of both aggregated and hyperthyroid mice. These include depletion of tissue norepinephrine stores, depletion of liver glycogen and hypoglycemia(5-8). As a result of the present study a further comparison can be made with regard to tissue electrolyte changes. In both aggregated and hyperthyroid mice amphetamine caused an increase in the sodium and a decrease in the potassium concentration in kidney and liver; there were no changes in the water or electrolyte content of skeletal muscle or brain. The electrolyte concentration in the plasma changed in a direction opposite to that in kidney and liver, that is, an increase in the potassium and a decrease in the sodium concentration.

The significance of the role that the amphetamine-induced electrolyte changes might play in the death of the aggregated or hyperthyroid mice is not clear. Since previous studies have described similar electrolyte shifts occurring after various types of trauma (14), it would appear that the changes reported in the present paper are part of general chemical changes that accompany any severe stress.

Summary. The enhanced toxicity of d-amphetamine in aggregated and in hyperthyroid mice is accompanied by similar changes in tissue electrolyte concentrations. These changes include an increase in the sodium and a decrease in the potassium contents of liver and kidney and a decrease in the sodium and an increase in the potassium concentrations in the plasma.

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Precipitin and Neutralizing Antibody Response Elicited by *Crotalus atrox* Venom-Antivenom Precipitate. (31117)

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Diphtheria toxin neutralized with antitoxin was used as an immunizing agent in 1898(1). Other reports followed which indicated that such antigen-antibody complexes were unique in that antibody formation to the toxin was enhanced when compared to using the toxin alone(2,3,4). Similar results were reported for tetanus and serum albumin complexed with specific antiserum(4,5,6,7,8).

This ability of insoluble antigen-antibody complexes, in enhancing antibody formation, could have a 2-fold advantage with extremely toxic antigens. First from the added protection gained by increased antibody, and secondly, because of slower release, it is likely that more antigen could be injected, bound to antibody, than could be given alone without deleterious effects. For example, the greatest difficulty experienced with producing rapid and effective immunity to snake venoms is inherent in the extreme toxicity of the venom and its high resistance to chemical and physical detoxifying agents. These factors dictate the use of venoms in extremely low doses over

a prolonged period to allow for the introduction of sufficient antigen to establish effective immunity.

One of the requirements for formation of an insoluble antigen-antibody complex is that the system be precipitable. Bier(9) showed that the quantitative course of the precipitin reaction is very similar for *Crotalus terrificus* venom-antivenom and diphtheria toxin-antitoxin systems. However, subsequent to an earlier report that rabbits immunized with cobra venom produce precipitating antibodies (10), it was reported that no constant relationship existed between antitoxic value and precipitating power of rabbit anti-cobra venom(11).

In the present investigation rabbits were inoculated with *Crotalus atrox* venom-antivenom precipitates, and the sera were analyzed for precipitating and neutralizing antibodies. Evidence is presented which shows that neutralizing, as well as precipitating, antibody was stimulated by the precipitate complex, suggesting that venom neutralizing antibody is a precipitin, and that the apparent non-relationship between precipitin content and antitoxic value is due to the antigen-antibody multiplicity of venom-antivenom systems. The data support the use of antigen-antibody complexes as a method for isolating specific antigens from a mixture for immunizing purposes.

Materials and methods. Antigen preparations and immunization procedure. C. atrox venom was extracted from a large number of snakes, maintained at the US Army Medical Research Laboratory, by allowing them to inject the venom directly through a latex diaphragm into an ice cooled container which was then frozen at —15°C. The pooled venom was then lyophilized and maintained in the dried state in a desiccator at —15°C. For rabbit inoculations the dried venom was brought directly into solution with 4 per cent sodium alginate adjuvant (Colab Laboratories, Chicago, Ill.). Unused aliquots were stored at —15°C and thawed, when needed, for additional inoculations. Venom antigen for precipitin and neutralizing antibody titrations was made up fresh, from the dried state, with saline.

Initial venom-antivenom complex was prepared by precipitating 1 ml commercial equine origin antivenom (Crotalidae polyvalent, Wyeth Laboratories, Inc., Marietta, Pa.) with a .3 mg *C. atrox* whole venom N (venom nitrogen) after preliminary titrations indicated this ratio gave maximum precipitation. The precipitate was washed three times with saline and then resuspended in 4% sodium alginate adjuvant.

Five New Zealand white rabbits, obtained locally, were each injected subcutaneously with 3 doses of 10 mg TP (total protein) initial venom-antivenom complex given one day apart (total 30 mg). The animals were rested for 7 days and the same series of injections was repeated. After a second rest period of one week, each rabbit was inoculated with a third series consisting of a total of 9 mg (dry wt) whole *C. atrox* venom given in 3 equal doses one day apart.

Blood was collected from each rabbit by cardiac puncture 8 days after the last inoculation, was allowed to clot at room temperature, and the serum was then separated by centrifugation. A preliminary quantitative precipitin titration determined that a ratio of 1 ml of this antiserum to 50 μg whole *C. atrox* venom N (slight antigen excess) produced maximum precipitation. Rabbit antivenom-venom complexes were then precipitated using this ratio, washed 3 times with saline and finally resuspended in Na alginate for the following experimental animal inoculations. Aliquots not used immediately were stored in the same manner as whole venom in adjuvant.

Immunization: primary response. Each animal in a group of 10 rabbits (Group I) was inoculated subcutaneously with a total of 0.48 mg whole *C. atrox* venom N. Three equal doses were given one day apart. At the same time, each of 10 rabbits (Group II) was inoculated with a total of 2.28 mg complex N also given in 3 equal doses. Based on the quantitative precipitin titration, the calculated maximum value for bound venom in the inoculated complex was equivalent to 0.36 mg whole venom N. Rabbits were bled from the marginal ear vein at periodic intervals after the last inoculation and the pooled serum, from two samples of five rabbits each,

was titrated as a single specimen for precipitating and neutralizing antibody. These results were averaged in the final data processing.

Immunization: secondary response. Group I and Group II rabbits were started on a second series of inoculations 34 days after the last dose of the primary series *via* the same route and schedule as the first series. Group I rabbits were given a total of 0.96 mg whole *C. atrox* venom N while each of the rabbits in Group II was given a total of 5.0 mg complex N equivalent to a theoretical maximum of 0.78 mg of bound whole *C. atrox* venom N. The animals were periodically bled from the marginal ear vein after the last inoculation and the serum was again analyzed for precipitating and neutralizing antibody. Three rabbits (Group III) were maintained and bled along with the experimental group throughout the first and second series as uninoculated controls. The pooled serum from these rabbits was also treated as a single sample.

Quantitative precipitin titrations. Complete details of the quantitative precipitin technique may be found in Kabat and Mayer (12). Antigen-antibody precipitates and antigen solutions were analyzed for protein N by the Lowry modification of the Folin-Ciocalteau procedure(13).

Neutralizing antibody determinations. Whole *C. atrox* venom in physiological saline was made up in a stock concentration of 2 mg per ml (dry wt), and .2, .4, .6 and .8 ml were added to 1.0 ml of pooled test serum from Groups I, II and III, and the volume of each dilution was brought to 4.0 ml with physiological saline. After 1 hour incubation at 37°C, 0.5 ml of each dilution was injected intraperitoneally into Swiss albino mice weighing between 18 and 20 g. In these titrations the pooled sera from all animals in their respective groups were treated as a single specimen. Five test animals were used for each venom-serum dilution containing 50, 100, 150 and 200 μg of venom. The number of live and dead mice were recorded during a 24-hour period and the LD_{50} was then calculated according to the method of Reed and Meunch(14).

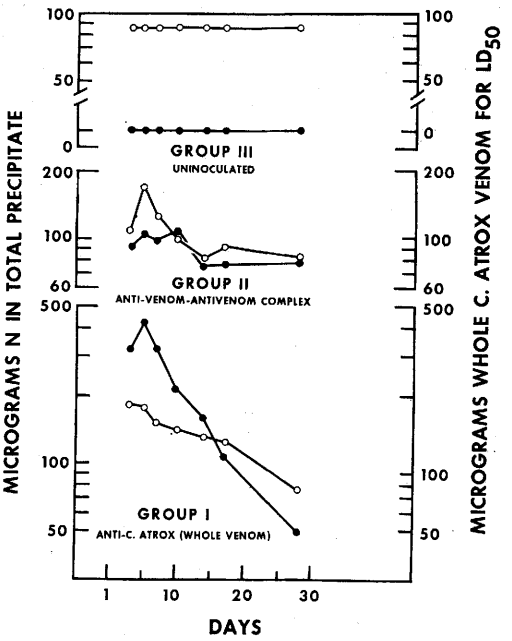

FIG. 1. Average precipitin response (● solid circles) and neutralizing antibody (○ open circles) in 10 rabbits inoculated with whole *C. atrox* venom (Group I), 10 rabbits inoculated with rabbit venom-antivenom complex (Group II), and 3 uninoculated controls (Group III).

Results. Primary response. Neutralizing antibody was measurable only on the second day following the last inoculation. Preliminary determinations showed that the quantity of whole venom in saline required for a one LD_{50} dose in mice was 75 μg (dry wt). In the presence of .125 ml of normal rabbit serum (Group III) 86 μg of the same toxin were required to produce a one LD_{50} dose in mice, suggesting slight neutralization of venom by normal rabbit serum. In the presence of .125 ml of sera from Groups I and II, 116 and 150 μg of toxin, respectively, were necessary for a one LD_{50} dose in mice. Thus, by subtraction, it is apparent that antiserum from Group I neutralized 41 μg of toxin and that from Group II neutralized 75 μg.

The same sera when tested by quantitative precipitation were found to be non-reactive with whole venom.

Secondary response. The data in Fig. 1 show that rabbits inoculated with whole venom (Group I) reached peak precipitin titer 5 days after the last dose of the second series

inoculations. Thereafter, there was a precipitous fall in antibody. The neutralizing antibody reached peak titer at 3 days and did not decline as rapidly. At this time 180 μg of whole venom in the presence of .125 ml of Group I serum were required to produce a one LD_{50} dose in mice as compared to 86 μg in the presence of .125 ml of serum from uninoculated controls (Group III). Rabbits inoculated with venom-antivenom complex (Group II) produced considerably less precipitins, but neutralizing antibody reached peak titer at 5 days. At this time 175 μg of whole venom in the presence of .125 ml of Group II serum were required for a one LD_{50} dose in mice.

Even though Group II serum was only slightly less than Group I serum in neutralizing antibody, the more rapid appearance and longer persistence of antibody in the latter group was typical for a secondary immune response, whereas the data for Group I was more in line with that expected from a primary response. This result was indicative of less antigenic stimulation in the Group II rabbits.

Discussion. A 2-fold increase in serum neutralizing antibody was elicited by injecting rabbits with whole *C. atrox* venom, and a slightly lesser increase was obtained with venom-antivenom precipitates. It is obvious that this yield would have been considerably amplified, possibly as much as 10-fold, had the venom been titrated against the concentrated purified globulins as is often done with antivenoms. Inoculations with whole venom resulted in considerably more precipitins than were elicited by the complex. From calculations based on the amount of crude venom added to rabbit antivenom and the total N precipitated a maximum of 1.14 mg bound venom N was given each rabbit of Group II in 6 doses, while 1.44 mg whole venom N was injected into each of the animals in Group I. However, at slight antigen excess not all the whole venom antigens added to the antivenom appeared in the final precipitate complex. Thus, the actual amount of bound venom injected into each rabbit of Group II was much lower than the calculated value of 1.14 mg and qualitatively different from whole venom. Glenn *et al*(15) reported that at least 16 precipitin bands occurred when whole venom-polyvalent (mixed species) horse anti-*crotalidae* systems were analyzed by double diffusion. With monovalent (single species) antivenoms Minton(16) showed that rattlesnake venoms are composed of at least 4-7 distinct antigenic fractions. Thus, it is evident that venom-antivenom titrations constitute multiple systems in which, at all dilutions, some systems are either at antigen or antibody excess. The result is that at equivalence, or at slight antigen excess, only a portion of the antigen mixture is precipitated with antivenom.

Quantitative immunochemistry is extremely difficult, to say the least, with a multiple antigen-antibody system. Also, in this study only the overall "equivalence point" was used as the immunogen. In spite of these limitations the data given above and the results in Fig. 1 show that the insoluble venom-antivenom precipitate was rich in lethal toxin and contained fewer antigens, than whole venom. Since antibody response is, in part, dose dependent it is likely that the higher precipitin titer of Group I animals resulted from the higher protein concentration, and the greater number of different antigens in whole venom as compared to the complexed venom. The nearly equivalent response of Group I and Group II rabbits in neutralizing antibodies may be explained by the greater specificity for lethal toxin of the injected complex. Thus, the implication that venom-antivenom precipitate is not equal to the immunizing potency of whole *C. atrox* venom is misleading. The data, in fact, suggest that venom bound to antibody, at equivalence, may even surpass an equivalent weight of whole venom as an immunogen for stimulating neutralizing antibody. There is evidence that venom neutralizing antibodies are precipitins. Furthermore, the neutralizing antibody of Group II more closely follows the precipitin pattern in that both reached peak titer at the same time. The observed increase in precipitins on the tenth day is not consistent with the progressive decline that usually occurs with pure antigen-antibody systems. This may occur because of the peculiarities inherent in using com-

plexed antigen, *e.g.*, the slow release of bound antigen by dissociation from the complex.

Summary. Rabbits immunized with insoluble *C. atrox* venom-antivenom precipitate produced less precipitins and nearly as much neutralizing antibody as those injected with whole venom. The data show that venom neutralizing antibodies are precipitins and that the apparent non-relationship between precipitin titer and antitoxic value is the result of the polyvalency of venom-antivenom systems. The value of using insoluble antigen-antibody precipitates to isolate specific antigens, from a mixture, for immunization is pointed out.

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Effect of Hydrocortisone Feeding on Concentration of Free Fatty Acids and Other Lipids of Rabbit Sera.* (31118)

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The chief purpose of this investigation was to study changes in the free fatty acids (FFA) concentration of rabbit serum as the serum lipids were modified by administration of hydrocortisone. We reported previously that addition of 20 mg of hydrocortisone/kg of stock rabbit diet markedly increased the serum triglyceride concentration(1). When the diet also contained cholesterol, the serum cholesterol as well as the triglyceride concentration rose to very high levels. Since the blood became very lipemic when cholesterol was added to the diet, cholesterol was omitted in this study in order to be able to work with less severe lipid changes. Two diets were employed. One was Purina rabbit chow to which hydrocortisone was added at a level of 30 mg/kg. The other was similar except that coconut oil was added at a 5% level. The coconut oil replaced an equal weight of Purina chow. Blood samples were collected in the morning. Food was allowed at all times.

Method. Free fatty acids were determined using the modification by Trout *et al*(2) of Dole's procedure(3). Phospholipid phosphorus was determined using the method of Outhouse and Forbes(4), following extraction with alcohol-ether. All basic references to the procedure for cholesterol and triglycerides were given previously(5).

Results. The experimental results are shown in Table I. The average and range are given in each case. It will be seen that a markedly elevated FFA was always associated with a high level of the other lipids. The presence of coconut oil accelerated the changes although its presence in the diet was not necessary to obtain elevated serum lipid concentrations.

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