BIKEN,S JOURNAL 4, 171-186 (1961)

Immunological and Biological Properties of a Subcellular Component of *Corynebacterium diphtheriae* Having the Capacity to Bind Antitoxin* **

MASAHIKO YONEDA AND MORIHIRO MATSUDA

Department of Tuberculosis, Research Institute for Microbial Diseases, Osaka University, Osaka
(Received for publication, August 22, 1961)

SUMMARY

An attempt to elucidate the intracellular events occuring during toxin production by *Corynebacterium diphtheriae* showed that there was a subcellular component which could specifically bind antitoxin.

The antitoxin binding capacity of various subcellular fractions of a toxinogenic strain (Park Williams No.8 Toronto Harvard) of *C. diphtheriae* was studied by the quantitative absorption test and the agar gel diffusion technique. All the fractions tested derived from toxin-producing cells (Fe^D cells) except the cell wall preparation bound antitoxin. The highest activity was always in a fraction precipitating at between 14,500 and 100,000 × g. No antitoxin binding activity was detectable in fractions derived from cells whose toxin production had been completely inhibited by excess iron in the culture medium (Fe^S cells).

Preincubation of the antitoxin binding fraction with horse diphtheria antitoxin resulted in a loss of the binding activity. However, activity was unaffected by preincubation with either normal horse serum or with anti heat-killed diphtheria bacilli rabbit serum. Neither ordinary buffers nor physiological saline at near neutral pH released more than 35 per cent of the antitoxin bound to the fraction.

Animal tests showed that 1 mg dry weight of thoroughly washed antitoxin binding fraction (Fe^D fraction/14500-100000) was not lethal in guinea pigs but gave an intradermal reaction to rabbits equivalent to 20 m.r.d. for standard diphtheria toxin. The reaction was neutralized by antitoxin. Antisera obtained by immunizing rabbits with five to twenty milligrams dry weight of the well washed fraction in incomplete Freund's adjuvants contained antibody which could precipitate crystalline diphtheria toxin and the antitoxin titers of the sera were in the range of 0.5 to 5.0 units/ml.

Disruption of the Fe^D fraction by sonic oscillation resulted in the release of macromolecules which did not precipitate at a force of 100,000 × g for two hours. The precipitation lines of a preparation of these macromolecules against diphtheria antitoxin in an Ouchterlony's plate fused, with spur formation,with a single precipitation line formed between crystalline diphtheria toxin and the antitoxin.

---

* An outline of this work was reported at the 6th Symposium on Bacterial Toxins (June 20, 1959) and at the 33rd Meeting of the Japanese Bacteriological Association (July 18, 1960).

** Aided by a grant from the Ministry of Education, Japan

The significance of these findings in the biosynthesis of diphtheria toxin is discussed.

INTRODUCTION

Since most toxin protein is found in the culture filtrate and only traces in the cells of *C. diphtheriae* (Raynaud, Turpin, Mangalo & Bizzini, 1954), it is not surprising that much attention has been directed towards the conditions and mode of release of the toxin. In 1942, Morton and Ganzalez suggested the presence of a site for diphtheria toxin formation in the cell. However, there is no work on the nature of their hypothetical site. With regard to the biosynthesis of diphtheria toxin protein, information on this would be of great importance in understanding, not only the mode of toxin biosynthesis but the intrinsic mechanism involved in the inhibitory effect of iron on the release of diphtheria toxin. During the course of our investigations on the intracellular events occuring during diphtheria toxin production, and particularly on the immunochemical properties of cell-free extracts of mechanically disintegrated diphtheria bacilli, we found that, when an extract of toxin-producing bacilli was centrifuged at 14,500 × g for 30 minutes and the resulting supernatant was mixed with a known amount of diphtheria toxin and then incubated with increasing amounts of antitoxin, the earliest flocculation occurred after a long incubation period, and in the tube containing much more antitoxin than that equivalent to the toxin added. This could not be observed with the supernatant of the same extract obtained by centrifugation at 100,000 × g for 60 minutes. Therefore, it seemed that there must be some component sedimentable at 100,000 × g which bound antitoxin. We therefore studied the characteristics of antitoxin binding and also some of the immunological and biological properties of the component in the hope that this component might be related in some way to the intracellular site of diphtheria toxin formation.

This paper reports the antitoxin binding capacity of various subcellular fractions of diphtheria bacilli including the cell wall. The specificity of the binding activity and the toxicity and antigenicity of an antitoxin binding component are also described.

MATERIALS AND METHODS

1. *Bacterial culture*

A substrain of Park Williams No. 8 Toronto Harvard strain of *Corynebacterium diphtheriae* was used throughout this work.

2. *Cultural medium*

The papain digest of beef muscle described by Pope et al. (1932) was employed as the basic medium. To one liter of this medium, 0.5 gr of $Na_2HPO_4 \cdot 12H_2O$ and 3.0 ml of 10 per cent $CaCl_2 \cdot 2H_2O$ solution were added and the pH was adjusted to 8.0. The medium was then heated at 95°C for several minutes and then filtered while hot through Toyo No.7 filter paper. After cooling, 2 ml of 10 per cent solution of L-cystine and 4 ml of a solution of vitamins and metals (Solution II,

Mueller and Miller, 1941) were added. The pH was adjusted to 7.8 and the medium was dispensed in 140 ml aliquots in 500 ml shaking flasks and autoclaved at 10 lbs for 15 minutes. Solutions of sterile maltose and iron were added just before inoculation. Nine ml of maltose solution (50 per cent) which had been deferrated by the calcium phosphate gel method (Mueller and Miller, 1941) were added to each flask. Iron was added to this medium as sterile solutions of 0.01 per cent and 0.1 per cent of $FeSO_4 \cdot 7H_2O$ in 0.1 per cent HCl in amounts of 0.75 ml of the former per flask, to give optimal toxin production and 1.4 ml of the latter, to cause complete inhibition of the toxin production.

3. *Cultural methods*

A 24 hour culture on a Löffler's slope was first inoculated into 150 ml of the medium, containing 0.1 μg Fe/ml and incubated at 35°C for 17 hours with shaking at 120 strokes per minute at an amplitude of 10 cm. Then 2.5 ml of the culture (optical density 25 at 590 mμ in a Universal type Coleman spectrophotometer) was reinoculated into each flask containing 150 ml of medium and incubated at 35°C for 20 hours with shaking as described above. At the end of incubation, the bacterial growth was usually equivalent to an optical density of 30 to 36 and the final pH was 8.3 to 8.6 regardless of the amount of iron added. The toxin content of cultures containing 0.75 ml of 0.01% $FeSO_4 \cdot 7H_2O$ solution/flask was usually 120 to 180 Lf/ml. No toxin was detectable by flocculation in cultures containing 1.4 ml of 0.1% $FeSO_4 \cdot 7H_2O$ solution.

4. *Disintegration of cells and preparation of subcellular fractions*

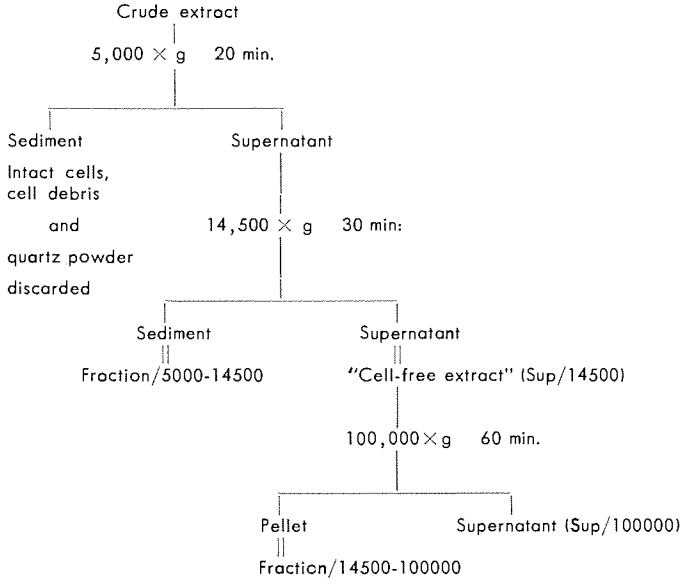


Fig. 1. Outline of Preparation of the Subcellular Fraction

After incubation, the whole cultures were immediately harvested and centrifuged at 5,000 × g for 20 minutes in the cold. The cells were then washed three or four times with physiological saline by centrifugation at the same speed. The washed packed cells were then disintegrated as follows: 100 gr cells mixed with about 300 gr of quartz powder (200 mesh) were ground in a mortar for about 30 minutes in the cold. The resulting muddy paste was mixed with 2 volumes of M/20 phosphate buffer (pH 7.4). The cell debris and quartz powder were removed by centrifugation

at 5,000 × g for 20 minutes. The turbid supernatant was then centrifuged at 14,500 × g for 30 minutes in the cold. The sediment thus obtained was designated as Fraction/5000-14500. The supernatant (Sup/14500) was further centrifuged in a Spinco Model L at 100,000 × g for 60 minutes and the resulting pellet was referred to as Fraction/14500-100000 (Figure 1). These fractions were prepared from both Fe^D and Fe^S cells and also from the Fe^S cells which had been suspended in Fe^D culture filtrate containing 200 Lf/ml of toxin and incubated at 32°C for 30 minutes. In the last case, disintegration of the cells were performed in the presence of 4 Lf toxin/gram wet weight of the packed cells and Fraction/14500-100000 from this disintegrate was used as a control (Fe^{S+tox} Fraction/14500-100000).

5. *Cell wall fraction*

A purified cell wall preparation was given to us by Prof. S. Kotani of the Depaı tment of Bacteriology, Nara Medical College. It had been prepared in his laboratory from toxin-producing cultures of the P.W. No.8 strain of *C. diphtheriae* by the method described by Kitaura *et al.* (1959).

6. *Toxin preparations*

Unless otherwise stated, the crude diphtheria toxin obtained from culture filtrates of the P.W. No.8 strain was used for the quantitative assay of antitoxin in all experiments. The flocculation units of the crude toxin were determined each time with standard antitoxin. For agar gel diffusion analyses, *crystalline diphtheria toxin* prepared by the method of Katsura et al. (1957) with modifications was used. The specific activity of the toxin was 3300 Lf/mg protein N and it gave a single precipitation line against crude horse antitoxin both in an Oakley's tube and in an Ouchterlony's plate. For the quantitative rabbit skin reaction test, *standard diphtheria toxin* No.108 from the Production plant of our Institute was employed as control. This has a toxicity of 450 m.l.d., 900,000 m.r.d. and 4000 × 20 Lr/2000 per ml.

7. *Sera*

1) *Antitoxin*

Antitoxin, containing 1300 flocculating units/ml was supplied by the Production plant of our Institute. A standard antitoxin containing 10 neutralizing units/ml was kindly provided by the National Institute of Health of Japan.

2) *Anti heat killed P.W. No. 8 bacilli rabbit serum*

The serum was kindly given by Dr. I. Fujimoto of the Department of Public Health, Osaka University Medical School.

3) *Anti Fraction/14500-100000 rabbit sera*

These were obtained by immunizing rabbits with washed Fraction/14500-100000, as described in the following section.

4) *Normal horse serum*

The normal horse serum employed in this study was kindly given by Dr. I. Fujimoto. It did not contain more than 1/1000 units of diphtheria antitoxin.

8. *Immunization of rabbits*

A small amount of serum was taken from white rabbits weighing about 3 kg for measuring the normal antitoxin contents. These animals then were immunized to the washed Fraction/14500-100000 of Fe^D, Fe^S and Fe^{S+tox} cells. They were also immunized to the similar Fe^D and Fe^S fractions after heat treatment at 70°C for 10 minutes. Injections were made as follows: In one series, rabbits were injected intramuscularly each week with 1.23 mg dry weight of the fraction in 4 ml of incomplete Freund's adjuvant (1951) for four weeks. In another series, rabbits were injected intramuscularly each week with 10.8 mg dry weight of the fraction for two weeks. The animals

were bled a week after the last injection and the sera were inactivated at 56°C for 30 minutes and stored at −20°C with 1:10,000 Merthiolate.

9. *Double diffusion precipitation in agar gel*

Precipitation reactions in agar gel were carried out by the double diffusion technique of Ouchterlony (1949) using 0.7 per cent Bacto Agar (Difco) dissolved in physiological saline containing 0.01 per cent Merthiolate.

10. *Dry weight measurement*

The dry weight of bacterial fractions was estimated by drying preparations to constant weight at 120°C.

RESULTS

1. *Antotixin binding capacity of various fractions*

Antitoxin binding capacity of two subcellular fractions, Fraction/5000-14500 and Fraction/14500-100000 from either Fe^D or Fe^S cells and a purified cell wall preparation of the former were tested. For measuring the binding capacity the quantitative absorption test was used. The procedure employed for preparing these materials is described in the preceding section.

Two hundreds mg (dry wt.) of each fraction and 230 mg (dry wt.) of the cell wall preparation were each suspended in 15 ml of buffered saline (0.9 per cent NaCl in M/100 phosphate buffer, pH 7.4) containing 15 units/ml of a standard horse diphtheria antitoxin. Tubes were incubated at 37°C for 15 minutes and the residual antitoxin in the supernatants, obtained by centrifugation of the preparations at the gravity used in the preparation of each material, was measured by flocculation with 15 Lf diphtheria toxin. The amount of antitoxin absorbed by these materials was calculated by subtracting the amount of residual antitoxin from the initial antitoxin added. The antitoxin binding activity was expressed as units per gram dry weight of material.

Table 1. Antitoxin Binding Capacity of Various Cellular Fractions of the P.W. No. 8 Strain of *C. diphtheriae*

Fraction	Antitoxin bound (unit/gr. dry weight)	
	Fe^D cells*	Fe^S cells**
Fraction/5000-14500	256	0
Fraction/14500-100000	681	0
Purified cell wall	0	—

* Cultures grown in an iron deficient medium for optimal toxin production.

** Cultures grown in a medium containing sufficient iron, to suppress toxin production.

The results are illustrated in Table 1. Only two fractions, Fraction/5000-14500 and Fraction/14500-100000 of Fe^D cells, absorbed antitoxin, and the binding

activity of the latter fraction was 2.6 times as much as that of the former. Using 70 to 900 mg (dry wt) of these fractions and 15 to 50 units/ml of antitoxin, similar experiments were made. The latter fraction always showed the highest activity. The capacity of the fraction in terms of units of antitoxin per gram dry weight was between 556 and 695. No measurable antitoxin was absorbed by similar fraction of Fe^S cells which had not released detectable amount of toxin during growth. It is interesting that the purified cell walls of Fe^D cells had no capacity to bind antitoxin.

2. *Antitoxin binding by Fraction/14500-100000 of Fe^D cells shown by Ouchterlony's agar double diffusion technique*

Aliquots of 56 mg dry weight of washed Fraction/14500-100000 of Fe^D cells and of 75 mg weight of unwashed Fraction/14500-100000 of Fe^S and of Fe^{S+tox} cells were mixed with 0.3 ml of horse diphtheria antitoxin. The concentration of antitoxin used was 40 units/ml for the former and 20 units/ml for the latter two fractions. The mixture was incubated at 37°C for 15 minutes, cetrifuged at 14,500 $\times$ g for 60 minutes and the supernatant thus obtained was tested against crystalline diphtheria toxin in an Ouchterlony plate. 0.2 ml aliquots of each suternapant and of antitoxin of each initial concentration were added alternately to the peripheral wells of the plate and the central well was filled with 0.2 ml of crystalline diphtheria toxin solution in buffered saline (pH 7.0), whose Lf/ml was equivalent to the control antitoxin units/ml used in each plate. The plates were then incubated at 37°C for 5 days and photographed.

The result is shown in Photograph 1. As can be seen in this photograph, the presence of Fe^S and Fe^{S+tox} fractions in antitoxin did not appear to affect precipitation of the antitoxin with toxin, while no precipitation was observed between the toxin-well and the well of antitoxin plus Fe^D fraction.

3. *Effect on the antitoxin binding capacity of pretreatment of Fe^D Fraction/14500-100000 with various sera*

113 mg dry weight of Fe^D Fraction/ 14500-100000 were mixed with 5 ml of normal horse serum, horse antitoxin containing 225 units and anti heat killed P.W. No. 8 bacilli rabbit serum respectively. These mixtures were incubated at 37°C for 15 minutes and the pellets obtained by centrifugation at 100,000 $\times$ g for 60 minutes were used for assaying the antitoxin binding capacity by the quantitative absorption test described in the preceding section. In the case of anti heat killed bacterial serum, the fration was treated repeatedly with 5 ml of the fresh antiserum until the fraction was saturated with the agglutinin. Usually two treatments were enough. Table 2 shows the change in the agglutinin titer of the antiserum during this procedure. It will be seen that appreciable amounts of agglutinin remain unabsorbed in the antiserum used for the second treatment of the fraction.

Table 2. Changes in the Agglutinin Titers of the Anti Heat-killed Diphtheria bacilli Rabbit Serum after Absorption with Fraction/14500-100000 Derived from Toxin-producing diphtheria bacilli

Anti heat-killed diphtheria bacilli rabbit serum	Agglutinin titer*
Before absorption	1 : 320
After the first absorption	$\leqslant$1 : 20
After the second absorption	1 : 160

* Two fold dilutions of a ten fold dilution of antiserum were made. The agglutinin titers of the antisera were determined against an equal volume (0.5 ml) of a suspension of the heat-killed diphtheria bacilli after incubation at 37°C for 2 hours and then standing overnight at room temperature.

Table 3 illustrates the change in the antitoxin binding capacity of Fe^D Fraction /14500-100000 before and after treatment with these sera. As can be seen in the table, fractions treated with normal horse serum or anti heat killed diphtheria bacilli rabbit serun still retain their original capacity to absorb antitoxin, while the fraction treated with horse diphtheria antitoxin loses this activity completely.

Table 3. Effect of Treatment with Various Sera on the Antitoxin Binding Capacity of Fraction/14500-100000 Derived from Toxin-producing Diphtheria bacilli

Preincubation with	Antitoxin bound (units/gr. dry weight)	
	Before preincubation	After preincubation
Normal horse serum	556	556
Anti heat-killed diphtheria bacilli rabbit serum	556	556
Antitoxic horse serum	556	0

4. *Effect of repeated washing on the reabsorption of antitoxin by antitoxin-absorbed Fraction /14500-100000*

Ten ml aliquots of a suspension of washed Fe^D Fraction/14500-100000 were put into four preparative centrifuge tubes of a Spince Model L ultracentrifuge. The dry weight of the fraction in each tube was 160 mg. After centrifugation at 100,000 $\times$g for 60 minutes, the supernatants were discarded, the pellets were drained completely and then resuspended in 13.5 ml of a preparation containing 15 units/ml of antitoxin. Tubes were incubated at 37°C 15 minutes. Three of the pellets obtained by centrifugation were washed once, twice and three times respectively, by alternate suspension and centrifugation. The fourth pellet was not washed. The four pellets were then again exposed to 13.5 ml of 15 units/ml antitoxin to measure their capacity to reabsorb antitoxin. As was expected from the preceding experiment, the unwashed pellet did not reabsorb any antitoxin. However, washed pellets reabsorbed appreciable and constant amounts of antitoxin irrespective of the number of times they were washed. 33.4 units of antitoxin were reab-

sorbed on each pellet which corresponds to about 35 per cent of the antitoxin units initially absorbed by the pellet. These results are summarized in Table 4.

Table 4. Effect of Repeated Washing on the Reabsorption of Antitoxin by Fraction/ 14500-100000* Derived from Toxin-producing Diphtheria bacilli

Number of washes	0	1	2	3
Antitoxin reabsorbed (units)	0	33.4	33.4	33.4
Antitoxin initially absorbed (units)	96.0			

* 160 mg dry weight for each aliquot.

5. *Toxicity of Fraction/14500-100000*

A preliminary test showed that intramuscular injections of 1 to 1.5 mg dry weight of thoroughly washed Fe^D Fraction/ 14500-100000 failed to kill 250 grams guinea pigs. However, it gave a typical intradermal reaction in rabbits which could be neutralized by antitoxin. Since the toxicity of the fraction was so low, the question arose as to whether the entity responsible for the intradermal reaction was trace of soluble diphtheria toxin contaminating the fraction. To test this, the effect on the toxicity in terms of intradermal reaction in rabbits of repeated washing of the fraction was studied.

About 400 mg dry weight of Fe^D Fraction/14500-100000 was suspended in 160 ml of M/100 phosphate buffer (pH 7.4), distributed in four Lusteroid tubes. The fraction was washed by repeated suspension and centrifugation in a Spinco Model L preparative ultracentrifuge. For centrifugation, new tubes were employed each time to avoid contamination by soluble toxin in the supernatant. A small aliquot (about 1 ml) was taken from both the resuspension and supernatant obtained before and after each centrifugal step to assay the toxicity. For measuring the toxicity, appropriate dilutions of these samples were made in buffered saline(pH 7.0) and a 0.1 ml aliquot of each dilution was injected intradermally into white rabbits. The toxicity of Fe^S and Fe^{S+tox} Fractions/14500-100000 was also studied in a similar way. As controls, 1 ml of each dilution was added by an equal volume of 10 units/ml standard diphtheria antitoxin, incubated at 37°C for 60 minutes and 0.1 ml of each mixture was injected intradermally into rabbits. The results are shown in Table 5. From the table, it will be noted that, after washing Fe^D fraction three times, there is no detectable toxin in the supernatant. However, a suspension of the fraction was found to be toxic even after washing it five times, and the toxicity after the final washing was about 1 m.r.d. per 50 μg dry weight. In the cases of Fe^S and Fe^{S+tox} fractions, on the other hand, their toxicity were considerably less than that of Fe^D fraction. These intradermal reactions were completely neutralized by diphtheria antitoxin.

Table 5. Change in Toxicity of Fraction/14500-100000 on Repeated Washing

	Number of washes	Fraction/14500-100000 suspension			Resulting supernatant	
		Dilution	μg d.w.** /0.1 ml	Skin reaction* (mm)	Dilution	Skin reaction* (mm)
Fe^D	3	× 1	144	17 N++***	× 1	16 n
		× 2	72	19 N++	× 2	13 R
		× 4	36	15 N+	× 4	10 r
	4	× 1	103	15 N++	× 1	—
		× 2	51.5	12 N++		
		× 4	25.8	13 N+		
	5	× 1	585	18 N+++		
		× 2	292.5	14 N++		
		× 4	196.3	13 N+		
		× 8	98.2	7 Rn		
		×16	49.1	11 R		
Fe^S	4	× 1	804	17 R	× 1	—
		× 2	402	13 R		
		× 4	201	11 r		
		× 8	100.5	8 r		
		×16	50.3	±		
		×32	25.2	—		
Fe^{S+tox}	4	× 1	804	18 R	× 1	—
		× 2	402	15 R		
		× 4	201	10 r		
		× 8	100.5	±		
		×16	50.3	—		

* These skin reactions were neutralized by diphtheria antitoxin.

** The dry weight of the Fractions was calculated from the optical density, using the factor 0.1 o.d. at 510 mμ≒134 ug d.w..

*** R : marked erythema r : slight erythema
N : marked necrosis n : slight necrosis

6. *Antigenicity of Fraction/14500-100000*

Fraction/14500-100000 from Fe^D, Fe^S and Fe^{S+tox} cells was washed five times with M/100 phosphate buffer (pH 7.4) by repeating centrifugation at 100,000 × g for 60 minutes and the washed fraction was injected into white rabbits as described in the section on *Materials and Methods*. The precipitating antibody in the antisera thus obtained was examined against crystalline diphtheria toxin both by the ring test and Ouchterlony's technique. Their neutralizing units were measured using standard diphtheria toxin No. 108. In addition to these antigen fractions, the antigenicity of fractions Fe^D and Fe^S after heat treatment at 70°C for 10 minutes

and of small amounts (500 and 1000 m.r.d. in the total dose) of the standard diphtheria toxin in an incomplete Freund's adjuvant were examined in a similar way. Photograph 2 illustrates the agar precipitation pattern showing the presence of precipitating antibody in the anti Fe^D Fraction/14500-100000 rabbit serum against diphtheria toxin. As can be seen, single precipitation lines were formed between the anti fraction serum and crystalline diphtheria toxin and these lines fused completely with the precipitation line between the toxin and horse antitoxin. The mean antitoxin titers of the pooled antisera of two rabbit groups injected with 4.9 and 21.6 mg dry weight of Fe^D fraction were 5 and 0.5 units respectively. No other antigen elicited either toxin-precipitating antibody or antitoxin production. Table 6 is a summary of the *in vivo* tests.

Table 6. Antigenicity of Fraction/14500-100000

Antigens	Total dose injected		Times of injection*	Antitoxin units of sera
	mg dry weight	m.r.d.		
Fe^D Fraction	4.9	100	4	5
Fe^D Fraction	21.6	430	2	0.5
Fe^D Fraction heated at 70°C for 10 min.	21.6	0	2	$\ll$1/100
Fe^S Fraction	21.6	$\ll$215	2	$\ll$1/100
Fe^S Fraction heated at 70°C for 10 min.	21.6	0	2	$\ll$1/100
Fe^{S+tox} Fraction	21.6	$\ll$215	2	$\ll$1/100
Standard diphtheria toxin No. 8		500	4	$\ll$1/100
		1,000	4	$\ll$1/100

* Intramuscular injections with incomplete Freund's adjuvant at weekly intervals.

7. *Agar gel diffusion analysis of the antitoxin binding material released from Fe^D Fraction/14500-100000 by sonic oscillation*

In order to characterize the immunochemical properties of the antitoxin binding principle bound to the Fe^D fraction sedimentable by centrifugation at 100,000 × g for 60 minutes, the fraction was sonicated and the material unsedimentable by centrifugation at 100,000 × g for 2 hours was tested for the reaction with antitoxin by agar gel diffusion techniques.

About 200 mg dry weight of Fe^D Fraction/14500-100000 were suspended in M/15 phosphate buffer (pH 8.2) and sonicated in an oscillator (Kubota, 10 KC). During this treatment, appropriate sized aliquots were removed at 20 minutes intervals and their optical density at 510 mμ was measured in a Universal type Coleman spectrophotometer. As shown in Figure 2, the optical density of the suspension decreased markedly with the time of oscillation and after about 40

minutes no significant reduction in the optical density was observed. The amount of protein released after 100 minutes sonic oscillation was determined by Lowry's method (1953) in the supernatant obtained after centrifugation of the suspension at 100,000 × g for 120 minutes. It was about 286 μg/ml. The supernatant of the untreated fraction, on the other hand, contained 57 μg/ml. Since the 280/260

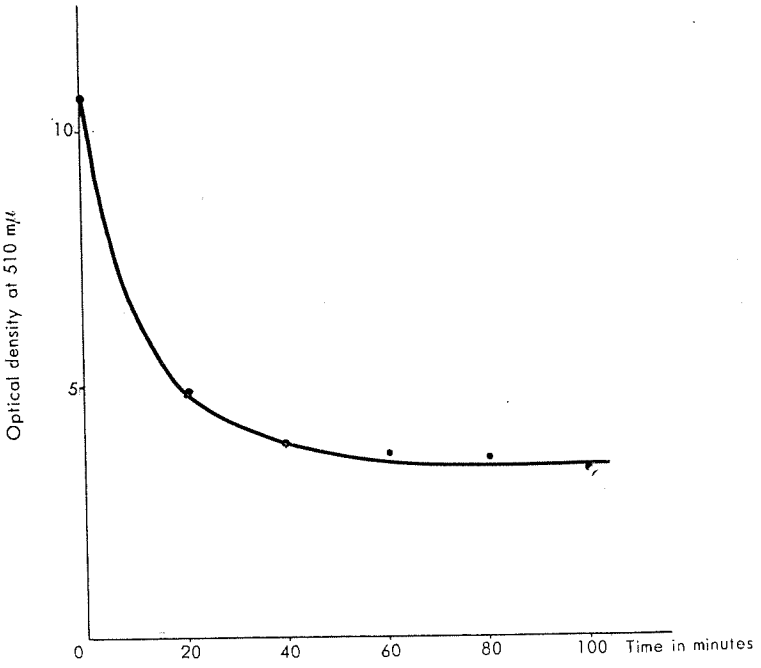


Fig. 2. Change in the Optical Density of the Suspension of Fe^{D} Fraction/14500-100000 during Sonication at 10 KC

mμ ratio was 0.79, it appears to contain relatively small amounts of nucleic acids (6.5 %). About 40 ml of the supernatant, obtained after 100 minutes oscillation, was precipitated by 70 per cent saturation with ammonium sulfate, dialyzed against M/15 phosphate buffer (pH 7.0) and the concentrated, dialyzed material was tested in an agar gel plate by Ouchterlony's technique. The form and arrangement of the reservoirs is shown in Photograph 3. It may be seen in the photograph that few lines developed between the wells for the test material and a crude horse antitoxin. Some of these lines fuse with spur formation, with a single precipitation line formed between the antitoxin and the crystalline diphtheria toxin wells. Besides these fusing lines, lines crossing a precipitation line between toxin and antitoxin were sometimes observed. As a control, a crude diphtheria toxin solution in phosphate buffer (pH 8.2) containing 50 Lf/ml was sonicated similarly and its pattern of precipitation was tested in an Ouchterlony's plate. However, no difference was

seen in the pattern before and after treatment.

DISCUSSION

Examination of the reaction between antitoxin and a cell-free extract (Sup/14500) of toxin-producing diphtheria bacilli revealed that there is a subcellular component (Fraction/14500-100000) which can bind antitoxin. A major objective of this study was to prove that it specifically binds diphtherial antitoxin. From the results described in the preceding sections there may be little doubt that this is so. First, the result on pretreatment of the fraction with various sera supports this. If the binding of antitoxin were merely non-specific, physical adsorption of the serum protein by the particular fraction, there would not be much difference in the binding capacity of antitoxin after pretreatment with various sera. Actually, however, complete loss of the capacity was seen after pretreatment with diphtheria antitoxin while the capacity was unaffected by other sera tested. Furthermore, saturation of the fraction with the agglutinin for heat killed P.W. No.8 diphtheria bacilli, which also agglutinates a suspension of the fraction, did not alter the capacity to bind antitoxin. Moreover, exaustive absorption with Fe^S fraction of a crude horse diphtheria antitoxin, which has also a strong agglutinating activity for both Fe^S and Fe^D fractions, resulted in the complete removal of the agglutinin but the flocculating units of the antitoxin were not reduced by this treatment. These results clearly indicate that the binding sites of Fe^D fraction for the agglutinin and for the antitoxin are entirely different. It is yet uncertain whether the binding sites for both antibodies are located in the same subcellular component. It may be possible that these fractions are grossly contaminated with the cell wall debris of the organism and that agglutination occurs merely between the debris and the agglutinin. The fact that an antitoxin binding capacity is absent from Fe^S fraction may eliminate the possibility of non-specific, physical adsorption of the antitoxin and provide a circumstantial evidence for the specificity of the antitoxin binding of Fe^D fraction. Very recently the authors (1961) have observed that the Fe^D Fraction/14500-100000 of both strains C4(β) and C7(β) of *C. diphtheriae*, which are toxinogenic and β prophage lysogenic, can bind antitoxin. However, similar fractions derived from cells of the non-toxinogenic, non-lysogenic strains C4 and C7, from which the former were derived by infection with the β phage (Groman, 1955; Barksdale and Pappenheimer, 1954) could not bind antitoxin. This indicates the association of the antitoxin binding component with a specific lysogenic state related to the toxinogenicity and also provides further circumstantial evidence for the specificity of the binding of antitoxin. Second, only a part of the antitoxin bound to Fe^D fraction was removable by repeated washings with 0.9 per cent saline or buffers at near neutral pH. In this case the degree of removal of antitoxin by washing was calculated by determining the antitoxin units reabsorbed by the washed fraction, which had been in contact with antitoxin. Calculations show that 65 per cent

of the bound antitoxin remained in the fraction irrespective of the number of times the fraction was washed. It appears therefore that about two-thirds of the antitoxin initially absorbed is quite firmely bound to the fraction just as in the case of other antigen-antibody complexes. The rest easily removable by washing may be regarded as antitoxin which is absorbed non-specifically by the fraction. However, it may be possible that the period (15 minutes) employed in this study for incubating Fe^D fraction in antitoxin might be too short for antitoxin molecules to bind firmly to all of the binding sites of the fraction. In any case, true answer must be obtained by using isotope labelled purified antitoxin and by determining the amount of bound antitoxin directly. Third, demonstration of the antitoxin in the sera of rabbits immunized with Fe^D fraction may be further evidence for the specificity of the antitoxin binding. One may argue, however, that the antitoxin might have been elicited either by a trace of soluble toxin contaminating the fraction or by the toxin non-specifically adsorbed to the fraction used for immunization. This appears not to be the case because immunization of rabbits with Fe^S or Fe^{S+tox} fractions, or with a small amount of crude diphtheria toxin containing two times as many m.r.d. as the total dose of Fe^D fraction, failed to elicit a precipitating antibody against crystalline diphtheria toxin, or even to cause formation of antitoxin as little as 1/100 units. Consequently, all of these findings indicate the presence of a specific antitoxin binding principle in Fe^D fraction. This is directly evidenced by the demonstration of the antigenic specificity, by agar gel diffusion techniques, of the material released from the fraction by sonication.

One of the characteristics of the antitoxin binding fraction is its low toxicity. As was shown previously, 1 mg dry weight of the fraction had no lethal effect on guinea pigs but gave an intradermal reaction in rabbits equivalent to 20 m.r.d. of standard diphtheria toxin. Assuming that one flocculation unit of diphtheria toxin is equivalent to 10^5 m.r.d. in terms of rabbit skin reactivity and provided that one unit of the antitoxin bound represents one Lf of ordinary diphtheria toxin, it can be calculated from Table 1 that 1 mg dry weight of the fraction should have 6.8×10^4 m.r.d., which is about three thousand times that actually found. In other words, the toxicity is only about $1/10^3$ times that of an equivalent amount of diphtheria toxin in terms of the antitoxin binding units. Therefore the Fe^D fraction appears to behave as if it were a diphtheria toxoid in its low toxicity and high specific antigenicity. Since no significant difference in toxicity was found between Fe^S and Fe^{S+tox} fractions, it may be that the toxicity of Fe^S or Fe^D fraction is not due to the exogenous toxin adsorbed to these fractions.

The antitoxin binding capacity and specific antigenicity are unaffected by repeated washing in the cold. This indicates that the active material responsible for these characters must be firmly bound to the fraction precipitated by centrifugation at $100,000 \times g$ for 60 minutes. But it was released from the fraction by sonic treatment as unsedimentable macromolecules by high speed centrifugation (100,000

× g for 2 hours). From the precipitation pattern of the molecules in agar gel (Photograph 3), it may be considered that the material is comprised largely of the macromolecules whose antigenic determinants are partly in common with those of the diphtheria toxin protein. However, a precise information of the immunochenical properties must await further purification of the material. A similar treatment of Fe^S fraction by sonication also resulted in the release of such macromolecules that are unsedimentable by centrifugation at 100,000 × g for 2 hours. But, in this case, no antigens whose specificity is in common with that of the diphtheria toxin protein were detected.

From the morphological point of view, the complete absence of the antitoxin binding capacity in the purified cell wall fraction of toxin-producing diphtheria bacilli is of great interest. This suggests that the binding material is associated with some inner structure of the organism such as the cytoplasmic membrane or subcellular particles. Experiments using protoplasts of the organism may be helpful to prove this point (Mori *et al.* 1961). Preliminary chemical analyses have recently shown that Fraction/14500-100000 is composed mostly of protein and ribonucleic acids. More recently, it has been found that highly purified ribonucleoprotein particles (ribosomes) of toxin-producing diphtheria bacilli can still bind antitoxin specifically (Matsuda, Hirai and Yoneda, 1961). In other organism, such as *E. coli*, the structural unit on which protein molecules are synthesized seems to be the ribosome (Roberts et al., 1958). Thus the antitoxin binding ribosomes may be the site of diphtheria toxin protein synthesis and the cross reacting material on the ribosomes could be an nascent form of the toxin protein. If this is so, diphtheria toxin protein will provide a valuable tool for the *in vitro* study of protein biosynthesis because it can be detected specifically in amounts as low as 0.00002 micrograms by the rabbit skin reaction. It was reported that the toxin was detectable in the cells of non-lysogenic, non-toxinogenic strains (C4 or C7) infected with a virulent phage B before the liberation of the phage (Barksdale, 1958). This means that the formation of the toxin synthesizing system may be initiated only by the incorporation of B phage DNA having genetic information of toxinogenicity. Thus, it may be also possible to study toxin protein biosynthesis *in vitro* in relation to genetic information.

The intrinsic mechanism involved in the inhibition of diphtheria toxin production by iron is still obscure. Considering from the fact that no antitoxin binding materials except a trace amount of toxin are present in Fe^S fraction, it appears that iron acts on a step of the toxin biosynthesis in cells. If this is true, therefore, following three possibilities may be considered for the inhibitory action of iron 1) A component having the capacity to bind iron may form some complex with toxin protein in the presence of iron, resulting in the masking of the specific antigenicity and the toxicity of the latter. This possibility has been originally suggested by Pappenheimer in his hypothesis(1947). Additoin of excess iron to cell free extracts

(Sup/14500) of toxin-producing diphtheria bacilli or to Fe^{D} Fraction/14500-100000 fails to eliminate the antitoxin binding capacity. It appears therefore that once formed, the antitoxin binding material bound to the ribosomal fraction is not masked merely by saturation with iron.

2) Iron may combine with template RNA for toxin protein, resulting in an alteration of the specificity of the protein replicated.

3) Iron may affect the DNA with genetic information on toxinogenicity in some way, resulting in a block of toxin protein synthesis.

However, the possibility that the inhibitory action of iron is directly involved in the release mechanism of the toxin from cells may not be ruled out.

ACKNOWLEGEMENT

The authors are grateful to Dr. T. Amano for his criticism and to Dr. W. L. Barksdale for his helpful discussion. They are also greatly indebted to Dr. S. Kotani for supplying the cell wall material, to Dr. I. Fujimoto, for various sera and to Mr. K. Takaku and Mrs. Y. Ikehata, for the standard diphtheria toxin and antitoxin.

REFERENCES

Barksdale, W. L. and Pappenheimer, A. M. Jr. (1954). Phage-host relationships in non-toxigenic and toxigenic diphtheria bacilli. *J. Bacteriol.* **67,** 220-232.

Barksdale, W. L. (1958). Phage multiplication and toxin production in *Corynebacterium diphtheriae*. *J. Gen. Microbiol.* **18,** x-xi.

Freund, J. (1951). The effect of paraffin oil and mycobacteria on antibody formation and sensitization. *Am. J. Clin. Path.* **21,** 645-656.

Groman, N. B. (1955). Evidence for the active role of bacteriophage in the conversion of nontoxigenic *Corynebacterium diphtheriae* to toxin production. *J. Bacteriol.* **69,** 9-15.

Katsura, T., Kato, I., Nakamura, H. and Koyama, J. (1957). Purification of diphtheria toxin. I Application of zone electrophoresis in a starch-supporting medium as a means of fractionation of toxin. *Jap. J. Microbiol.* **1,** 213-224.

Kitaura, T., Hirano, T., Kotani, S., Inui, S., Higashigawa, M. and Tsuchitani, T. (1959). The chemical composition of the cell wall of *Corynebacterium diphtheriae*. *J. Nara Med. Assoc.* **10**, 45-48. (in Japanese).

Matsuda, M., Hirai, T. and Yoneda, M. (1961). Studies on the mode of production of diphtheria toxin. II. Some properties of a subcellular component of toxin-producing diphtheria bacilli having the capacity to bind antitoxin and the invariable association of the formation of the component with a specific lysogenic state. The 8th Symposium on Bacterial Toxins (June 21, 1961).

Mori, Y., Kato, K., Matsubara, T. and Kotani, S. (1961). The "protoplast" of *C. diphtheriae*. The 8th Symposium on Bacterial Toxins. (June 21, 1961).

Morton, H. E. and Ganzalez, L. M. (1942). On the site of formation of diphtheria toxin. *J. Immunol.* **45,** 63-67.

Mueller, J. H. and Miller, P. (1941). Production of diphtheria toxin of high potency (100 Lf) on a reproducible medium. *J. Immunol.* **40,** 21-32.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Ouchterlony, Ö. (1948). Antigen antibody reactions in gels. *Akriv. Kemi. Mineral. Geol.* **26 B,** 1-9.

Pappenheimer, A. M. Jr. (1947). Diphtheria toxin III. A reinvestigation of the effect of iron on toxin and porphyrin production. *J. Biol. Chem.* **167,** 251-259.

Pope, C. G. and Smith, M. L. (1932). The routine preparation of diphtheria toxin of high value. *J. Path. Bact.* **35,** 573-588.

Raynaud, M., Turpin, A., Mangalo, R. and Bizzini, B. (1954). Croissance et toxinogenèse. *Ann. Inst. Pasteur* **87,** 599-616.

Roberts R. B., Ed. (1958). Microsomal particles and protein synthesis (Pergamon Press, New York, N. Y. , 168).

Yoneda, M. and Matsuda, M. (1961). A subcellular component of *Corynebacterium diphtheriae* having the capacity to bind antitoxin. *Nature* **192**, 4803, p. 642.

EXPLANATION OF PHOTOGRAPHS

Photograph 1. Agar gel precipitation pattern demonstrating the antitoxin binding by Fe^D Fraction/14500-100000.

TOX 20, TOX 40 : 0.2 ml of 20, 40 Lf/ml of crystalline diphtheria toxin.

AT 20, AT 40 : 0.2 ml of 20, 40 units/ml of horse diphtheria antitoxin.

Fe^DFr.+AT 40 : 0.2 ml aliquot of the supernatant of a mixture of 0.3 ml of 40 units/ml of antitoxin and 56 mg dry weight of Fraction/14500-100000 of cells from cultures grown in an iron deficient medium for optimal toxin production.

Fe^SFr.+AT 20 : 0.2 ml aliquot of the supernatant of a mixture of 0.3 ml of 20 units/ml of antitoxin and 75 mg dry weight of Fraction/14500-100000 of cells from cultures grown in a medium containing sufficient iron to suppress toxin production (Fe^S cells).

Fe^{S+tox} Fr.+AT 20 : 0.2 ml aliquot of the supernatant of a mixture of 0.3 ml of 20 units/ml of antitoxin and 75 mg dry weight of Fraction/14500-100000 of Fe^S cells disintegrated in the presence of 4 Lf toxin/gram wet cells.

Photograph 2. Agar gel precipitation pattern demonstrating the presence of toxin precipitating antibody in the anti Fe^D Fraction/14500-100000 serum.

ANTI Fe^DFr. : 0.2 ml of anti Fe^D Fraction/14500-100000 rabbit serum obtained by four intramuscular injections of the washed fraction, 4.9 dry weight in total dose, with incomplete Freund's adjvant at 7 days intervals.

AT 10, AT 20, AT 40 : 0.2 ml of 10, 20, 40 units/ml of horse diphtheria antitoxin.

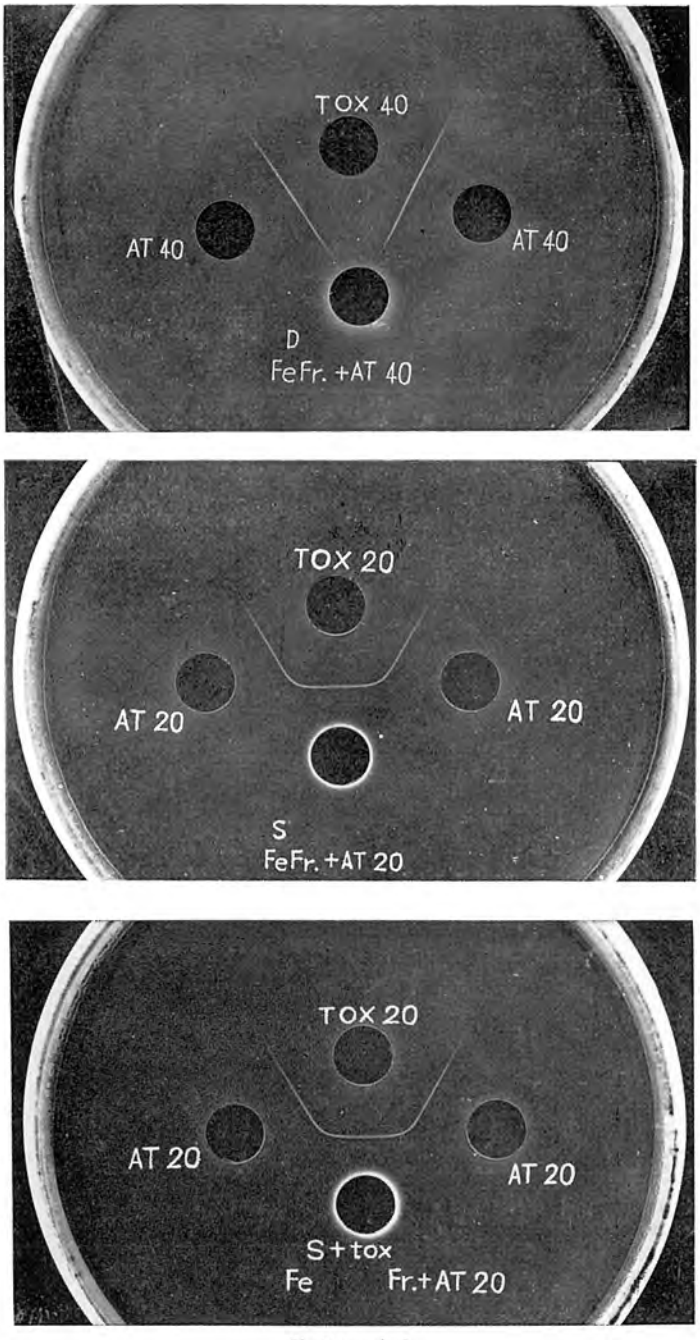
TOX 10, TOX 20, TOX 40 : 0.2 ml of 10, 20, 40 Lf/ml of crystalline diphtheria toxin.

Photograph 3. Agar gel precipitation pattern demonstrating the presence of cross reacting materials in Fe^D Fraction/14500-100000.

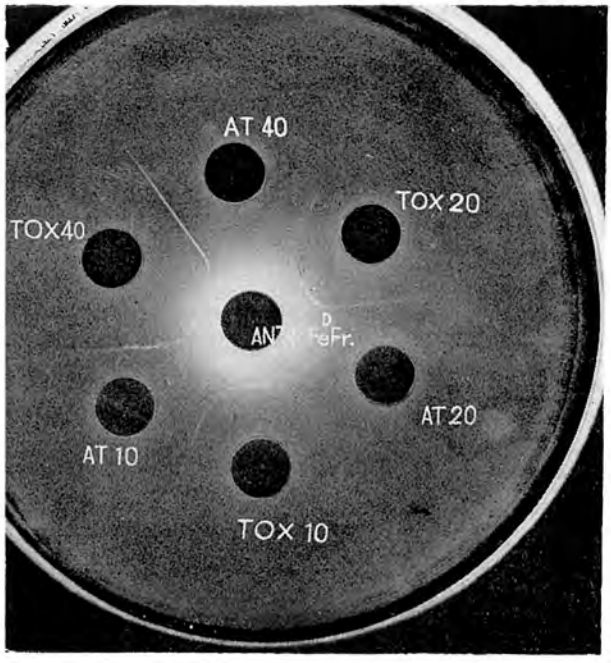
TOX 10 : 0.2 ml of crystalline diphtheria toxin (10 Lf/ml)

AT 10 : 0.2 ml of horse diphtheria antitoxin (10 units/ml)

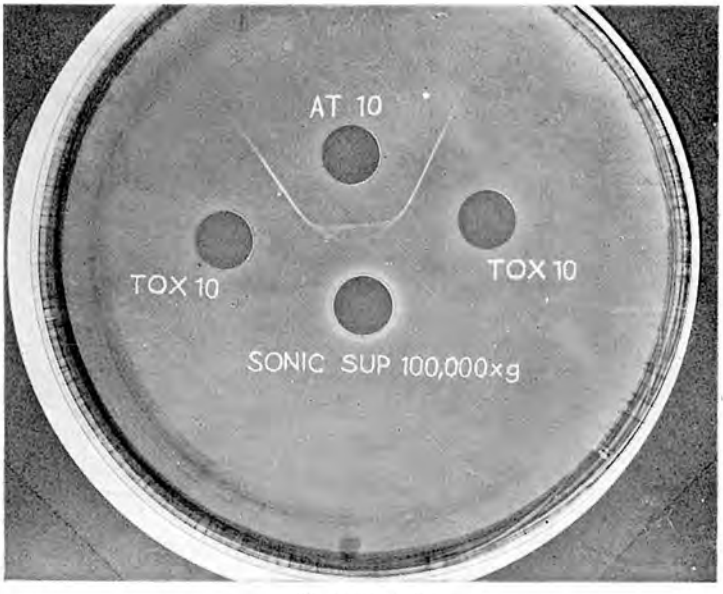
SONIC SUP 100,000×g : 0.2 ml of concentrated and dialyzed 100,000×g supernatant obtained after sonic oscillation of Fe^D Fraction/14500-100000 (3.8 mg protein/ml)



Photograph 1.



Photograph 2.



Photograph 3.