SNAKE VENOM IN RELATION TO HÆMOLYSIS, BACTERIOLYSIS, AND TOXICITY.

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CONTENTS.

INTRODUCTION. GENERAL CONSIDERATIONS CONCERNING HÆMOLYSIS AND BACTERIOLYSIS.

[I have long desired that the action of venoms upon blood should be further examined. I finally indicated in a series of propositions the direction I wished the inquiry to take. Starting from these the following very satisfactory study has been made by Professor Flexner and Dr. Noguchi. My own share in it, although so limited, I mention with satisfaction.—S. WEIR MITCHELL.]

The following research, which is presented at this time in abstract, was conducted under a grant from the Bache fund of the National Academy of Sciences. It forms the first instalment of a new study

of venoms upon which we have been engaged during a year past and which, it is hoped, will be continued during another year or longer.

While these studies are still incomplete, the data here given have been worked out in detail and may therefore be accepted as final. On account of the large number of tables which will be given in the final publication, and the many drawings necessary to illustrate properly the text, more or less delay in bringing out the full work will be inevitable. But inasmuch as the results of the studies form an integral part of the work on hæmolysis and bacteriolysis, which is now attracting so much attention among bacteriologists and pathologists, and as they contain certain facts of fundamental importance bearing on the theory of these phenomena, it seems best not to delay publication until the entire series of researches shall have been completed.

At present we shall not give the full bibliography. Since the fundamental studies of Weir Mitchell and his collaborators,[1] the effects of venom upon the blood and the nervous system of animals have been generally recognized. The rapid putrefaction which sets in after poisoning with venom was also explained by Welch and Ewing's[2] observations on the loss of bactericidal power of the serum of such poisoned animals. The close relationship of the poison with certain toxins of bacteria and of higher plants was shown by the discovery of Sewall,[3] of Calmette[4] and of Fraser[5] that animals could be immunized from the effects of venom and that they yielded an active antitoxin. That the poison of venom is not simple, but that it consists of a complex of constituents of a proteid nature was proven by Mitchell and Reichert. The time, therefore, seemed ripe for a further study of the physiological effects of venom upon the blood, upon bacterial life, and upon tissues, in the light of the recent studies upon various kinds of immunity.

For the purpose of these studies dried venom has been employed.

[1] *Smithsonian Contrib. to Knowledge*, 1860, xii, and 1886, No. 647.
[2] *Lancet*, 1894, i, p. 1236.
[3] *Journal of Physiology*, 1887, viii, p. 203.
[4] *Ann. de l'Institut Pasteur*, 1894, viii, p. 275.
[5] *British Med. Journ.*, 1895, i, p. 1309.

Fortunately several kinds were available—through the kindness of Prof. Reichert, that of the rattlesnake (*Crotalus adamanteus*); of Dr. Joseph McFarland, that of the water mocassin (*Ancistrodon piscivorus*) and of the cobra (*Naja tripudians*), and of Messrs. Mulford & Co., that of the copperhead (*Ancistrodon contortrix*). We wish to express to these gentlemen our great appreciation of their kindness.

Before presenting the matter of our studies, it seems best to preface what we have to say with a brief statement of some of the facts and views relating to the phenomena of hæmolysis and bacteriolysis. In this preface we shall not distribute credits for the work or the views here embodied, inasmuch as this will be done in the complete publication. Only so much will be said as is necessary for an understanding of the experimental data relating to venom which are to follow.

By hæmolysis is meant solution of the blood-corpuscles. The term is usually applied to solution of the red corpuscles, but the white cells are also subject to a similar solution. It is, therefore, correct to speak of hæmolysis when both kinds of cells, erythrolysis when the red cells only, and leucolysis when the white cells alone are dissolved. In this solution of the red cells, which is the type of hæmolysis, the hæmoglobin is separated from the stroma of the corpuscles. The separation of hæmoglobin by hypotonic solutions and through the action of destructive chemical substances is not considered in this article. Hæmolysis as here employed refers to such separation through the action of complex agents derived from living plants or animals. This form is distinguished as biologic hæmolysis. Of all such agents the most active are found in the blood plasma or serum of alien animal species. Others are the products of cellular activity, such as venom, certain toxic products of bacterial growth, as tetanolysin, staphylotoxin, etc., and still others are yielded by some of the higher plants, as crotin from *Croton tiglium*.

The most familiar examples of hæmolysis are supplied by the effects of the transfusion of animal blood into man. It was early discovered that the practice was dangerous for the reason that the red corpuscles of the host were dissolved by the foreign blood. This effect was quickly seen to be due to the serum of the alien blood and it was observed to take place with equal readiness *in vitro*. The blood of animals

also is hæmolysed by foreign sera—the red corpuscles of the rabbit, for example, being dissolved readily by dog's serum. Some sera have very high dissolving power, the most active thus far known being that obtained from the eel, which is correspondingly toxic. After admixture of the corpuscles and foreign serum, solution does not occur immediately. The corpuscles first run together, become clumped, or, as we now denominate the effect, agglutinated. The dissolving effect of venom upon corpuscles is also preceded by a similar agglutination, as was first shown by Mitchell and Stewart.[6]

There is much similarity in the phenomena of agglutination and of lysis as observed in blood-corpuscles with appearances seen in connection with bacteria. The Gruber-Widal reaction of agglutination, which has served so well in the diagnosis of typhoid fever and other bacterial infectious diseases, is of a similar nature. Moreover, under certain conditions solution of the agglutinated bacteria may also occur, when bacteriolysis more or less analogous to hæmolysis results. The well-known Pfeiffer phenomenon, in which cholera spirilla undergo disintegration and solution in the peritoneal cavity of the immunized guinea-pig is the classical example of bacteriolysis.

The studies of the past two or three years upon the allied phenomena of bacteriolysis and of hæmolysis have not only demonstrated their fundamental similarity, but provided chemical explanations of the processes involved. Pfeiffer observed that the serum of immune animals caused agglutination only of the bacterial species used for immunization; he believed that for complete solution of the bacteria the mixture must be brought into the living body, and for this purpose he chose the peritoneum of the guinea-pig. Somewhat later Metchnikoff and Bordet discovered that the same effect could be produced *in vitro* by the addition to the immune serum of a small quantity of peritoneal exudate, or even of the fresh serum of an animal. From this experiment it could be inferred that for agglutination of bacteria certain bodies were required which were in the immune serum; but for solution still other substances contained in part in fresh serum were requisite. That this second substance might also be present in the original immune serum could now be shown; and it was also demonstrated that it is of a very labile nature and quickly disappears spontaneously. The addition of fresh serum or exudate restores it. It may be destroyed by raising the temperature of the fluid to 56° C.

A great advance in our knowledge of cytolysis was made when it was

[6] *Trans. College of Physicians of Philadelphia*, 1897, 3. s., xix, p. 105.

discovered that immunization to blood and body cells gives rise to the production of lysins. Just as in bacterial immunization lysins for the special bacteria employed are yielded, so also are evolved analogous substances for red and white blood cells, for epithelial cells, spermatozoa, etc. Indeed the number and variety of lysins that can be produced experimentally are limited only by the number and variety of animal cells available. Blood cells of one animal may be used to produce a lysin in the body of another animal of the same species—isolysin; in another species—heterolysin; and success has in rare instances followed the re-injection of withdrawn blood through which autolysins have been produced. Nor is the production of lysins the only result of the injection of cellular structures. Preceding the solution, clumping of the cells takes place from which it may be concluded that agglutinins are also formed.

The factors required for producing solution of cells are similar to those for causing solution of bacteria under like conditions. Only when the lytic serum is very fresh will solution be effected; the addition, however, of peritoneal exudate or fresh normal serum to immune serum which has lost the solvent property, suffices to restore it.

A consideration of the preceding facts shows that the agglutinating principle is distinct from the dissolving one. This consideration also indicates that more than one body is necessary to bring about solution either of bacteria or of animal cells.

An analysis of the phenomena suggests that at least two substances are requisite. One is stable and contained in the immune sera (whether for bacteria or animal cells); the other is labile, and while originally contained in the immune sera, it is lost spontaneously. This latter substance is a normal constituent of the lymph and blood plasma, for it can be restored by the addition of these fluids.

Experiments conducted in a very convincing way by Ehrlich and Morgenroth indicate: (1) that a special principle is concerned in agglutination—the so-called agglutinin; and (2) that two principles are concerned in lysis. These principles are different in origin. One—that which is stable—is the product of immunization, and, on account of certain combining properties possessed by it, they call it the 'intermediary body.'[1] The other is normally present in the body juices but

[1] Ehrlich has recently suggested the name 'ceptor,' in place of 'intermediary body.' According to the manner of action he distinguishes 'uniceptors' and 'amboceptors.' Bordet calls this body 'substance sensibilisatrice;' Metchnikoff 'fixator;' P. Mueller, 'copula.'

is easily destroyed by heat and tends to disappear spontaneously when the fluids are removed from the body. This latter principle, on account of the complemental nature of its action, they propose to call the 'complement.'[8]

There is conclusive experimental evidence that, although the intermediary body unites first with the cells—bacterial, blood cells, etc.—this substance by itself cannot bring about solution. But after the union of this intermediary body with the cells the complement is capable of being brought into action, through this intermediation, so that solution takes place. The union of intermediate body and cells is conceived to take place through certain combining (haptophore) groups present in the cells and in the intermediary substance; while the complement is linked through similar combining (haptophore) groups possessed by the intermediary body and itself. The intermediary body, therefore, carries two sets of combining or haptophore groups: one for the cells and the other for the complement (complementophilic group). The complement possesses in addition to such a corresponding haptophore group, another group which exhibits fermentative properties (zymotoxic or toxophore group), through the action of which solution of cells takes place.

This conception of lysis applies not solely to that produced by immunization, but the same factors are believed to be operative in the solution of blood cells or of bacteria by normal blood sera. Here also an intermediary body and a complement are brought into action; the only difference being that, in the one case, the intermediary body is produced through artificial immunization, and in the other, it is present normally, but whether because of some insidious and unperceived change, similar to but slighter than artificial immunization, is not known.

It would carry us too far afield to give in detail the elaborate views of Ehrlich and his co-workers as to the origin of the intermediary bodies. Suffice it to say that they are the products of immunization by bacterial or other cells, and are believed by them to be yielded by certain constituents of cellular protoplasm within the body, designated as 'lateral chains,' which through their haptophore groups are capable of combining with the haptophore groups of protoplasmic constituents of the bacteria or the cells used for the immunization. When dealing with the toxic constitution of venom more will be said concerning this aspect of the subject.

[8] This body is called 'alexin' by Bordet, and probably agrees in part with the body of the same name described by Buchner Metchnikoff calls it 'cytase.'

VENOM-AGGLUTINATION.

For the study of agglutination all the available varieties of venom were employed. Several kinds of animal blood—from the dog, rabbit, guinea-pig, sheep, ox, pig, *Necturus*, and frog—were tested. Either the blood was defibrinated or the specimen consisted of the corpuscles separated by centrifugalization and washed six times, as a rule, in 0.8% normal saline solution (washed corpuscles). The different venoms showed slight differences only in the degree of agglutination, with the exceptions of the action on the blood of *Necturus* and the frog, which are not affected in weak solutions but show agglutination in stronger solutions (2%).

The usual method consisted in dissolving in normal saline solution dried venom in strengths ranging from 0.01% to 10%—the last that of *Crotalus adamanteus*. The phenomena of agglutination appear rapidly in favorable solutions, while in very weak solutions a delay of some minutes up to one hour may be noted. The corpuscles which come together thus slowly do not show the great modification of shape that is characteristic of those that fuse more completely and quickly. In a general way it may be said that the several varieties of dried venom with which we experimented gave, when employed in the strength of 0.5%, what are to be regarded as maximal agglutinations for mammalian corpuscles. Active agglutination still takes place in 0.2% solutions, while weaker ones either produce no change at all or show an imperfect fusion.

The morphological changes need not be described here as these have been fully dealt with by Mitchell and Stewart.[9]

The value of the use of washed corpuscles comes especially from the fact that the succession of lytic phenomena is eliminated. Agglutination, therefore, may be studied purely. For this purpose a 5% solution of the corpuscles in normal salt solution was employed. That complete agglutination has no effect upon subsequent solution (lysis) of the corpuscles will be shown when treating of the latter phenomena. On the other hand, distinct differences in susceptibility

[9] Loc. cit.

to agglutination have been observed. Of the mammalian blood thus far employed the red corpuscles of the rabbit may be said to be highly susceptible, while those of the guinea-pig, dog, sheep, swine, and ox were less responsive in about the order given.

The use of defibrinated blood permitted observation upon the succession of phenomena of agglutination and hæmolysis. In general it may be said that the first effect of the venom is the production of agglutination to be followed by solution after a variable interval, depending on the kind and strength of the venom and on the temperature. There are, however, notable exceptions in that the range of lytic activity of venoms is greater than that of the agglutinating property. Very weak solutions of venom which no longer cause agglutination may still be capable of producing solution.

Moreover, on account of the action of two sets of factors in defibrinated blood—one tending to produce agglutination, and the other solution of the red corpuscles—the degree of agglutination is here less marked than in the washed cells where no lysis occurs. This difference is explained by the fact that a part of the corpuscles go into solution before agglutination can take place; and hence the extent of precipitation and fusion varies inversely with the susceptibility to lysins. As a consequence, dog's corpuscles which are more easily hæmolysed by venom than any others of the animal bloods tested by us show the least degree of agglutination. The rapidity of agglutination in any case is not affected by ordinary temperatures. Hence a low temperature (0° C.) permits, even in defibrinated blood, the separation of the phenomena of lysis and those of agglutination. At this temperature defibrinated dog's blood behaves as do the washed corpuscles, the amount of precipitation being, therefore, greater than at the temperature of the room or of the thermostat.

The agglutinating power of venoms is destroyed by temperatures of 75° to 80° C. maintained for thirty minutes.

VENOM-HÆMOLYSIS.

Unless mention is especially made the same group of animals was employed in these studies as in the foregoing. The venom solutions

varied from 5% to 0.0001%, depending upon the source of the corpuscles and the variety of venom. The venoms differ in hæmolytic power as follows: cobra most active; water mocassin, copperhead, rattlesnake, in less degree in the order named. A similar variation in susceptibility to the reaction could be distinguished in the different mammalian bloods employed. Thus dog's blood was most quickly and easily hæmolysed and it responded to the greatest dilutions, while the corpuscles of the ox were the least susceptible. The intermediate animals were in about the following order: sheep, guinea-pig, pig, and rabbit. On the other hand, the blood of *Necturus* is acted upon slightly after longer periods, and frog's blood almost not at all after equally long periods.

The extent of variation in the response of the different bloods is considerable. Very strong solutions of venom (5%) are needed to cause hæmolysis of corpuscles of the ox, but such great strengths are without action upon rabbit's corpuscles, although they are still capable of producing rapid solution of dog's or sheep's corpuscles. Taken altogether solutions averaging 0.2 per cent of venom have proven the most favorable for bringing out the hæmolytic effect upon blood generally.

Defibrinated Blood.—In making the tests with defibrinated blood uniform mixtures were employed. In all experiments 5 per cent of blood was added to the venom solutions, and the mixtures were kept at temperatures varying from 36° to 37° C.

The differences in activity of venoms are shown by the following series in which minimal active solutions are given for several kinds of blood.

Dog's blood hæmolysed by solutions of *Crotalus* venom 0.001%; copperhead 0.0005%; water-mocassin 0.0002%; cobra 0.0001%

Sheep's blood hæmolysed by solutions of *Crotalus* venom 0.002%; copperhead 0.0005%.

Guinea-pig's blood hæmolysed by solutions of *Crotalus* venom 0.002%; copperhead 0.001%.

Swine's blood hæmolysed by solutions of *Crotalus* venom 0.002%; copperhead 0.001%.

Rabbit's blood hæmolysed by solutions of *Crotalus* venom 0.005%; copperhead 0.002%.

Rabbit's blood hæmolysed by solutions of water-mocassin venom 0.002%; cobra 0.001%.

Ox's blood hæmolysed by solutions of *Crotalus* venom 0.05%; copperhead 0.02%.

Effect of Heat upon Hæmolytic Power of Venoms.—Temperatures of 75° to 80° C. for thirty minutes have no effect upon the hæmolytic action of any kind of venom. From 90° to 96° C. *Crotalus* venom in solution suffers a moderate reduction in hæmolytic power, while the remaining venoms are entirely unaffected at these temperatures. After heating to 100 C. for fifteen minutes the dissolving power of cobra, mocassin, and copperhead venom in solution is slightly reduced.

Effect of Venoms upon Washed Blood-Corpuscles.—In no instance were the washed blood-corpuscles hæmolysed by venom. Agglutination occurs as already described. But if the separated serum is restored to each of the several kinds of blood-corpuscles treated with venom, lysis takes place.

Certain important differences may be noted. Thus if the quantity of serum added to rabbit's corpuscles exceeds the normal, quicker and more complete solution occurs than is noted in defibrinated blood. A similar but less striking action may be observed with the blood of the guinea-pig. This action depends upon the union first effected between the red corpuscles and the intermediary body of venom, the latter later combining with the introduced complement of the serum so that solution takes place. That this is the mechanism of the action the succeeding experiments prove. But before taking them up another set of phenomena must be briefly considered.

Rabbit's and guinea-pig's washed blood-corpuscles (hereafter termed washed corpuscles) are quickly dissolved by fresh dog's serum. Dog's corpuscles are but little acted upon by fresh rabbit's serum and not at all by guinea-pig's serum. Rabbit's and guinea-pig's sera are about equally hæmolytic for each other's corpuscles. *Necturus'* serum is highly hæmolytic for rabbit's, dog's, and guinea-pig's corpuscles. Frog's serum is less destructive for the mammalian cor-

puscles mentioned than that of *Necturus*, but is still very active. *Necturus*' serum and frog's serum are slightly and equally active on each other's corpuscles.

This dissolving action is not noted at freezing temperatures. If, therefore, washed corpuscles which have been treated with serum for thirty minutes at zero temperature are separated by centrifugalization or precipitation, the complement in the serum is unaffected, while the intermediary body will be found to have been removed from the serum by the corpuscles. In this way the complement for a particular species of corpuscles, free from the intermediary body, can be obtained. The addition of such complement-containing serum to venomized washed corpuscles of the same species brings about hæmolysis, while the addition of fresh washed corpuscles to the treated serum, from which the intermediary body for them has been removed, is unattended by solution.

The action of complements, freed from any intermediary body by this means, upon venomized corpuscles of different species has also been studied. The results are of interest. The procedure is as follows: Let us suppose that it is desired to test the effects of rabbit's serum upon venomized dog's corpuscles. The rabbit's serum is first treated with washed dog's corpuscles in the cold to withdraw all combining intermediary bodies; the clear serum having been separated is now added to the venomized corpuscles, when solution of a slow and limited nature occurs, thus showing that there exists in the dog's corpuscles a limited number of receptors capable when venomized of uniting with rabbit's complement.

Controls for these experiments were made in the following manner: Any serum treated with alien corpuscles at zero temperature and then separated from the corpuscles by centrifugalization has become inactive for this kind of fresh washed corpuscles (tested for dog's, guinea-pig's, rabbit's, and *Necturus*' corpuscles). However, if to the inactive mixture the same variety of serum heated to 58° C. is added (this serum containing intermediary body but without complement) solution takes place.

The degree of interaction of different species of sera minus inter-

mediary bodies upon different species of venomized washed corpuscles is shown by the following series, in which copperhead venom is used throughout. 1 cc. of 0.2% solution of venom is mixed with 0.05 cc. of washed corpuscles and 0.5 cc. of complement, with these results:

Dog's corpuscles and rabbit's complement = slow and imperfect hæmolysis.

Dog's corpuscles and guinea-pig's complement = slight hæmolysis, more marked than preceding.

Rabbit's corpuscles and dog's complement = rapid and imperfect hæmolysis.

Rabbit's corpuscles and guinea-pig's complement = weak and imperfect hæmolysis.

Guinea-pig's corpuscles and dog's complement = rapid and imperfect hæmolysis.

Guinea-pig's corpuscles and rabbit's complement = slow and imperfect hæmolysis.

Dog's corpuscles and *Necturus'* complement = slight and imperfect hæmolysis.

Guinea-pig's corpuscles and *Necturus'* complement = no action.

Rabbit's corpuscles and *Necturus'* complement = no action.

Venom solution treated with dog's, rabbit's, and guinea-pig's washed corpuscles in succession gives up to each a part of its intermediary bodies. No one kind of corpuscle is capable of fixing the entire content of intermediary bodies. The supernatant fluid probably contains still other intermediary bodies capable of fixation by still other corpuscles. If to the several kinds of venomized corpuscles here mentioned different complements are added, then, as shown in the foregoing series, lysis will or will not take place, depending on the nature of the complement employed; but so long as the complement is foreign to the corpuscles it never causes complete solution.

From these results the following conclusions are warranted: (1) Venom contains several or many intermediary bodies. (2) These bodies show specific affinities for certain complements. In addition to this there is evidence that the many susceptible corpuscles contain, besides specific haptophore groups for intermediary bodies, certain common haptophore groups, which are shared, perhaps, by all vulnerable corpuscles.

Combined Action of Venom and Ricin. Relation of Agglutination and Hæmolysis.—Agglutination produced by venom does not affect lysis. But on the other hand when lysis takes place quickly, agglutination may fail or may appear imperfectly. The principles causing the two phenomena are distinct in the manner of combination and in action. That the agglutinative and the lytic principles are different is now proven; and there is evidence that they act upon different constituents of the red cells. Thus, if ricin, a strong agglutinator, is permitted to act upon red corpuscles for periods under thirty minutes, then upon the addition of venom lysis ensues in about the average time and proceeds normally. If, however, the ricin has acted for two or more hours, then solution by venom still takes place, but the stroma of the corpuscles remains in the bottom of the test-tube as a white conglutinated mass. From this it appears that agglutination brings about a kind of coagulation of the stroma, from which, through the action of the hæmolysin in venom, hæmoglobin has been released. Ricin is without action upon venom itself, and conversely ricin is equally unaffected by venom.

VENOM-LEUCOLYSIS.

In the blood snake-venom causes destruction of the leucocytes as well as of the red cells. In order to ensure a more accurate study of its action upon the white blood-cells, these were obtained in larger quantities and without admixture of red cells by injecting positively chemotactic substances into the pleural and peritoneal cavities of the rabbit. For this purpose cultures of *B. megatherium* killed by heat were injected into the pleural cavity and sterile bouillon into the peritoneum.

From eighteen to twenty-four hours after the introduction of *B. megatherium* into the pleural cavity, fluid rich in leucocytes may be withdrawn by means of capillary tubes without sacrificing the animal. The same procedure employed twenty-four hours after the injection of bouillon yielded a fluid less rich in leucocytes, and consequently this second method was not extensively employed.

The leucocytes in the fluids thus obtained could be separated ac-

cording to size and granulation into lymphocytes (20 to 25% of the total leucocytes), finely granular, medium-sized cells (60%), larger non-granular cells (3%), still larger irregular and coarsely granular cells (4%), and others again somewhat smaller but showing very coarse granules (6%). The susceptibility to the destructive effects of venom varied somewhat for the different cells. Those of the largest size with coarse granules are most quickly affected; next to these come the finely granular varieties, the lymphocytes showing the least injury of all.

In studying the changes taking place in the leucocytes under the influence of venom a warm stage (37° C.) was used, the edges of the cover glasses having first been sealed with vaseline. The venom solutions varied from 10% to 0.002%. The weakest effective solution was that of cobra venom (0.002%), whereas in the case of the rattlesnake and of the mocassin, 0.002% and 0.005% respectively, caused definite changes.

Only the granular cells showed motility. Weak active solutions are without immediate effect on motion but begin to manifest an inhibiting action after about one hour, the controls being still motile at the end of two hours or longer. After the motility ceases, the cells in general, except the lymphocytes, show increased granulation due to the appearance of coarser and more numerous granules in the protoplasm, the nuclei coincidently becoming more distinct. After six hours the majority of the largest granular cells have already disintegrated, the nuclei having been liberated. After twenty-four hours most of the medium-sized granular cells have suffered disintegration, while the lymphocytes show but slight and inconspicuous changes. Stronger solutions, varying from 0.2% to 10%, cause instant cessation of motility and rapid agglutination without distinction of variety of cells. Within five to thirty minutes thereafter dissolution sets in, affecting first the largest, then medium-sized cells, and finally the small lymphocytes.

There are variations in the activities of the several venoms and in the completeness of solution of the cells. Rattlesnake venom is far less active than that of the cobra. Thus in 2% solutions cobra venom

causes complete solution in thirty minutes while that of the rattlesnake requires two hours to bring about the same result.

The effects upon washed leucocytes differ from those described in that venom solutions cause agglutination, but with the production of only very slight lysis.

Are the Hæmolysins (Erythrolysins) Identical with Leucolysins?—Copperhead venom (1 mg. in 4 cc. of normal saline) was treated with washed rabbit's red corpuscles at the thermostat temperature for thirty minutes, until the supernatant fluid after centrifugalization was without action upon defibrinated rabbit's blood. This solution when brought into contact with leucocytic fluid was without agglutinating action upon the cells while still causing their solution in about thirty minutes. On the other hand, the parallel experiment in which venom solution was treated with washed leucocytes yielded a fluid still active for defibrinated blood.

The conclusions from these experiments are as follows:

(1) Venom contains principles which are agglutinating and dissolving for white blood-corpuscles.

(2) The agglutinating principles may be identical for both white and red cells.

(3) The dissolving principle for leucocytes is distinct from that for red cells.

(4) In order that solution of venomized leucocytes shall occur a complement-containing fluid is required.

(5) The several varieties of white cells of the rabbit's blood show different susceptibilities to the action of venom.

VENOM-TOXICITY.

For the study of the toxic principles copperhead venom was chiefly employed. The animal selected for these experiments was the guinea-pig. The method of procedure was the following: We first determined for the particular sample of venom to be used the minimal lethal dose. This was found to be 0.3 mg. for a guinea-pig weighing from 250 to 300 grammes, death resulting within 24 hours. A dose of 0.6 mg. caused death in from two to three hours, and of 0.9 mg. in from 30 to 45 minutes.

Our special purpose was the determination of the existence of neutralizing substances for venom in the tissues of the body. The following tissues and organs were employed: brain, liver, spleen, kidney, voluntary muscle, adrenal gland, and blood. For this purpose the tissues were first washed in tepid, sterile, normal saline and a weighed quantity (2 grammes) was taken. This was triturated in a sterile mortar and mixed in test tubes with three times the minimal lethal dose (M. L. D.) of venom.

The mixture was now placed in the thermostat where it remained for one hour. It was then centrifugalized and the supernatant fluid was injected into the guinea-pigs. In the course of the experiment the original volume of the venom-solution suffered a loss amounting on an average to one-third of the whole volume. There should, therefore, after this subtraction remain behind at least twice the minimal lethal dose ($2 \times$ M. L. D.).

In the case of the blood, washed corpuscles were employed in excess, the other steps remaining the same as in previous experiments.

The results of these experiments are as follows:

Control	Dead in	45 minutes.
Brain	"	19 hours.
Blood	"	3 hours and 50 minutes.
Adrenals	"	2 hours and 35 minutes.
Spleen	"	2 hours and 10 minutes.
Liver	"	1 hour and 30 minutes.
Kidney	"	1 hour and 55 minutes.
Muscles	"	1 hour and 30 minutes.

In the next experiment 2 M. L. D. of venom were employed. The control died in five hours. The animals receiving the venom solution treated with the organs, etc., reacted as follows:

Brain	Survived.	
Blood	Dead in	28 hours.
Liver	"	19 hours.

Relation of Neurotoxic to Hæmolytic Principle.—That these two principles are distinct is rendered probable by the effects of washed red-corpuscles and of brain tissue respectively upon the toxicity of

venom. Blood-corpuscles remove little or perhaps none of the toxic constituent that brain cells do away with *in toto*. The proof of difference can, however, be brought in another way: Four M. L. D. of venom were treated with an excess of red corpuscles and the supernatant fluid was injected into a guinea-pig; death ensued in 30 minutes. The same quantity of venom having been treated with four grammes of brain emulsion, the supernatant fluid injected into a guinea-pig caused death in 48 hours. The experiments in which 9 M. L. D. of venom were used resulted, (1) in the case of the blood in death in 25 minutes; and (2) in the case of the brain (7 grammes of brain having been employed) in 25 hours.

The supernatant fluid from the brain emulsion was strongly agglutinating and hæmolytic for defibrinated blood, while that from the washed corpuscles had lost all these properties. The supernatant fluid from the brain emulsion, when treated with an excess of washed corpuscles, re-centrifugalized and the fluid then injected, is non-toxic for guinea-pigs.

These experiments show: (1) that the neurotoxic and the hæmolytic principles are physiologically distinct; (2) that while the chief toxic constituent unites with the nerve cells, in multiple M. L. D. from which the neurotoxic principle has been removed a quantity of hæmolysin may be contained sufficient to bring about fatal intoxication.

These results are in keeping with the views expressed by Ehrlich and supported by Wassermann and Takaki's experiments on the fixative power of cells for certain groups of toxic substances. They tend, therefore, to support the hypothetical considerations of Ehrlich on which he bases his well-known lateral-chain theory of immunity. Expressed in the terms of this hypothesis brain cells may be said to contain the receptors for the neurotoxic constituent of venom, whereas blood cells furnish the receptors for the hæmolytic principle; these receptors are distinct and specific, and are not contained to any considerable amount, and perhaps not at all, in the liver and kidney cells and, if at all, in small quantity only in adrenal cells. Walter Myers found that the adrenal cortex possessed a very feeble combining power

for cobra venom, most marked in mammals (sheep) in which the cortex of the organ is well developed. He also observed little effect from the adrenal of the guinea-pig.

EFFECTS OF VENOM UPON BACTERICIDAL PROPERTIES OF BLOOD SERUM.[10]

The animals employed were the dog, rabbit, and *Necturus;* the venoms belonged to the cobra, moccasin, copperhead, and rattlesnake, and the bacteria were *B. typhi*, *B. coli*, and *B. anthracis.* The method consisted in (1) introducing venom into the animal and drawing the blood from the femoral artery into sterile Nuttall bulbs; (2) permitting the blood from the normal animals to enter Nuttall bulbs in which the venom solution was contained; (3) admixture of the venom in sterile solution (heated for 4 days to 56°-60° C.) with separated serum.

The bactericidal effects of the normal sera were first established. Rabbit's serum is highly destructive for *B. typhi* and *B. anthracis* and least for *B. coli.* Dog's serum is highly destructive for *B. typhi. Necturus'* serum is also very destructive to *B. typhi* and *B. coli.* It is without marked effect on *B. anthracis.*

Serum Venomized in vivo.—Cobra venom was most active. Blood from rabbits which had received 10 mg., taken 57 minutes after the injection, showed great loss of bactericidal properties.

Experiment LXXI.—1 cc. venomized serum employed.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	1841	4254	7152
After 1 hour	767	3125	4130
" 3 hours	2488	13460	4320
" 6 "	Innumerable	Innumerable	17280
" 24 "	"	"	Innumerable

The controls for this experiment showed complete destruction of all bacteria, or, as in a few experiments, of all except *B. coli,* which

[10] In order to determine whether any effect is produced on the growth of bacteria by the presence of venom in culture media, varying small quantities of venom were added to nutrient agar-agar. The bacteria—*B. anthracis, B. coli, and B. typhi*—grown upon these tubes underwent rapid involutions and exhibited marked plasmolysis, as compared with control tubes of the same organism.

showed considerable diminution until after six hours when increase began.

Experiment LXX.—30 mg. rattlesnake venom injected; blood taken after 45 minutes. 1 cc. venomized serum employed.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	838	9940	5750
1 hour	756	6240	3654
3 hours	2930	Increase	6219
6 "	Increase	Innumerable	About 10000
24 "	Innumerable	Innumerable	Innumerable

Blood mixed with Venom in vitro.—In this series rabbits only were employed. The venom solutions were placed in Nuttall's bulbs and the blood from the femoral artery was permitted to stream into them. In each experiment 6 mg. of venom were mixed with 20 to 30 cc. of blood. Coagulation was very slow or completely inhibited and the serum was obtained when necessary by centrifugalization. It invariably contained hæmoglobin.

Experiment LXXIII.—1 cc. of venomized serum employed.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	1644	4580	4085
1 hour	2080	10760	4870
3 hours	18930	149740	24730
6 "	Innumerable	Innumerable	Innumerable

Experiment LXXII.—1 cc. venomized serum employed.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	736	3720	1275
1 hour	407	2340	920
3 hours	860	22210	8720
6 "	5220	Innumerable	Innumerable
24 "	Innumerable		

This series of experiments may be open to the criticism that the increased nutritive value of the serum because of the hæmoglobin present may have been the cause of the effects noted; as a control,

therefore, peptone was added to the serum in the proportion of 6 mg. of peptone to 20 cc. of serum.

Experiment LXXX.—Peptone added to rabbit's blood: 1 cc. employed.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	1043	5120	7430
1 hour	193	2240	1534
3 hours	87	578	71
6 "	22	520	262
24 "	0	Innumerable	About 20000

From this experiment it follows that improvement in nutritive value reduces bactericidal effect but in far less amount than is noted in the parallel case of venom.

That the nutritive change is unimportant is shown by the first experiments, in which the poisoning was done *in vivo* and also by those to follow in which venom was added directly to the separated serum.

Experiment LXXXV.—Rabbit's serum 1 cc. with rattlesnake venom 1 mg.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	745	3990	5430
1 hour	594	4667	3136
3 hours	4486	12120	43430
6 "	About 100000	Innumerable	About 200000
24 "	Innumerable		Innumerable

Experiment LXXXII.—Dog's serum 1 cc. with copperhead venom.

	Venom 6 mg. *B. typhi*	Venom 1 mg. *B. typhi*	Control; norm. ser. *B. typhi*
Immediate	8860	3572	5808
1 hour	21120	6525	584
3 hours	65250	14950	184
6 "	Innumerable	Innumerable	92
24 "			0

In order to determine the least quantity of venom required to remove the bactericidal properties of the serum varying quantities of copperhead venom were employed. Dog's serum was chosen with *B. typhi.* In each case 1 cc. of serum was used.

Experiment LXXXVII (a).—1 cc. dog's serum and varying amounts of copperhead venom.

	Venom 1/2 mg.	1/5 mg.	1/10 mg.	1/20 mg.	1/50 mg.
Immediate	5970	3070	4290	4940	3350
1 hour	6240	3960	1830	2620	920
3 hours	12810	10000	6730	1350	593
6 "	Innumerable	100000	13140	172	15
24 "		Innumerable	Innumerable	About 10000	0

From this it may be concluded that the specimen of venom employed by us destroys the bactericidal properties of dog's serum when added in the proportion of 1/20 mg. of venom to 1 cc. of serum; and that 1/50 mg. in the same quantity of serum is practically without action.

In view of these positive results the partial inaction of venom upon *Necturus* serum is both remarkable and important.

Experiment CII.—1 cc. *Necturus* serum employed.

	B. anthracis	*B. coli*	*B. typhi*
Immediate	4215	5127	8350
1 hour	6950	453	233
3 hours	18340	84	12
6 "	50170	4	0
24 "	Innumerable	0	

Experiment CIII.—Same as above and copperhead venom 6 mg.

	B. coli	*B. typhi*
Immediate	5237	3846
1 hour	615	394
3 hours	95	11
6 "	180	2
24 "	2950	3105

Control: *Necturus* serum 1 cc. + peptone 6 mg.

	B. coli	*B. typhi*
Immediate	9270	4530
1 hour	3810	577
3 hours	632	92
6 "	78	2
24 "	1850	927

Experiment CIV.—*Necturus* serum 1 cc. + copperhead venom 1 mg.

	B. coli	*B. typhi*
Immediate	6240	7360
1 hour	1170	833
3 hours	583	96
6 "	25	13
24 "	0	0

It may be remarked that *Necturus* is highly refractory to venom. An animal weighing 250 grammes received without effect 0.05 grm. venom, equivalent to 160 M. L. D. for the guinea-pig.

The effect of heat upon venom in relation to its action upon the bactericidal properties is of interest. For this purpose cobra, rattlesnake, moccasin, and copperhead venoms were studied. Temperatures varying from 75° to 90° C. were employed, and the heated venoms were mixed with the streaming blood in Nuttall's bulbs and with the separated serum. The venom was kept at the lower temperature (75°) for 30 and the higher (90°) for 15 minutes.

The heated venom acts just as the unheated except in the case of rattlesnake venom, the effect of which is somewhat diminished at the higher temperature, 90° C.

The Mechanism of the Action of Venom upon Serum.—That the bactericidal action of serum depends upon the intermediary body and complement seems established. That the influence of venom upon this property does not depend upon changes in the nutritive value of the serum the foregoing experiments prove conclusively. It is therefore possible that venom acts injuriously upon the intermediary body or the complement or upon both bodies at the same time. The complement is destroyed by heating serum to 56°-58° C.—a temperature which does not affect the intermediary body.

Experiment XCVIII.—To test effect of venom on the intermediary body.

(1) Copperhead venom 1/20 mg.; rabbit's serum 1 cc., and rabbit's serum heated to 58° C., 1 cc. (2) Control; rabbit's serum heated to 56° C.

	(1) *B. typhi*	(2) *B. typhi*
Immediate	4990	5320
1 hour	5800	7800
3 hours	18840	12400
6 "	Innumerable	Innumerable

Experiment XCIX.—To test effect on the intermediary body.

(1) Copperhead venom 1/10 mg.; dog's serum 1 cc.; dog's serum heated to 56°, 1 cc. (2) Control; serum heated to 56° C.

	(1) *B. typhi*	(2) *B. typhi*
Immediate	2270	3440
1 hour	2680	3950
3 hours	71950	12800
6 "	Innumerable	Innumerable

From these experiments the conclusion can be drawn that venom is without action upon the intermediary body contained in dog's and rabbit's serum.

The next experiment was to determine whether any action was exerted by venom upon the complements of these sera. For the purpose of obtaining the serum-complement free from the intermediary body, the rabbit was treated with dog's serum heated to 56° C. In this way the anti-intermediary-body was obtained, which, when heated to 56° C. (to remove rabbit's complement) and added to fresh dog's serum neutralized the action of the latter upon rabbit's corpuscles. From this it could be concluded that the intermediary body of the dog's serum was neutralized by the anti-intermediary-body contained in the immunized rabbit's serum, leaving behind the pure dog's complement in the fluid.

Experiment XCIX (a).—Action on complement.

(1) Fresh dog's serum 1 cc., and copperhead venom 1/10 mg., and dog's complement 1 cc.[11] (2) Control, dog's serum 1 cc., and 1/10 mg. venom.

[11] To obtain these complements fresh dog's or rabbit's serum was treated with rabbit's or guinea-pig's serum containing the anti-intermediary-body which was heated

	(1) *B. typhi*	(2) *B. typhi*
Immediate	5270	4360
1 hour	930	5980
3 hours	28	25410
6 "	15	Innumerable
24 "	0	

A similar experiment in which anti-intermediary-body for rabbit's serum was produced in the guinea-pig gave practically identical results except that when 1/10 mg. of venom was employed the neutralizing effect of this quantity on the complement was also exerted upon the second quantity of complement added.

Experiment XCVIII (a).—Copperhead venom; fresh rabbit's serum 1 cc., and rabbit's complement 1 cc.

	B. typhi 1/10 mg. venom	*B. typhi* 1/20 mg. venom
Immediate	4590	3280
1 hour	3740	1360
3 hours	1850	730
6 "	4900	110
24 "	Innumerable	0

From the experiments under the present heading the following conclusions are warranted:

(1) All venoms when used in suitable quantities destroy the bactericidal properties of many normal blood sera.

(2) The manner of this destruction consists in the fixation of the serum-complements by the venoms.

(3) Venoms have no action upon the intermediary bodies of serum.

(4) If the venom is incapable of uniting with the serum-complements (*Necturus*) then the original bactericidal properties remain unaffected by the presence of the venom.

EFFECTS OF ANTIVENIN ON HÆMOLYSIS AND BACTERIOLYSIS.

Through the kindness of Dr. McFarland we secured a small vial of Calmette's antivenin. This was used to test the restraining action

upon venom hæmolysis and venom anti-bacteriolysis. The antivenin was first proven to be non-hæmolytic for rabbit's corpuscles and to improve slightly the nutritive value of fresh rabbit's serum.

Erythrolysis by cobra venom on rabbit's corpuscles is prevented if neutralization by antivenin is effected. Thus 2 mg. of venom + 1 cc. of antivenin is still lytic although action is retarded; 1.5 mg. of venom + 1 cc. of antivenin caused slight hæmolysis after 24 hours, while 1 mg. + 1 cc. was without action.

In the case of rattlesnake venom 1 cc. of antivenin neutralized 3 mg. of the poison.

Leucolysis was affected in approximately the same degree as in the case of erythrolysis.

The effect on bacteriolysis is equally marked. When cobra or rattlesnake venom is treated with a neutralizing quantity of antivenin and fresh serum is added the resulting fluid behaves in a manner similar to that of the control mixture of normal fresh serum and antivenin.

Antivenin, therefore, neutralizes venom and removes both the hæmolytic and the anti-bacteriolytic actions.