate Fractions of 2nd Absorbed Serum

Toxin	2nd absorbed serum		Flocculation				Kf(min)
1.0ml	% Ammonium sulfate	30~35	6 units	10 F_1	14	18	22'
			$+_1$	$+_4$	−	−	
		35~40	6 units	10 F_1	14	18	45'
			$+_1$	$+_4$	−	−	
		40~45	6 units	10	14	18	95'
			$+_1$	$+_4$	−	−	
		45~50	6 units	10 F_1	14	18	145'
			$+_1$	$+_3$	−	−	
	Unabsorbed serum		6 units	10 F_1	14	18	115'
			$+_1$	$+_4$	−	−	

F_1: Initial flocculation tube
Toxicity: +death, number indicates survival days; −no toxicity
Toxin: 160 L+/10/ml

of Sephadex G-200 and eluted with M/75 phosphate buffered saline (pH 7.2) at a flow rate of 3.3 ml per hour.

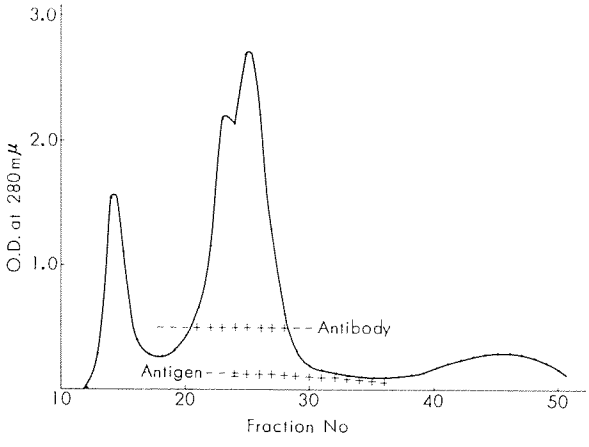


Fig. 2. Sephadex G-200 Column-Chromatogram of 2nd Absorbed Serum

Figure 2 summmarizes the results obtained. Two protein peaks appeared in the chromatogram and most of the excess antigens were present in the second of these which was mainly composed of antitoxin. Thus, it would be very diffucult

to separate the antigens from the antitoxin in the *2nd absorbed serum* by this procedure.

3) *Zone-electrophoresis*

Zone-electrophoresis using starch as a supporting medium was carried out in a horizontal type apparatus as described by Kunkel and Slater (1952) with a slight modification. Concentrated *2nd absorbed serum* was dialyzed against veronal buffer (pH 8.6, $\mu = 0.1$). Then 3.0 ml of this solution (1.0 per cent protein) was used. The electrophoretic pattern obtained in the packed starch plate is shown in Figure 3.

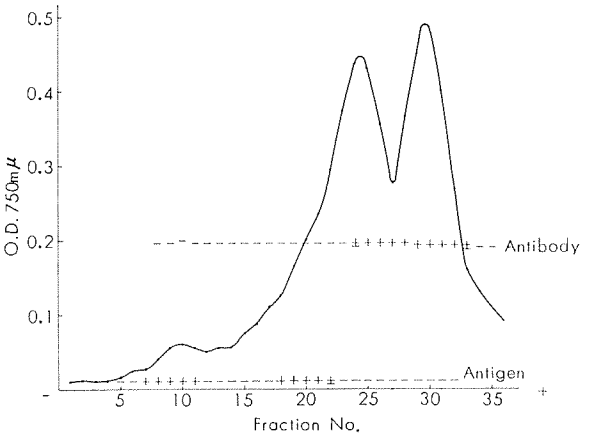


Fig. 3. Zone-electrophoretic Diagram of 2nd Absorbed Serum

As is seen from this figure, one minor and two major protein peaks appeared.

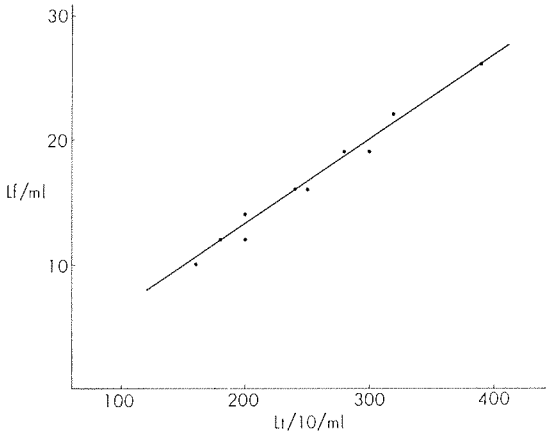


Fig. 4. Relation between the L + /10 Values of Various Toxins and their Lf Values Determined with 2nd Absorbed Serum

Antitoxin was detectable only in the last major peak by the ring test, while excess antigens were detected both in the minor peak and in the more slowly moving part of the 1st major peak. This clearly indicates that excess antigenic components in the *2nd absorbed serum* can be separated by zone-electrophoresis without affecting the antitoxin in the serum.

4. *Relation between the in vivo titers (L + /10) of various fresh toxin preparations and their in vitro values (Lf) determined with absorbed serum*

The flocculation titers of toxin preparations with various L + /10 doses were assayed by Ramon's method using the 30-45 % ammonium sulfate fraction of the *2nd absorbed serum* and the relationship between these *in vivo* and *in vitro* values was examined quantitatively. The results are summarized in Figure 4.

This figure shows that there is a linear relationship between the L +/10 and Lf values of these toxins. The correlation coefficient was calculated from these ten samples to be 0.96 at the significance level of 0.05 and the critical value to be 0.82 to 0.99. A similar linear relationship between the combining power (Lb) and the Lf values of tetanus toxoids was obtained in a separate experiment when the latter were determined with the *2nd* or *3rd absorbed serum.*

DISCUSSION

The present work clearly demonstrates that removal of non-specific flocculating antibodies from commercial pepsinized tetanus antitoxin serum can be satisfactorily accomplished by a stepwise absorption procedure with a concentrated culture filtrate of a toxinogenic strain of *Cl. tetani* grown in a medium supplemented with excess glutamate. The success of this method is entirely due to the application of such a culture filtrate as this antigen for absorption, for its antigenic composition was shown to be indentical with that of the toxin preparations except that the toxin content of the former was almost negligible. Indeed, after two or three absorptions with this filtrate, none of the precipitaing antibodies except antitoxin were detectable by Ouchterlony's immunodiffusion method, as clearly seen in Photographs 3 and 4 and the yield of the absorbed serum was more than 90 per cent in terms of antitoxin units. This is in rather sharp contrast with the result of Moloney *et al.* (1944) whose yield of absorbed serum was only 28 per cent. The very low value of their yields would not be surprising since they employed crude tetanus toxin for the removal of heat labile non-specific flocculating antibodies from tetanus antitoxin serum.

Provided that the absorbed serum contains antitoxin as a single precipitating antibody, it is to be expected that the serum would give a single flocculation zone with any tetanus toxin preparation and the flocculation would represent a specific reaction of tetanus toxin with antitoxin. Actually, this is the case. As shown in Table 5, only a single flocculation zone was observed and the supernatant of the initial flocculation tube contained negligible amounts of both the toxin and antitoxin indicating that the tube represents a neutralizing point. In addition, a

quantitative precipitation test with the *3rd absorbed serum* and crude tetanus toxin gave a typical precipitation curve representing the pattern of a single antigen and antibody system. Morevoer, the flocculation point, which in Ramon's method varies with the amount of antitoxin employed in the test, was in the region of equivalence of the curve.

The linear relationship between the L + /10 of the toxins and their Lf values using *2nd* or *3rd absorbed sera* gives strong circumstantial evidences for the idea that this flocculation is due to a true reaction of toxin with antitoxin and further provides strong support for the suitability of these absorbed sera for use in *in vitro* titrations of tetanus toxin or toxoids. Indeed, using 2nd or 3rd absorbed tetanus antitoxin serum, various toxin and toxoid preparations have been assayed successfully by Ramon's method in our institute. The mean value of L + /10 per Lf of various toxins calculated from Figure 4 was found to be approximately 16, a value which should be 10. However, as was reported by Relyveld *et al.* (1957) in diphtheria antitoxin the ratio appears to depend upon the proportions of T and γ antitoxins in the serum employed. This may be the case in tetanus antitoxin serum also and the value of 16 may be characteristic of the tetanus antitoxin serum (Batch No. 128) employed for the present absorption. It is thus possible to test various tetanus antitoxin sera and to select the antitoxin having the ratio of 10 for convenience.

REFERENCES

Glenny, A. T., and Stevens, M. F. (1938). The labortaory control of tetanus profilaxis. *J. Roy. Army Med. Corps*, **70,** 308-310.

Goldie, H., Parsons, C. H., and Bowers, M. S. (1942). Titration of tetanal toxins, toxoids and antitoxins with the flocculative test. *J. Infect. Dis.*, **71,** 212-219.

Kubota, K, Yamamoto, A., Tsukamoto, M and Murata, R. (1960) A study of the medium for the toxin production of *Cl. tetani*. *Baichi-Kizai ni kansuru Kenkyu*, 1, **18,** (in Japanese).

Lowry, O. H., Rorebrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J. biol. Chem.*, **193,** 265.

Moloney, P. J. and Hennessy, J. N. (1944). Titration of tetanal toxins and toxoids by flocculation. *J. Immunol.* **48,** 345-354.

Murata, R. Kubota, K. and Kawashima, S. (1952). Studies on the titration of tetanus antitoxins by flocculation. *Japanese J. of Med. Sci. & Biol.*, **6,** 331-340.

Prevot, A. R. (1938). Discrimination graphique entre flocculation vraie et paradoxale dans le titrage de la toxine tetanique par la methode de Ramon. *C. R. Soc. Biol.*, **127,** 1166-1168.

Ramon, G., (1937). Sur la floculation dans les melanges de boillon tetanique fitre et de serum antitetanique. *C. R. Soc. Biol.*, **124,** 414-416.

Tsunashima, I., Sato, K., Shoji, K., Yoneda, M. and Amano, T. (1964). Excess supplementation of certain amino acids to medium and its inhibitory effect on toxin production by *Cl. tetani*. *Biken J.* (in press).