reagents, precipitants, adsorbants or whatnot. It accomplishes a separation of insulin, as prepared commercially, into active and inactive protein-like substances the isoelectric points of which are so close together that fractional precipitation as ordinarily practised is almost impossible.

Crystals, the chemical and physiological properties of which are being investigated, are formed from the active sediment, either by cision or by compound formation.

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The Ramon flocculation test in relation to the antigenic value of diphtheria toxoid (anatoxin).

By ABRAHAM ZINGHER.

[*From the Bureau of Laboratories, Department of Health, New York City.*]

In a series of publications, Ramon[1] and Glenny, Hopkins and Pope[2] have claimed that the antigenic value of diphtheria toxoid (anatoxin) can be determined by the flocculation test described by Ramon. Since anatoxin, as defined by Ramon, is completely atoxic, the L+ and Lo cannot be used for determining the neutralizing and antigenic values of such a preparation. There are three other tests, however, that can be utilized:

1. The flocculation reaction of Ramon;
2. The combining value for antitoxin, as shown by the neutralization test;
3. The immunizing value in guinea pigs.

The flocculation reaction of Ramon, as will be shown below, is not a true index of the antigenic value of toxoid (anatoxin). Baecher, Kraus and Lowenstein[3] have also come to the same conclusion as a result of their work with guinea pigs.

In a recent communication Nelis[4] states that while anatoxin

[1] Ramon, G., *Ann. de l' Inst. Pasteur*, 1925, xxxix, 1-21.

[2] Glenny, A. T., Hopkins, B. E., and Pope, C. G., *J. Path. and Bact.*, 1924, xxvii, 261.

[3] Baecher, S., Kraus, R., and Lowenstein, E., *Zeitschr f. Immunitats f.*, 1925, xlii, 350.

[4] Nelis, P., *C. R. de la Soc. de Biol.*, 1925, xcii, 1112.

preparations, which contain the largest amount of toxoid, have the best antigenic value, yet this property does not depend exclusively upon its presence in the anatoxin. He found that toxins inactivated by different agencies and in which toxoid could not be demonstrated any longer by its neutralizing power for antitoxin were still capable of producing immunity, although the appearance of the immunity was somewhat retarded. Such agencies were: prolonged exposure to incubator temperature, action of ozone on toxin, and toxins inactivated by sodium oleate (0.04 per cent) or by quinine bichlorhydrate (0.01 gm. to 50 M. L. D. of toxin).

In this connection it is of interest to note that Bronfenbrenner and Reichert[5] working with *Botulinus* toxin found that "a young toxin, though it be physiologically more potent than an older one and productive in animals of a highly antitoxic serum, may not produce precipitins; whereas a filtrate from a culture old enough to contain presumably a relatively high concentration of bacterial protein in addition to toxin produces a flocculating as well as antitoxic serum when used for immunization of animals." They conclude that the use of the flocculation reaction for the *in vitro* titration of *Botulinus* antitoxin is limited by the fact that the flocculating power is not strictly parallel to toxicity, but depends upon the presence of bacterial proteins in the antigen.

The combining value for antitoxin is shown by the addition of an excess of antitoxin and then by titrating the unneutralized antitoxin by toxin. According to Glenny, Pope and Waddington,[6] this method has one drawback: These observers claim that toxin has a tendency to dissociate the antitoxin from the toxoid, and then to combine with the antitoxin. This is due to the greater affinity of antitoxin for toxin than for toxoid.

The third method is by testing the immunizing value of toxoid (anatoxin) in guinea pigs. One human dose is given to a series of 10 or 12 guinea pigs. After four weeks a Schick test is made with 1/50 M. L. D. toxin on the denuded lateral side of the abdomen. Note is taken of the local reaction after 48-72 hours. The positive reactors are retested after 2 weeks on the opposite

[5] Bronfenbrenner, J., and Reichert, P., PROC. SOC. EXP. BIOL. AND MED., 1925, xxii, 391.

[6] Glenny, A. T., Pope, C. G., and Waddington, H., *J Exper. Path. and Bacteriol.*, xxviii, 279.

side of the abdomen. The percentage of negative reactors at each test will serve as a guide of the antigenic value.

The immunizing results in the guinea pigs are determined not only by the skin test with toxin but also by injecting the negatively reacting animals with amounts of toxin varying from 5 to 100 M. L. D., *i. e.,* 5, 10, 25, 50, 75 and 100 M. L. D. Such tests I made recently on 10 guinea pigs injected with 0.5-1 cc. of Ramon's anatoxin. Five of the animals gave a slight positive skin reaction to 1/50 M. L. D. at the end of 6 weeks; the others were negative. A second skin test made 2 weeks later with a similar amount of toxin showed that 9 of the 10 guinea pigs gave negative reactions. These animals were injected with doses of toxin varying from 2.5-100 M. L. D. The guinea pigs receiving 2.5, 5 and 10 M. L. D. showed no local reactions. Those receiving 25 to 50 M. L. D. had local induration and necrosis, but recovered. After 75 M. L. D. the animal survived for 8 days; after 100 M. L. D. the guinea pig died after 48 hours. Even 0.2 cc. of Ramon's anatoxin protected after a period of 6 weeks three guinea pigs against 50 M. L. D. of toxin, although the animals showed considerable local induration at the site of the toxin injection.

THE FLOCCULATION REACTION OF RAMON

This depends upon the addition of graded amounts of antitoxin to a definite amount of toxin. The tube showing the most complete neutralization will be the first one to show flocculation. The rapidity of the appearance of the reaction depends upon the strength of the toxin, the temperature of the waterbath or incubator, the reaction of medium, etc.

In preparing our toxoid with formalin we noted that the preparation first became opaque and that a precipitate formed after the toxoid remained for a few days in the thermostat. This settled out later in the ice box as a heavy sediment. *This precipitate carried down with it the flocculable substance.* After filtration through a Berkefeld, the clear filtrate tested with the Ramon test showed no flocculation. It had, however, retained all its immunizing value. Such a filtrate gave as good immunity results as the unfiltered toxoid when the amounts injected were equal.

The precipitation in the toxoid was due to the action of the formalin upon the toxin to which 0.4 per cent tricresol (alcresto)

had been added as a preservative. Toxin without tricresol showed no precipitation upon the addition of formalin. The addition of formalin in amounts increasing from 0.1 per cent to 0.75 per cent produced varying degrees of opacity, depending upon the amount of formalin added. When phenol is used as a preservative, the precipitate formed may be very slight or absent. Ramon adds no preservative to the diphtheria toxin, and has never noted such a precipitate.

Immunity Results in School Children Showing the Relation Between the Ramon Flocculation Test and the Antigenic Value of Diphtheria Toxoid (Anatoxin).

Preparation	Berkfeld Filtration after formalin treatment.		L f Per cc. Toxoid	Doses.		Clinical Results.	
	For Flocculation Test	For Active Immunization		Number	Amount cc.	No. Tested	Per Ct Neg. on Schick Retest
1—Toxin 589	No		4 units				
2—Toxoid 589	Yes		0				
3—Toxoid 2 C	Yes	Yes	0	3	0.5	226	79.0-95.0
4—Toxoid 10	Yes	No	0	3	1.0	124	98.0
5—Toxoid 7	Yes	No	0	3	.25	359	58.0-86.0
6—Anatoxin, Ramon	No	No	10	3	0.5-1.0	205	98.0
7—Toxoid 437	No	No	2.5-3.5 0	3	0.05-0.1	1500	84.0-94.0
8—Toxoid 377	No	No	2.0 0	3	0.07-0.1	1200	27.0-63.0

The table shows that Toxin 589 gave a flocculation test with 4 units of antitoxin per cc. After the addition of formalin (0.25 per cent) the typical cloudiness and precipitate developed. Berkfeld filtration cleared the toxin. No flocculation occurred when antitoxin was now added in amounts varying from 12 to 1 units to one cc. of toxoid. This preparation (Toxoid 589) was not used for human immunization.

Toxoid 2 C was similarly treated with formalin and cleared by Berkfeld filtration. *No flocculation was noted with the cleared filtrate. Three doses of 0.5 cc. each immunized from 79 to 95 per cent of children.*

Toxoid 10 was passed through the Berkfeld filter for the flocculation test, but used for human immunization without filtration. No flocculation was noted. Of the injected children, 96 per cent became immune with 3 doses, each 1 cc.

Toxoid 7 was not passed through the Berkfeld filter for the flocculation test. No flocculation was noted. For immunization the cloudy preparation was used. Three doses of 0.25 cc. only immunized from 58 to 86 per cent of the injected children.

Anatoxin (Ramon) consists of several preparations sent to me by Ramon. No Berkfeld filtration was necessary as the preparation was clear. The flocculation test showed that 10 units of antitoxin gave the initial flocculation with 1 cc. of the anatoxin. Injected into guinea pigs in doses of 0.5 to 1.0 cc., 90 per cent of the animals gave a negative skin reaction to 1/50 M. L. D. of toxin after 8 weeks, a preliminary test having been made 2 weeks previously.

Among children, 98 per cent gave a negative Schick test 6 weeks after the third dose of anatoxin. The doses were 0.5 cc., 0.5 cc., and 1.0 cc., given at intervals of two weeks.

The flocculation and animal immunization tests on Toxoids 437 and 377 were kindly carried out for me 6 months ago by Ramon, by Glenny of the Burroughs, Wellcome & Co. Laboratories, and by Moloney of the University of Toronto. These observers obtained somewhat different results. With Toxoid 437 the Lf value was found to vary between 2.5-3.5 units per cc. A recent test showed that the toxoid had lost its power to flocculate with antitoxin, although the immunizing value for guinea pigs was not impaired.

Toxoid 377, according to Moloney, had a flocculating value of

20; according to Glenny of 2; according to Ramon 0.5 unit of antitoxin only produced a cloudiness after 24 hours with 1 cc. of toxoid.

In guinea pigs, according to Banzhaf, Toxoid 437 in 0.1 cc. dose immunized (negative Schick test) only 15 per cent of the animals, and Toxoid 377 in 0.1 cc. dose 50 per cent of the animals. According to Moloney 0.2 cc. of Toxoid 437 immunized 2 out of 3 guinea pigs and 0.2 cc. of Toxoid 377, all of 3 guinea pigs; he concludes, however, "that the immunizing power of the two toxoids for guinea pigs is about the same." According to Glenny, 1 cc. of Toxoid 437 immunized guinea pigs in 15 to 18 days; 1 cc. of Toxoid 377 in 19 to 21 days. The first (437) required 2 to 3 previous Schick tests. According to the animal results Banzhaf found Toxoid 377 about 3 times as efficient as Toxoid 437; Moloney found them of equal value and Glenny found Toxoid 437 to be more efficient than Toxoid 377.

In the active immunization of children I obtained strikingly different results, using the two toxoid preparations in doses of 0.05-0.1 cc. Of 1500 children injected in different schools with three doses of Toxoid 437, from 84 to 94 per cent gave a negative Schick retest; of 1200 children injected with Toxoid 377, 27 to 63 per cent gave a negative Schick retest.

CONCLUSION.

The flocculation reaction does not appear to be an index of the antigenic value of a diphtheria toxoid (anatoxin).

The reaction is probably a specific bacterial precipitation phenomenon.