Diphtheria Antitoxin: Antigen-Combining and Toxin-Neutralizing Properties of Papain Fragments

Abstract. *Papain-digested rabbit antibodies to diphtheria toxoid were separated into three fragments. Fragments I and II combined with toxoid in vitro and neutralized toxin. The ratios of these activities were not the same for fragments I and II, indicating that antitoxic and accessory antibody may be distributed unequally between two classes of antibody.*

Porter has shown (*1*) that papain digestion of rabbit antibody produces univalent subunits (fragments I and II) which combine with, but do not precipitate, antigen. We now report the use of Porter's technique in determining whether univalent fragments of rabbit diphtheria antitoxin can neutralize toxin in vivo.

Three weekly injections of 100 Lf (limit of flocculation) units (*2*, p. 156) of partially purified diphtheria toxoid (*3*) in complete Freund's adjuvant were given to each of three rabbits. The rabbits were bled 3 weeks after the last injection. A single injection of toxoid in adjuvant was given to each of the rabbits 1 week after bleeding; the rabbits were bled again 1 week after the injection. The six serums were kept separate throughout the experiments. Gamma globulin was precipitated from the serums with 35 percent saturated ammonium sulfate, dialyzed, and lyophilized. No protein other than γ-globulin was detected by paper electrophoresis.

Papain digests were separated on carboxymethylcellulose columns (*1*). The eluate was collected in 5-ml fractions; selected fractions were tested for antigen-combining ability and antitoxic activity.

Divalent antibody activity against diphtheria toxoid was determined by passive hemagglutination with bis-diazotized benzidine reagent (*4*). The untreated γ-globulin preparations agglutinated the sensitized cells in concentrations of 0.08 to 2.0 μg/ml, whereas no chromatographic fraction caused agglutination. Univalent antibody activity was demonstrated as follows: A passive hemagglutination test (*4*) was performed with serially diluted fractions replacing the antibody dilutions of the original method. After a 2-hour incubation at room temperature, 0.1 ml of untreated γ-globulin at 100 times the minimal agglutinating concentration was added to each tube. Subsequent lack of agglutination in a particular tube indicated an excess of univalent fragments. With this technique fractions from peaks I and II inhibited the reaction between untreated antibody and antigen-sensitized cells; fractions from peak II were inert. The inhibitory titers of peak II fractions were many times higher on a unit-weight basis than those of peak I (Fig. 1).

Antitoxic activity of the fractions and the untreated antibody was demonstrated by the capacity to neutralize the amount of diphtheria toxin causing necrosis after injection into the skin of rabbits (*5*). Dilutions of the chromatographed fractions and untreated γ-globulin were prepared in 0.2-ml volumes, and 0.1 ml of diphtheria toxin (1/100 L+ unit) (*2*, p. 155) was added to each dilution. After 2 hours at room temperature, 0.1 ml of the mixture was injected into a site on the back of a normal rabbit. Our test dosage of toxin alone (1/300 L+ unit) produced a 20-mm circle of erythema with central necrosis in 3 days. Antitoxic activity (Table 1) is expressed as the number of L+ units neutralized by 1 mg of fraction protein or γ-globulin protein as determined from the minimal concentration completely inhibiting the

Table 1. Papain digests of diphtheria antitoxin; relation between hemagglutination inhibition and antitoxic activity.

Material tested	Antitoxic (L+ activity* units)	Hemagglutination inhibition activity† (μg)	Ratio, antitoxic: hemagglutination activity
Rabbit RV5, first bleeding			
Untreated γ-globulin	0.66		
Peak I ‡	.62	0.013	48
Peak II	1.9	.059	32
Rabbit RV5, second bleeding			
Untreated γ-globulin	0.84		
Peak I	.48	.012	40
Peak II	.87	.098	8.9
Rabbit RV8, first bleeding			
Untreated γ-globulin	0.64		
Peak I	.87	.016	54
Peak II	.72	.077	9.5
Rabbit RV8, second bleeding			
Untreated γ-globulin	0.96		
Peak I	.96	.033	29
Peak II	2.84	.240	12
Rabbit RV34, first bleeding			
Untreated γ-globulin	0.58		
Peak I	.66	.019	35
Peak II	1.32	.071	19
Rabbit RV34, second bleeding			
Untreated γ-globulin	1.32		
Peak I	0.66	.027	24
Peak II	2.64	.252	11

* Number of L+ units of diphtheria toxin neutralized by 1 mg of whole antibody γ-globulin or chromatographic fraction. † The number of micrograms of protein of whole antibody γ-globulin inhibited by 1 μg of protein in the test fraction. ‡ All peaks in the table represent the average of six chromatographic fractions.

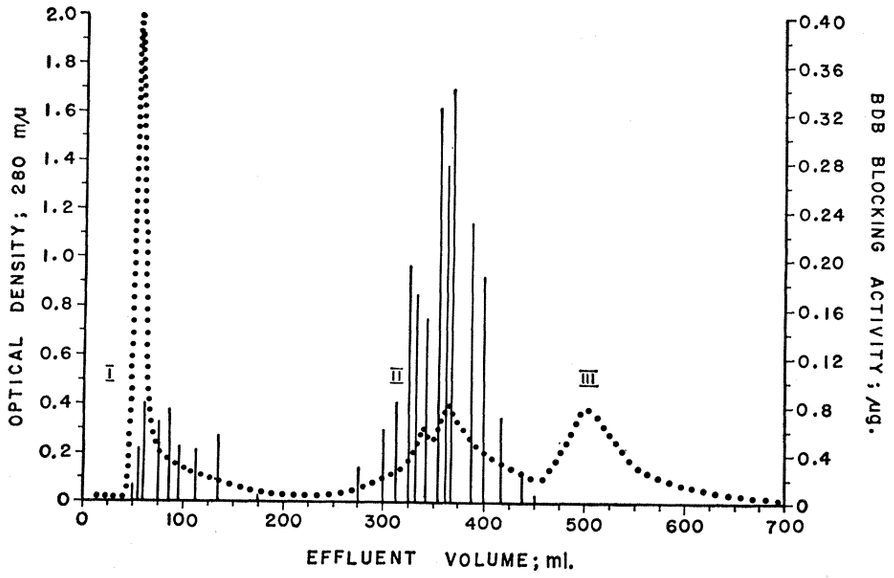

Fig. 1. Fractionation of papain-digested rabbit antiserum to diphtheria toxoid on carboxymethyl cellulose. The columns were eluted with 0.01*M* acetate buffer (*p*H 5.5) until the effluent volume was 200 ml, and then subjected to linear gradient elution with acetate buffer (*p*H 5.5) to a limit of 1.0*M*. The curve shows optical density at 280 mμ. The vertical bars indicate the inhibition of hemagglutination by fractions (micrograms of untreated γ-globulin inhibited by 1 μg of protein in the fraction).

necrotic reaction. Fractions from peak II had a significantly higher antitoxic activity than fractions from peak I; no fraction tested had less antitoxic activity than untreated γ-globulin.

Table 1 shows the average antitoxic and hemagglutination inhibitory activity of fractions from peaks I and II and the ratios of these activities in the six serums studied. Current evidence indicates that digestion of a single antibody molecule will yield either fragments I and III or fragments II and III (*6*). Antibody of one specificity may reside in fragment I and of another specificity in fragment II (*6*). Our data support these concepts; the difference in the ratios for peaks I and II may reflect a similar difference in the distribution of antibody specific for the toxic site as opposed to that directed against other antigenic sites on the toxin molecule.

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References and Notes

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Daily Rhythmicity of Corpus Allatum and Neurosecretory Cells in Drosophila melanogaster (Meig)

Abstract. *Adult females of* Drosophila melanogaster *show a bimodal daily rhythmicity in the function of neurosecretory and corpus allatum cells. This result was obtained by measuring the size of the nuclei and determining the pattern of neurohormone secretion.*

Previous experiments with insects seemed to indicate that neurosecretory and corpus allatum cells undergo cyclic changes during 24 hours (*1*). This has been shown either by measuring the nuclear volume by using a biological test or by just observing the amount of neurosecretory material in the cell. For further investigations it seemed desirable to measure also the amount of neurosecretion quantitatively and to work with genetically defined material under standardized conditions, which can be realized rather easily with *Drosophila*.

For these experiments I used highly inbred stocks of *D. melanogaster* and kept them at 25°C under a cycle of 12 hours of light and 12 hours of darkness. Flies, 3 to 5 days old, were killed every 3 hours and treated either according to methods of Gabe (*2*) or Gomori (*3*) for neurosecretory material, or Flax and Himes (*4*) for RNA. Sections of 2-μ or 4-μ thickness were cut with a cooled microtome knife; these sections had to be dried for 3 days on the albumin-coated slides before staining. After being embedded, the long and short diameters of the nucleus were measured by means of a camera lucida; the values were multiplied with each other, because the product gives a less arbitrary indication of the nuclear size than the attempt to calculate the volume.

The amount of neurosecretory material was determined from the absorption in the neurosecretory cells of the pars intercerebralis (*5*) after staining with paraldehyde fuchsin (PAF) or chromehematoxylin phloxine. The absorption spectrum of PAF-stained protein has its maximum at 550 mμ so that the green emission from a mercury vapor lamp could be used as an appropriate light source. The slide was projected by a microscope on a screen with a 0.0625-cm diameter hole opening to a photoelectric element. The absorption was measured once near the nucleus ("circumnuclear"), then toward the axon at the periphery of each cell. By calculating the ratio of these two values some variations in the amount of staining are eliminated, while a good indication is given of the secretory process.

The size of the nucleus of the neurosecretory cells of the pars intercerebralis stained with paraldehyde fuchsin (Fig. 1*a*) shows two peaks during 24 hours: a rather steep peak 3 hours before dawn and another, somewhat smaller but broader, 3 hours before dusk. The curve for the corpus allatum nuclei stained with Azure B (Fig. 1*b*) appears to be similar in the general

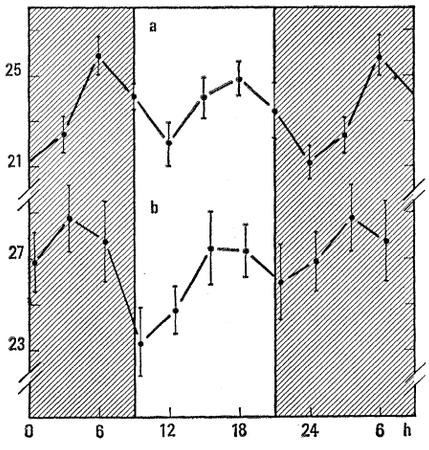

Fig. 1. Nuclear size of neurosecretory cells (*a*) and corpus allatum cells (*b*). Ordinate: product of longer and shorter diameter; abscissa: time in hours. Every point is the mean obtained from measurements on ten female flies, from each of which 15 to 20 nuclei were measured. Points of the dark phase are plotted twice on the same curve; vertical lines indicate the standard error.

shape, except that maxima and minima are occurring 3 hours earlier. The rather high variance in the corpus allatum curve may be due to individual differences in body size, slight differences in the conditions of development, and differences in the phase of the oscillation.

The distribution of neurosecretory

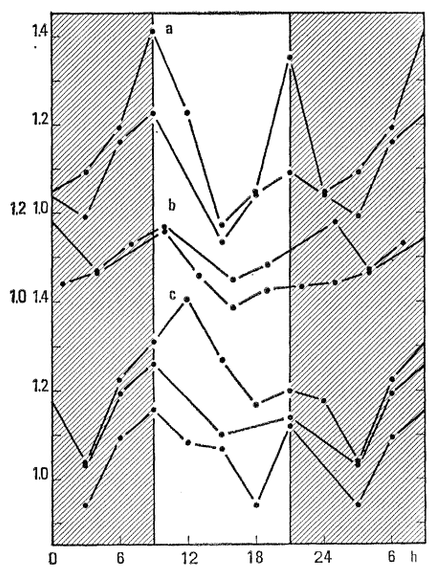

Fig. 2. Absorption of stained neurosecretory material. (*a*, *b*) Stock No. 103; *a* stained after Gabe (*2*), *b* after Gomori (*3*); (*c*) Stock No. 101, stained after Gabe. Ordinate: ratio between circumnuclear and peripheral absorption; abscissa: time in hours. Points of the dark phase are plotted twice on the same curve.