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PROBLEMS OF ANTIVENENE STANDARDIZATION REVEALED BY THE FLOCCULATION REACTION

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SYNOPSIS

Mixed with either of the specific venoms, refined antivenene (anti-*Bitis arietans-Naja flava*) will show optimal flocculation at a number of different venom : serum ratios. This is the natural consequence of individual antigen-antibody reactions in a heterogeneous system.

Toxin may be recovered from floccules formed at three optimal *N. flava* venom: serum ratios by heating the floccules at 70°C for ten minutes at pH 2. These three immunologically distinct heat-stable toxins have been called the α, β, and γ toxins of *N. flava* venom.

The flocculation reaction obtained with the viperine venom cannot be interpreted in terms of protective potency.

Most venom-antivenene systems fail to conform with the " law of combination in multiple proportions ", i.e., they give non-linear titration curves. The shape of such curves, convex towards the dose axis, is readily explained by the simultaneous titration of multiple toxin-antitoxin systems.

In the particular case of *N. flava* venom-antivenene, the titration curve obtained with whole venom is determined by the relative anti-α, anti-β, and anti-γ contents of the serum. Further study may pave the way to the standardization of the anti-α, anti-β, and anti-γ potencies of sera, but attempts at standardizing antivenene potency against any venom are futile unless the toxic " make-up " of the venom is understood.

The cross-protection afforded by sera produced against either *N. flava* venom, or the venom of *Sepedon haemachates* is limited but indicates an antigenic similarity which may be clarified by work in progress.

Precipitation in mixtures of venom and antiserum from rabbits was originally observed by Lamb [21] and has since been amply confirmed. It is especially interesting to note that, as early as 1909, Calmette & Massol,[6] who worked with cobra venom and sera from three immune horses, described all the essential features of the particular type of precipitation which is now known as the Ramon flocculation reaction. In 1904 Lamb [22]

had recorded that the reaction between Indian cobra venom and antisera from horses was either weak or absent but reference to tables IX and X in his publication indicates that the precipitation obtained with serum from one of the horses was, in fact, an optimal flocculation. Agreement between potency estimates obtained by flocculation and by animal experimentation has been observed with anti-*Vipera russelli* serum,[14] with anti-*Crotalus terrificus* serum,[2, 4] and with serum against *Bothrops jararaca* venom.[3] Schöttler [25] expressed the view that the concordance observed with the two latter venoms is coincidental. Other workers' experience with other venoms has led to similar conclusions; for example the flocculation obtained with *Vipera aspis*, *Cerastes cornutus*, and *Naja tripudians* venoms as antigens has been found not to reflect the in vivo potency of the respective antisera.[7, 8, 9, 23] With regard to venoms with which we in these laboratories are particularly concerned, Grasset and co-workers [19] recorded :

" In the case of the sera of horses immunized with puff adder and Cape cobra anavenoms we have been able to demonstrate a specific flocculation reaction, and if, as mentioned by Calmette, it does not give a true assessment of the two constituents, it certainly assists in affording an accurate estimate of the zone of neutralization and of indicating the specificity of the venom-antivenene reaction."

Speaking of *Bitis arietans* venom-antivenene mixtures, Grasset [16] has stated :

" When series of mixtures are prepared to correspond closely to the neutralization range, an opalescence is observed which appears usually within a few minutes of their preparation. The opalescence increases rapidly and usually results in an ' original ' flocculation as in the case of diphtheria toxin-antitoxin mixtures . . . A similar flocculation is observed quite frequently in the *N.* [*Naja*] *flava* venom serum mixtures but is usually delayed, taking place after a few hours and is of less heavy character. In unpublished investigations by the author on the possible use of this phenomenon in a titration method, the same conclusion was reached as that expressed by Calmette (1908) in the case of *N. tripudians* venom-antivenene titration. The phenomenon of original flocculation in specific venom-antivenene mixtures takes place in a zone close to the neutralization point but does not allow such a close, accurate titration as that obtained from the inoculation, with the same series of mixtures, of experimental animals such as mice."

The present study deals with the flocculation which occurs when " polyvalent " horse sera are mixed with the specific venoms (*N. flava*, *B. arietans*), i.e., the venoms which served as antigens for the horses. Unmodified sera from immune horses show with either venom optimal flocculation at more than one, and usually at several, venom : serum ratios; such multiple " points " of optimal flocculation are always more readily demonstrable when refined antivenene is employed. This complexity is a natural consequence of individual antigen-antibody reactions in a heterogeneous system. All snake venoms have many different actions both in vivo and in vitro, and the presence of many venom components is usually assumed to explain these actions. These components are named

after their effect, for example, haemorrhagin, haemolysin, proteolysin, rennin-like factor, etc., which, although convenient, may be misleading because very little is known about most of these components and their effects. Some of them may be responsible for more than one of the observed actions but, on the other hand, a given effect may well be caused by more than a single component. Most, if not all, reactions of venom can be inhibited by antiserum, and under suitable conditions any component might be expected to flocculate with its antibody. The many individual flocculation reactions which take place in a *B. arietans* venom-serum system cannot, at the present stage of our knowledge, be interpreted in terms of protective potency against its biologically active components, lethal or otherwise. It was hoped that the flocculation reaction in an *N. flava* venom-serum system would prove easier to interpret because this venom behaves as if it contains but one lethal toxin, often referred to as " neurotoxin ", and because there has been reason to place a fair amount of trust in the results of protective anti-*Naja* potency assays in animals. However, this system is also very complex. Pepsin-refined sera, as we shall see, may flocculate at no less than eleven different venom : serum ratios. Unmodified sera from horses undergoing immunization will rarely give more than two separate flocculation reactions within a range of ratios which, with reason, could be expected to cover the reaction between the lethal toxin and its antibody. However, the titre determined in mice and the points of optimal flocculation alter apparently independently of each other when individual horses' sera are followed during immunization. Because the toxin portion of *N. flava* venom is remarkably heat- and acid-stable it has been possible to demonstrate that it is composed of three immunologically distinct flocculable components. The methods employed are not new; it has long been recognized that toxin is recoverable from neutral mixtures of venom and antiserum [5] and from the floccules formed in such mixtures.[6] Calmette & Massol [6] recovered almost all the original (presumably Indian) cobra venom activity by heating acid-dissolved floccules at 72°C for one hour. The findings of these early workers have frequently been quoted in support of the view that no change is brought about in antigen by its union with antibody, but otherwise seem to have been neglected although they open up a fruitful approach to the study of elapine venoms.

Materials

Venoms

The venoms used were *N. flava* venom from reliable suppliers of venom to the South African Institute for Medical Research; *N. naja* venom purchased from an Indian drug house of repute; and *Sepedon haemachates*

and *B. arietans* venoms collected in this laboratory. Samples from bulk stocks of dry powdered venom were used throughout these experiments; variation among snakes may thus be disregarded.

Antisera

The antisera used were S 7432 and S 7837, two batches of concentrated polyvalent antivenene (anti-*B. arietans-N. flava*), refined by Pope's method,[20] as issued by this Institute for therapeutic use; and R 809, a refined and concentrated anti-*S. haemachates* serum, derived from one horse.

Animals

Laboratory-bred white mice weighing 16-18 g were used.

Methods

Determination of optimal flocculation ratios

Venom-antivenene mixtures were made to flocculate in rubber-stoppered test-tubes (3 inch × $^3/_8$ inch or 3 inch × $^1/_4$ inch (76 mm × 9.5 mm, or 76 mm × 6.3 mm)) maintained mechanically in gentle motion in a water-bath at 45°C.[24] In *N. flava* venom-antivenene mixtures the antigen : antibody ratios for optimal flocculation of the several individual antigen-antibody systems it contains are independent of whether antigen (β or Ramon procedure) or antibody (α or Dean & Webb procedure) is kept constant in the mixture, and either procedure was used according to convenience. With the materials and methods used, the Kf is small for the points of flocculation with high venom : serum ratios; it increases with decreasing ratio and the interval before flocculation of the venom fraction which requires relatively most serum is about 60 minutes or longer.

Collection of floccules in bulk

Two ways of approach were open when the venom : serum ratios for optimal flocculation had been determined. The one, which for convenience will be referred to as method A, consisted in mixing larger quantities of venom and serum in the exact proportions required for flocculation, one mixture for each of the individual reactions observed, and in estimating the amount of toxin liberated from the isolated floccules and supernatant fluids by the acid-heat treatment described on page 357. The second approach, method B, consisted in starting with a solution of venom and adding the calculated small amount of serum required for flocculation at the highest optimal ratio. After removal of these floccules by centrifugation the supernatant fluid could again be flocculated at the next, lower venom: serum ratio by the addition of more serum. The exact amount of serum to be added was determined by a pilot flocculation test on mixtures of the

supernatant and serum, and checked by calculation. Similarly the other flocculable fractions could be removed one by one by stepwise addition of the correct amounts of serum. All these floccule-fractions and the final supernatant fluid were examined for toxin after acid-heat treatment.

Liberation of toxin from mixtures and floccules

Earlier experiments had indicated that some of, but not all, the lethal *N. flava* toxin could be set free from washed floccules either by a short heating in acid solution or by the addition of trichloracetic acid, which does not precipitate this venom. At the time, this was ascribed to incomplete liberation of venom, and the first step, therefore, was to determine the most efficient way of recovering toxin from its combination with antibody. To this end samples of a serum-venom mixture were exposed to different treatments. The mixture contained serum in excess of the amount required for complete in vivo neutralization, and the amount of toxin recovered would indicate the least proportion of toxin that could be expected to be liberated if floccules, and not serum-venom mixtures, were exposed to the same treatment. The effect of various combinations of hydrogen-ion concentration, temperature, and trichloracetic acid concentration was examined. Heating at 70°C for ten minutes at pH 2, i.e., the method of Calmette & Massol,[6] was found to be the simplest and most efficient way of liberating all the toxin from a solution in which cobra venom had combined with antibody. This method has therefore been used in the experiments to be reported here. The floccules were separated from the supernatant fluid by centrifugation (2,000 revolutions per minute (r.p.m.), radius 16.5 cm, for 15 minutes, unless otherwise stated), washed twice with about twenty times their volume of a 0.9% solution of sodium chloride, suspended in a small volume of this saline, dissolved by the addition of a drop of concentrated hydrochloric acid, and transferred quantitatively into a measuring cylinder. The solution was brought to pH 1.8-2.0 (glass electrode) and, after a final volume adjustment, the solutions were heated in stoppered glass vessels. The temperature was brought rapidly to 70°C and maintained at between 70°C and 72°C for ten minutes. The supernatant fluids were heated under the same conditions after dilution with an equal volume of saline or distilled water in order to avoid gel formation. No precipitation of protein takes place under these conditions and the toxic solutions may be tested (flocculation, toxicity assay, titration against antivenene) without further treatment other than neutralization by the incorporation of buffer solution (veronal or phosphate) in the test mixtures.

Toxic potency assays

The intravenous median lethal dose (LD_{50})—24 hours—was estimated graphically (experiments 1 and 2) or arithmetically (other experiments)

by the probit method.[13] When not specifically stated, and excepting preparations possessing only traces of toxin, the limits of error of the LD_{50} were ± 10% or less for a probability of 0.95. The expression of toxicity in per cent, found in the text and tables, requires some explanation. The effect of the LD_{50} of the various toxic solutions has been accepted as identical with the effect caused by 0.0074 mg (the LD_{50}) of the original dry *N. flava* venom. In other words, the toxicity of a given solution may be said to be equivalent to the toxicity of a solution of the original venom of a certain concentration. This experimentally ascertained equivalent venom concentration has been expressed in per cent of the maximum possible concentration which could have been expected if all the venom present in the original reacting mixture had been recovered in the preparation under test. This is, in fact, based on a standard-curve method of toxicity estimation which, despite its shortcomings, was adopted for practical reasons which are obvious when the number of tests involved are considered. It should, however, be mentioned that the identical *N. flava* venom examined on two earlier occasions, and again at the conclusion of the present experiments, gave the LD_{50} estimates recorded in table I which show excellent agreement. The data presented here may thus be accepted with some confidence, although it should be pointed out that in comparing the LD_{50} of the original venom with the LD_{50} of the test preparations it has been assumed, without immediate proof, that the observed responses have been caused by the same toxic principle. The justifiability of this assumption will be discussed later.

TABLE I. LD_{50} OF ONE SAMPLE OF NAJA FLAVA VENOM ESTIMATED ON THREE OCCASIONS

Date	Best estimate (mg)	5% fiducial limits (mg)
November 1950	0.00746	0.00793 — 0.00702
January 1951	0.00714	0.00745 — 0.00685
December 1952	0.00742	0.00770 — 0.00715

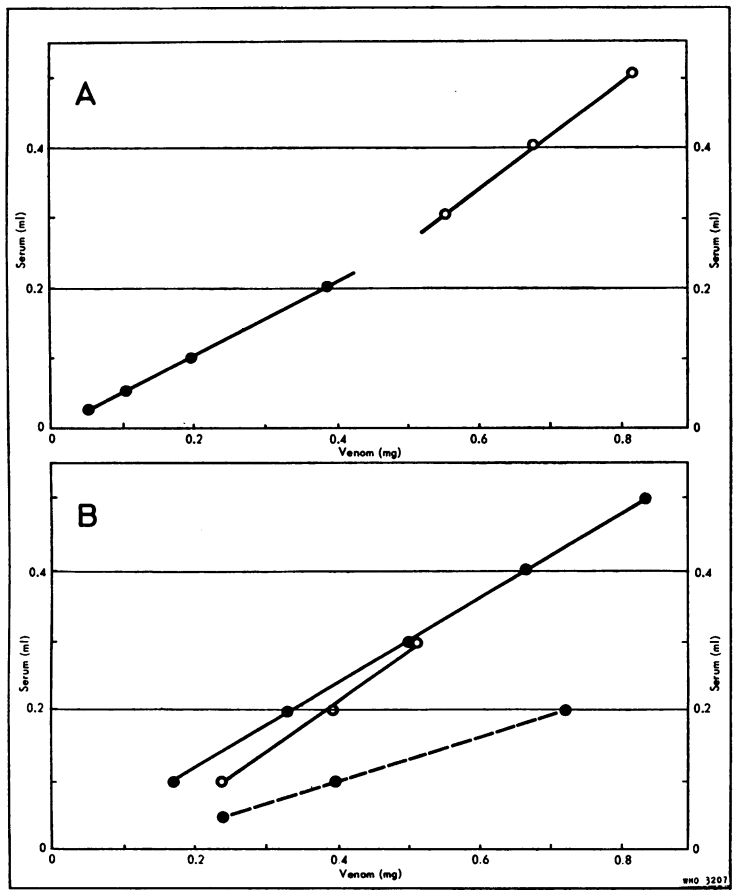
Limited use has been made of the graded response method [11] by which the test preparations were compared directly with the original venom employing a six-point design and five mice per point.

Antitoxic potency assays

A constant amount of serum was mixed with graded amounts of the toxic material (crude venom or fractions obtained from the floccules) and, after half an hour's contact at room temperature, the mixtures were injected intravenously into mice. These assays were carried out at several fixed levels of serum dose. The actual serum and venom concentrations tested

are indicated on the accompanying charts. The increments in venom dose were from 5% to 10%. Four mice (fig. 1 (A) and curves A and C in fig. 2 and 3) or two mice (fig. 1 (B) and curve B in fig. 2 and 3) were employed per venom-serum mixture according to the amounts of available material.

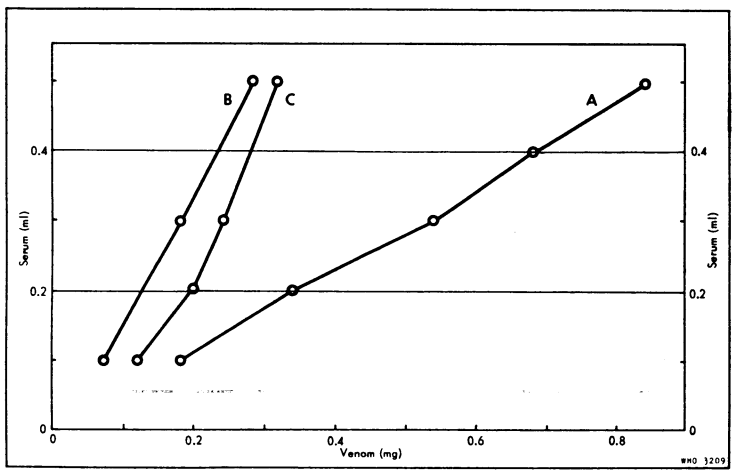
FIG. 1. CURVES OBTAINED BY TITRATION OF S 7837 AGAINST NAJA FLAVA VENOM



A = against whole venom B = against toxic components α, β, γ
—●——●— = points obtained with α toxin (B) or with toxin alleged to be α (A)
—○——○— = points obtained with γ toxin (B) or with toxin alleged to be γ (A)
—●—–—●— = points obtained with β toxin

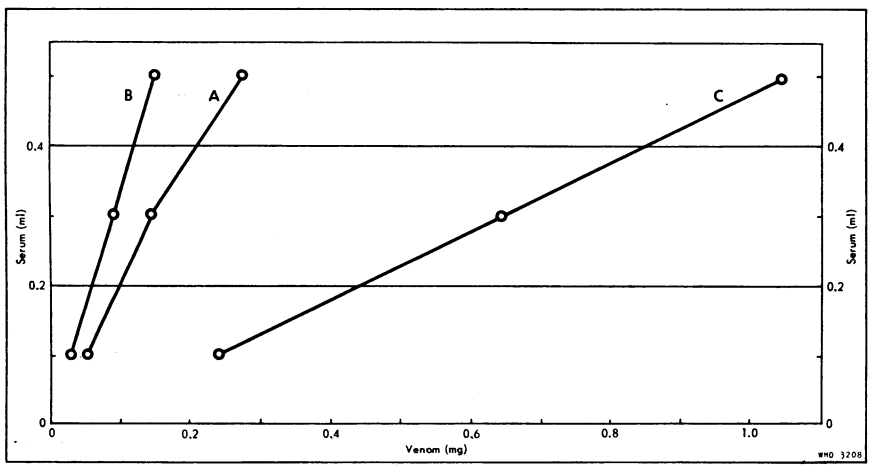
The end-point, majority survival, was read after 24 hours and was extremely sharp except in the titration of S 7432 against *S. haemachates* venom (fig. 2, curve C).

FIG. 2. CURVES OBTAINED BY TITRATION OF S 7432 AGAINST NAJA FLAVA VENOM (A, SPECIFIC), NAJA NAJA VENOM (B), AND SEPEDON HAEMACHATES VENOM (C)



The LD_{50} for *N. flava* venom was 0.0074 mg, for *N. naja* venom 0.0083 mg, and for *S. haemachates* venom 0.026 mg.

FIG. 3. CURVES OBTAINED BY TITRATION OF R 809 AGAINST NAJA FLAVA VENOM (A), NAJA NAJA VENOM (B), AND SEPEDON HAEMACHATES VENOM (C, SPECIFIC)



The LD_{50} for *N. flava* venom was 0.0074 mg, for *N. naja* venom 0.0083 mg, and for *S. haemachates* venom 0.026 mg.

Experiment 1

N. flava venom and S 7432 were found to flocculate at the following ratios (mg venom : ml serum), 1.00, 2.00, 6.40, and 32.00; higher ratios were not examined in this experiment. The floccules and supernatant fluids at these ratios were obtained by method A and examined. Somewhat more than 50% of the toxin could be accounted for in the floccules (see table II), and those formed at the two lower ratios contained the bulk of it.

TABLE II. PERCENTAGE RECOVERY OF TOXIN FROM FLOCCULES AND SUPERNATANT FLUIDS OBTAINED BY METHOD A AT DIFFERENT RATIOS (mg VENOM : ml SERUM)

Ratio	Percentage toxin recovered from	
	floccules	supernatant
32.00	1	95
6.40	1	89
2.00	15	99
1.00	37	59

Experiment 1, venom : *Naja flava* ; serum : S 7432

The sum of the toxicities of each set of floccules and supernatant fluid agrees fairly well with the expected value, 100%, when allowance is made for experimental error. The sum at ratio 2.00 (114%) is suspiciously large but a repeat experiment in which only the floccules and supernatant fluids obtained at the two lower ratios were examined gave the following result. At ratio 2.00 the floccules yielded 19% and the supernatant 72% of the total toxin, at ratio 1.00 the corresponding values were 37% and 45% respectively. As only between 50% and 60% of the toxin could be accounted for in the floccules an explanation was sought. The " missing " toxin might flocculate at a hitherto unobserved venom : serum ratio, different from those examined, or the time allowed for complete flocculation might have been insufficient. It was also thought likely that the centrifugation (about 2,000 r.p.m. for 15 minutes) had been inadequate for the complete separation of floccules and supernatant fluid, particularly at the low venom : serum ratios where the mixture is rather viscous. Should these explanations prove invalid, it might be necessary to postulate the presence of non-flocculating toxin or antibody. The possibility of unnoticed optimal flocculation ratios was examined by repeated tests in which the intervals between examined ratios were narrowed to about 5%. To extend the examined range much beyond a ratio of 32 seemed unnecessary because the venom : serum ratio of the

mixture, just neutral in the mouse, had been determined as less than 2.0 (see fig. 2). However, it was discovered that the ratio 1.00 was unhappily chosen because the floccules formed in a mixture of this composition probably consisted of components from two close but distinct flocculation systems, the one with an optimal ratio of 1.08, the other with one of 1.32. These floccules obviously had to be examined separately but it was considered preferable to choose another serum which gave a better separation of the flocculation points. All the available refined antivenenes were derived from the pooled sera of many horses and varied little from batch to batch with regard to the relative position of the different points of optimal flocculation. However, one batch, S 7837, gave five well-separated flocculation ratios and was used in the next experiment.

Experiment 2

Method B gave the results recorded in table III. The application of this method was facilitated by an increasing Kf with decreasing venom: sreum ratio; this diminished the chance of contaminating the floccules

TABLE III. PERCENTAGE RECOVERY OF TOXIN FROM FLOCCULES OBTAINED BY METHOD B AT THE RATIOS (mg VENOM : ml SERUM) SHOWN, AND FROM FINAL SUPERNATANT FLUID

Source of toxin		Toxin recovered (%)
Floccules at ratio	20.00	1 - 2
Floccules at ratio	6.40	1
Floccules at ratio	3.20	2
Floccules at ratio	2.40	27
Floccules at ratio	1.38	37
Floccules at ratio	0.80	5
Final supernatant		38

Experiment 2, venom : *Naja flava* ; serum : S 7837

collected at a certain ratio with floccules from the next, lower, ratio. The optimal ratio 2.40 shown in table III was not actually observed in the preliminary flocculation test because it was obscured by " spread " of the flocculation in the tube containing the reagents in the ratio 3.20 to the adjoining tubes. However, after removal of the floccules formed at ratio 20.00, then the floccules at ratio 6.40, and finally the floccules at ratio 3.20, it was seen that the resulting supernatant not only flocculated at the expected ratios 1.38 and 0.8, but also at a higher venom : serum ratio. This " new " optimal ratio was determined by calculation as 2.40. The results recorded

in table III are in agreement with those of the previous experiment. The three lower ratios accounted for most of the toxin, here nearly 70%; the bulk of the balance was found in the final supernatant, and only negligible amounts in the floccules removed at the high venom : serum ratios.

To expect a lethal fraction to flocculate at a higher venom : serum ratio than those examined in this experiment seemed unreasonable, yet the possibility could not be excluded. The venom might, for instance, contain a lethal fraction so dominant in antigenicity that high concentrations of antibody would be produced by horses stimulated with whole venom. The sera S 7432 and S 7837 did not flocculate at lower ratios than those examined but, by extending the range examined upwards to include ratios corresponding to between 2,000 and 3,000 mg of venom per ml of serum, the following higher ratios of optimal flocculation were observed with S 7837 : 50, 80, 180, 400, and 2,100. Floccules formed at these ratios were all examined (method A) and found to be atoxic; all the toxin could be accounted for in the supernatant fluids (the toxin estimates ranged from 90% to 100%). The failure to recover all the heat-stable lethal toxin in the floccules was thus more likely to be due to incomplete flocculation or incomplete harvesting of the floccules. The next experiment was designed to test this point.

Experiment 3

The limited stocks of the serum (S 7837) employed in experiment 2 had to be reserved for potency assays and the three major toxic fractions were isolated by method B from a solution of 200 mg of venom by flocculation with S 7432. The technique was altered slightly to make the conditions more favourable for complete recovery of the floccules. The mixtures were shaken gently for about five hours at 45°C, left for 24 hours at room-temperature, and thereafter in the refrigerator for five days, before the floccules were separated by centrifugation (2,000 r.p.m. for one hour), and washed. The three fractions and the final supernatant fluid were treated and tested in the usual way. The larger total amount (82%) of toxin liberated from the floccules in this experiment and the correspondingly smaller amount (16%) of toxin demonstrable in the supernatant fluid support the conclusion that all the venom's heat-stable toxin is found in the three flocculable fractions demonstrated here. These three immunologically distinct fractions have provisionally been called α, β, and γ toxins of *N. flava* venom in the order of toxic importance as indicated in table IV. Some of the other flocculable venom fractions yielded small amounts of toxin (see tables II and III) but can at most play a minor role in cobra-venom poisoning. These minor fractions are not heat-stable, in the sense in which this expression is used in the present study; this was borne out by an experiment in which S 7837 was flocculated with a solution

TABLE IV. PERCENTAGE RECOVERY OF TOXIN FROM FLOCCULES, OBTAINED BY METHOD B AT THE RATIOS (mg VENOM : ml SERUM) INDICATED, AND FROM FINAL SUPERNATANT FLUID, AND THE ANTIBODY-COMBINING-POWER OF THESE PREPARATIONS IN PER CENT OF MAXIMAL VALUE

Source of toxin	Toxin recovered (% of total venom toxicity)	Antibody-combining-power (% of maximal value)
Floccules at ratio 2.00 (β toxin)	19.7	71
Floccules at ratio 1.32 (α toxin)	62.1	75
Floccules at ratio 1.08 (γ toxin)	4.1	50
Final supernatant fluid	16.3	—

Experiment 3, venom : *Naja flava* ; serum : S 7432

of venom which had been heated at 70°C for ten minutes at pH 2. By comparison with an unheated control solution it was seen that the venom : serum ratios for the minor fractions were considerably increased, whereas the antibody-combining-power of the α, β, and γ toxins remained unaltered. The antibody-combining-power of the three toxins was determined by flocculation with S 7837, and in table IV the results have been expressed in per cent of the maximal possible values—i.e., the antibody-combining-power of whole venom in respect of the three individual fractions. These results indicate that nearly three-quarters of both the α and the β toxin, and about half the γ toxin, had been recovered in the floccules. The ratios at which the α and γ toxins flocculate in mixtures of whole venom and S 7432 are so nearly identical that it would be surprising if contamination of the α preparation with some γ toxin had been avoided under the conditions of this experiment. Indeed, a flocculation test carried out with the α preparation showed clearly that in addition to its α toxin it contained an amount of γ toxin equivalent to about 38% of the γ toxin present in the original serum-venom mixture. This was ascertained by the " blending " technique. The residual toxicity of the final supernatant fluid was probably due to the small " missing " fractions of the three toxins. It might be suggested that a flocculation test involving " blending " and the acid- and heat-treated supernatant fluid as antigen would have cleared up this point, but the relatively large amount of heat-denatured serum protein in the supernatant fluid was found to interfere with the interpretation of the flocculation reaction, being precipitated at pH values and concentrations which would allow visible flocculation to occur.

Experiment 4

The purpose of this experiment was to obtain the three toxins in sufficient quantities for use in antitoxic potency assays. S 7432 was employed and the fractions flocculating at the venom : serum ratios 2.00 (β toxin),

1.32 (α toxin), and 1.08 (γ toxin) were collected by method B. The activities of these fractions and the final supernatant fluid were tested after liberation of the toxin, and the results are recorded in table V. The floccules and supernatant fluids were well separated by centrifugation but no attempt was made to increase the yield of toxin by prolonged flocculation, which might lead to loss of purity, particularly of the α preparation.

TABLE V. PERCENTAGE RECOVERY OF TOXIN LIBERATED FROM FLOCCULES, OBTAINED BY METHOD B AT THE RATIOS (mg VENOM : ml SERUM) INDICATED, AND FROM FINAL SUPERNATANT FLUID, AND THE ANTIBODY-COMBINING-POWER OF α, β, AND γ PREPARATIONS IN PER CENT OF MAXIMAL VALUE

Source of toxin	Toxin recovered (% of total venom toxicity)	Antibody-combining-power (% of maximal value)
Floccules at ratio 2.00 (β toxin)	11.4 (11.3) *	63
Floccules at ratio 1.32 (α toxin)	53.7 (58.0) *	63
Floccules at ratio 1.08 (γ toxin)	3.7	40
Final supernatant	18.6	—

* The estimates in brackets are based on the graded response method
Experiment 4, venom : *Naja flava* ; serum : S 7432

The figures recorded in the last columns of tables IV and V have been included because they offer a means of estimating approximately how much of each of the three toxins was recovered in the floccules and how much was left behind. Taking as example the data for the β toxin in table V, it will be seen that the antibody-combining-power test indicates that only 63% of the total amount of this toxin had been recovered from the floccules, and with this information it may be estimated that the venom's total content of β toxin is responsible for $11.4 \times 100/63 = 18.1\%$ of its toxicity. Table VI is based on the data in tables IV and V " corrected " in this way. The potencies of the α and β preparations examined in this experiment were estimated by the quantal response method and by the graded response method [11] employing the logarithm of death-time as response metameter. It was found that the individual graded response curves for the α toxin, the β toxin, and the reference standard solution of venom showed no significant deviation from linearity, nor was there significant deviation from parallelism among the lines. The potency estimates obtained were in complete agreement with those based on the quantal response (table V). The data obtained for the γ toxin by the graded response method indicated that the response curve was significantly steeper than those observed with the other two fractions and the standard solution. This suggests another mode of action of the γ toxin. The elucidation of the part played by these

toxins in the symptoms produced by cobra venom will require detailed study, but the evidence so far gathered indicates that the toxins here referred to as the α, β, and γ toxins are responsible for the lethal effect of the venom, and that the α and β toxins have similar actions because (*a*) the gross symptoms produced by whole venom and by either of these fractions are indistinguishable; (*b*) their graded response lines are parallel and, moreover, parallel with the response line obtained with whole venom;

TABLE VI. THE TOTAL AMOUNTS OF α, β, AND γ TOXINS PRESENT IN NAJA FLAVA VENOM EXPRESSED IN PER CENT OF THE TOXICITY OF WHOLE VENOM

Toxin	Data from table IV	Data from table V
β	27.7	18.1
α	82.8	85.2
γ	8.2	9.3

See text for details

(*c*) as reference to table VI will show, the sum of the toxicities of the α and β preparations expressed in per cent agrees reasonably well with the value, 100%, which could be expected if these two toxins were responsible for the effect produced by the LD_{50} of whole venom. If a group of mice is given an LD_{50} of either α or β toxin, or of whole cobra venom, an hour or more may pass before symptoms of intoxication become obvious; from then onwards the symptoms gradually increase in severity. The mice move sluggishly if disturbed and all have respiratory difficulties, but about half will recover, gradually but completely; the other half of the group will die, death being preceded by a shorter or longer period in which the mouse rests flat on the belly with slow and forced diaphragmatic respiration. The interval before death varies from two to 24 hours. Given twice the LD_{50} the animals pass through the same symptoms more quickly, all die, and the mean time-interval before death is about 50 minutes. Animals receiving γ toxin behave differently. An LD_{50} of γ toxin produces immediate restlessness, and respiratory and general discomfort so severe that all the animals appear moribund. However, although about half the animals die in approximately 15 minutes, the others recover rapidly and completely. Twice the LD_{50} of this toxin leads to immediate violent symptoms and early death in all the animals, the mean time-interval before death being about five minutes.

Discussion

Grasset [15] showed that specific antivenenes titrated against *N. flava* venom at multiple levels of serum dose yield linear titration curves. Although

this is undoubtedly the rule, the presence of three different toxins in the venom would make it surprising if all sera, without exception, should behave in this regular fashion. Fig. 1 (A) illustrates the titration curve obtained with S 7837 and *N. flava* venom, and may serve to show that irregularities do occur. This curve is obviously not linear but shows a clear kink and, in fact, is considered to be composed of two individual linear titration curves. The part indicated by full points in fig. 1 (A) is undoubtedly the titration curve obtained with the α toxin and its antibody, and the part indicated by circles the titration curve for γ toxin and its antibody, as the relative toxicities of the toxins and their optimal flocculation ratios suggest. Fig. 1 (B) is an attempt to reconstruct the actual finding shown in fig. 1 (A) from data obtained with S 7837 and the preparations of α, β, and γ toxins of experiment 4. The serum was titrated at multiple dose levels against each of the three toxin preparations. The points obtained with the α and β toxins form linear titration curves and those obtained with the γ toxin do not deviate seriously from linearity. The LD_{50} of venom (0.0074 mg) and the LD_{50} in ml of the three toxins are known. On the basis of the data in table VI, it has been assumed that, in order to produce a given effect by means of its γ toxin alone, venom would have to be given in approximately ten times the normal dose. Similarly the dose would have to be about 100/80 or about 100/20 times larger if its lethal effect depended on either α or β toxin respectively. Accepting this as an approximation, the volumes of the three toxins neutralized by different amounts of serum may be converted into mg of whole venom and recorded, as has been done in fig. 1 (B).

Irregularities of *N. flava* venom-antivenene titrations are uncommon because the titration end-point is usually determined by either the α or γ toxin, and the reaction between γ toxin and its antibody may not be noticed if for technical reasons the titration cannot be carried out at a sufficiently high level. This is generally the rule when unmodified sera are examined. The flocculation test indicated a relatively high concentration of anti-γ in S 7432, and the titration curve (fig. 2, curve A) may well be considered linear. The β toxin will at no level affect the end-point in titrations when the serum's anti-β concentration is high, as it is in the case of S 7432 and S 7837.

The various biologically active components of venom are not of equal toxicological importance but all, or almost all, are antigenic and may call for the establishment of units of activity and corresponding units of antibody activity, even if this at present is utopian. Most commercially produced antivenenes are polyvalent, i.e., they can neutralize the toxic components of the venoms secreted by several species of snakes. The therapeutic value of such sera should, ideally, be ascertained by comparison with a series of standard preparations representing all the venoms against which the serum is active—not only the specific venoms used as antigens in the production of the sera but also other venoms against which the sera are alleged to be

active. The difficulties are, however, manifold and most work has been concerned with the standardization of activity against the specific venoms only.

Finlayson & Grobler [12] established a provisional unit of anti-*N. flava* venom potency and suggested a method of titration which could be improved by employing mixtures containing higher concentrations of venom and serum, as in the method recommended by Grasset.[17] As it stands, the method could serve a useful purpose if, in addition, minimum requirements of potency were established. Logically, it has become necessary to consider standardization of protective potency against each of the three toxins which here have been called the α, β, and γ toxins of *N. flava* venom. This might well be possible but would require the preparation of reasonably pure toxins in fair quantities. The standardization of a serum's protective potency against the lethal effect of *B. arietans* venom is less likely to be solved in the near future, because investigations (unpublished) carried out in this laboratory have shown that most sera titrated against this venom at multiple dose levels give non-linear titration curves. This apparent failure to conform with the " law of combination in multiple proportions " is a feature which many venom-antivenene systems have in common. The types of titration curve published by Schöttler,[25] who worked with venom from snakes belonging to the genera *Crotalus* and *Bothrops*, are analogous to those obtained with African viperine venoms such as those of the two important species *B. arietans* and *B. gabonica* tested against specific antivenene. Such curves do not owe their particular shape to non-avidity of the sera (the curvature is convex towards the dose axis), but kinks and curvature of the titration line are natural consequences of simultaneous titration of a number of individual antigen-antibody systems, different toxins being responsible for the end-point at different levels of titration. This is the obvious explanation of the irregularities so commonly observed when venom-serum systems are titrated in animals.

Another question in need of reconsideration is the cross-protective (paraspecific) action of antivenenes. Anti-*N. flava* serum is known to protect experimental animals against the effect of *N. naja* venom, and anti-*N. naja* serum confers protection against *N. flava* venom.[1, 18] Were this cross-protection absolute, one could conclude that both these venoms possess the α, β, and γ toxins, although not necessarily in the same proportions. The evidence is, however, too scanty to permit this conclusion. The observations made by Calmette & Massol [6] point to one major fraction in the cobra venom they examined (probably that of *N. naja*) because practically all the toxin could be accounted for in the only floccules observed which were formed at a ratio corresponding to the in vivo neutral point. It can safely be assumed that this toxin has an antigenic counterpart in *N. flava* venom. Curve B in fig. 2 shows that S 7432 has some, not very marked, neutralizing effect on *N. naja* venom. This venom and S 7432

flocculate at five different optimal ratios, within the limited range of ratios examined, but no toxin could be demonstrated in floccules at four of these ratios, and the concentration of toxin was too low to permit examination of the floccules formed at the fifth, and lowest, venom : serum ratio. Ringhals (*S. haemachates*) venom is of particular interest in South Africa because of the ubiquity of this snake. This venom is neutralizable by anti-*N. flava* serum, just as anti-*S. haemachates* serum confers protection against both the *Naja* venoms mentioned here; [10, 16] this cross-neutralization, however, is of limited nature. Curve C in fig. 2 illustrates the anti-*Sepedon* potency of S 7432; the end-point reading was not sharp but the curve is obviously not linear and suggests a lethal factor in ringhals venom against which S 7432 offers little protection. Several ratios of optimal flocculation were demonstrable with ringhals venom and S 7432, but again no toxin could be detected in the floccules. Fig. 3 has been included to demonstrate that anti-*S. haemachates* serum, which offers good protection against the specific venom (curve C), is of limited value against both *N. flava* venom (curve A) and *N. naja* venom (curve B). Tested against the preparations of the three *N. flava* toxins, 0.3 ml R 809 was found to protect a mouse against 4, but not against 6, LD_{50} of γ toxin, and against 10, but not against 20, LD_{50} of either α or β toxin. This indicates similar toxins, or at least similar antigens, in the *Sepedon* venom. Bulk preparation of monovalent sera are needed for further study, but most African elapine venoms show some antigenic similarity, and it is hoped that work in progress will clarify the relationship among their heat-stable toxins and lead to the preparation of these toxins in a pure state, using floccules as starting material.

RÉSUMÉ

Le mélange de sérums antivenimeux purifiés (anti-*Bitis arietans-Naja flava*) et l'un ou l'autre des venins spécifiques qui ont servi à leur préparation, présente un optimum de floculation à divers niveaux, correspondant à différents rapports venin:sérum. Ce fait est la conséquence naturelle de réactions individuelles antigène/anticorps au sein d'un système hétérogène.

Il est possible de récupérer de la toxine dans le floculat, à trois niveaux optimums de mélange venin-sérum *N. flava*, par chauffage à 70°C pendant 10 minutes, à pH 2. Ces trois toxines thermostables, qui sont immunologiquement distinctes, ont été désignées comme toxines α, β, et γ du venin de *N. flava*.

La réaction de floculation obtenue avec le venin de vipère ne peut être considérée comme une indication du pouvoir protecteur du sérum.

La plupart des systèmes venin-sérum antivenimeux n'obéissent pas à la loi des proportions multiples, c'est-à-dire que la courbe de titrage n'est pas une droite. La forme de ces courbes, convexes du côté de l'axe des doses, s'explique facilement par la titration simultanée de plusieurs systèmes individuels toxine-sérum antitoxique, différentes toxines déterminant le point final à divers niveaux de titrage.

Dans le cas du venin-sérum antivenimeux de *N. flava*, la courbe de titrage obtenue avec le venin complet est déterminée par la teneur relative du sérum en composants anti-α, anti-β et anti-γ. Des études ultérieures permettront sans doute d'aborder la question de la standardisation de l'activité anti-α, anti-β, et anti-γ des sérums, mais les tentatives en vue de standardiser l'activité antivenimeuse des sérums par rapport à un venin quelconque seront vaines, tant que l'on ne connaîtra pas la « structure » toxique du venin.

La protection croisée que confèrent les sérums antivenimeux préparés soit contre *N. flava*, ou *Sepedon haemachates* est limitée; mais le fait qu'elle existe indique une similarité antigénique que les travaux en cours permettront peut-être de préciser.

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