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ON EXTRACELLULAR AND INTRACELLULAR VENOM ACTIVATORS OF THE BLOOD, WITH ESPECIAL REFERENCE TO LECITHIN AND FATTY ACIDS AND THEIR COMPOUNDS.[1]

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INTRODUCTION.

Snake venom forms a hæmolytic compound with pure lecithin when it is shaken with a chloroform solution of this substance.[2] The same is true of the venom of bee.[3] Judging from this important discovery, linked with the fact that the serum as well as the corpuscles of blood yield upon hot alcoholic extraction a large quantity of lecithin, Kyes was led to deduce that the venom activating substance of blood is lecithin. This theory has, however, some rather serious difficulties in explaining certain fundamental phenomena which are observed in venom hæmolysis. In spite of the fact that all serums or corpuscles do yield upon extraction nearly uniform amounts of lecithin, certain kinds of serums possess no activating property; and the susceptibility of the corpuscles of different bloods exhibits a wide variation according to the species of animals. In some instances the corpuscles are completely refractory to the action of venom. Speaking quantitatively there is no relation between the amount of lecithin and that of the venom activating substance of serum or corpuscles. If we are forced to hold lecithin responsible for venom hæmolysis in normal serum or blood corpuscles we must assume that lecithin exists in different states in different bloods, the explanation adopted by Kyes to account for the phenomena observed.

This hypothesis appears to furnish an easy solution of the phenomena, but it requires, in the meanwhile, its verification by experi-

[1] Received for publication April 15, 1907.

[2] Kyes, *Berl. klin. Woch.*, 1903, xi, 956, 982.

[3] Morgenroth and Carpi, *Berl. klin. Woch.*, 1906, xliii, 1424.

mental evidence. The first question will be whether lecithin ever exists as an available compound in the activating serum and in the venom-susceptible corpuscles. To decide this point it is absolutely necessary to exclude other activators than lecithin from the activating serum or susceptible corpuscles. This having been done, we can compare the degree of firmness of lecithin-containing compounds of various kinds of serums and corpuscles, including the originally activating and non-activating serums and corpuscles. Even if we find that in one set of serums there is present an available lecithin compound, and not in the other, we shall then face a new problem, namely, whether the fact of availability or the looseness of the lecithin compound in the former serums is applicable to the whole blood or is to be attributed to a particular lecithin compound found only in the serum, but not in the blood corpuscles.

The part which lecithin may play in venom hæmolysis caused by fresh serum is not quite clear, inasmuch as serum contains still other complementary substances, which, doubtless, play their own rôles. A sharply differential method for venom hæmolysis caused by lecithin and by certain other complementary substances is required to clear up the question to what extent lecithin is responsible for the production of hæmolysis by venom in unmodified serums.

Before recording the results of my present investigations relating to the points already referred to above, as well as to some other questions to be introduced and considered later in this paper, a brief statement of the recent development of our knowledge concerning venom hæmolysis will make the necessity and object of the present work more obvious.

Snake venom produces hæmolysis only through the aid of a secondary substance. Thus Flexner and Noguchi[4] first demonstrated that the blood corpuscles of certain species of animals undergo venom hæmolysis when there is present suitable serum, and believed that complement of such serum is the activating principle. This was, however, found not to be always the case, as Kyes[5] discovered that there are instances where the corpuscles are dissolved without the addition of serum. The cause of the independent susceptibility of these corpuscles was ascribed to the presence of intracellular activator in these cells. The general characteristics of intracellular activators agreed with those of serum complement; Kyes called them endocomplements. At the same time lecithin

[4] Flexner and Noguchi, *Jour. of Exper. Med.*, 1902, vi, 277.
[5] Kyes, *Berl. klin. Woch.*, 1902, xxxix, 886, 918.

was found to be capable of activating venom with striking readiness, but Kyes distinguished this form of venom hæmolysis from that caused by the complementary substances of certain fresh serums or by the endocomplement of certain susceptible corpuscles, by their resistance to high temperature and their activity at 0° C., in which lecithin excelled the other. He also confirmed the important observation of Calmette[6] of the acquisition of power or the increase in strength of the venom activating property of various serums after heating to 65° C. and higher. This phenomenon was attributed by Keyes to the liberation of lecithin by the action of heat. While adding still further evidences to the occurrence of venom-complement hæmolysis, Keyes and Sachs[7] withdrew the opinion that endocomplement was a thermolabile complement, and presented a new view that it is really lecithin contained in the stroma of the susceptible corpuscles. The reason why the stroma of venom-resistant corpuscles does not react with venom was explained by the hypothesis that lecithin exists in an unavailable form in these corpuscles. In his third communication Kyes[8] succeeded in preparing a compound of venom and lecithin (lecithid) and threw suspicion on that mode of venom hæmolysis in which complement is thought to take a part.

Kyes suggested the possibility that any injurious substance may modify the corpuscles so as to render available the lecithin otherwise inaccessible to the venom. But this assumption has never been proven experimentally. My previous experiments[9] show lecithin to be by no means an inert compound, although its activity is likely to be underestimated on account of the slow reaction-time. Further, I was led to consider certain oleic compounds as well as oleic acid as venom activating agents.[10] This point has an important bearing on the present investigation of the venom activating substances of normal serum and susceptible corpuscles. Although no relationship between the lipolytic and the hæmolytic properties of venom and phytogenous toxalbumens have been established, yet the discovery by Neuberg and Rosenberg[11] of the lipolytic property of various venoms and bee poison is a fact of great interest. Ricin, which is a powerful lipolyzer,[12] forms a strong hæmolysin when mixed with free lecithin,[13] a fact important in that it shows that ricin does not unite with the lecithin in the integral corpuscles or serum. Apparently lecithin in the native condition in these substances is unattackable by ricin.

Hence it appears that we are again in the dark as to the real nature of venom hæmolysis. We are entirely unable to answer the question whether complement and certain complement-like bodies of serum have any part in venom hæmolysis, and whether lecithin

[6] Calmette, *Compt. rend. d. l'Acad. d. Sciences,* 1902, cxxxiv, 1446.

[7] Kyes and Sachs, *Berl. klin. Woch.,* 1903, xl, 21, 57, 82.

[8] Kyes, *Berl. klin. Woch.,* 1903, xl, 956, 982.

[9] Madsen and Noguchi, Oversight over det Kongl. Danske Vedenskabernes Selskabs Forhandlungen, 1904.

[10] Noguchi, *Jour. of Exper. Med.,* 1906, viii, 87.

[11] Neuberg and Rosenberg, *Berl. klin. Woch.,* 1907, xliv, 54.

[12] Pascucci, *Hofmeister's Beiträge,* 1906, vii, 457.

[13] Neuberg and Rosenberg, *loc. cit.*

is really present in available form for venom in activating serum and susceptible corpuscles. The sum of what we know at present is that blood serum and corpuscles yield large amounts of lecithin upon alcoholic extraction, that venom can form a powerful hæmolytic compound with free lecithin, that the activating property of serum and venom susceptibility of the corpuscles have no direct relation to their lecithin content, that certain activating serums contain venom activating principles which in some respects closely resemble complement, that certain chemicals, which may be present only in certain bloods as normal constituents, can produce a form of venom hæmolysis hardly to be distinguished from that caused by normal serum.

A ready way to clear up this confusing point is to discover an agent possessing the elective inhibitory action upon one or other of the venom activating principles. In course of my study[14] on the anticomplementary action of various acids, alkalies and salts, I found, among others, that various salts of the alkali earths inhibit complementary action without altering the serum amboceptors. Calcium chloride,[15] when used in a dilute solution, is most suitable to remove the complementary action of serum. This salt was employed in the present work to inactivate serum complement, as it has no marked destructive action upon venom amboceptor, when used in a strength of $\frac{1}{10}$ N, or weaker. On the other hand, calcium chloride has no anti-activating power against lecithin. Venom lecithid retains its hæmolytic activity in a medium containing calcium chloride. When the amount of lecithin, or venom lecithid, is very small the salts retard complete hæmolysis, but have no ultimate effect on the process.

In addition to this differential agent an ethereal extraction of venom activators prepared from an active serum or the stroma of susceptible corpuscles was employed as a means to distinguish the lecithin-like activator from the complement-like ones. This method was especially useful for determining the protein-lecithid nature of the activator of certain serums and all heated serums. I shall return later to a discussion of this point.

[14] Noguchi, read at the meeting of the American Society of Bacteriologists, New York, Dec. 28, 1906.

[15] 0.85 per cent. of this salt is isotonic.

VENOM ACTIVATORS OF FRESH SERUM.

To determine whether the venom activating property of fresh serum is removed by calcium chloride, 0.5 c.c. of the serum under consideration was mixed with 0.5 c.c. of $\frac{1}{10}$ N. solution of this salt, and the volume made 2 c.c. with 0.9 per cent. salt solution. The washed corpuscles were added in proportion of five per cent. After an incubation of half an hour at 37° C., 0.1 c.c. of 0.4 per cent. solution of cobra venom was introduced, and the whole incubated for several hours at 37° C. and left at room temperature for the rest of the night. The result was read off, as a rule, within twenty-four hours. The corpuscles and serum came from the same blood; but sometimes the washed corpuscles of a second blood were employed. This last combination is, of course, possible only when the serum in question has no hæmolytic action upon the corpuscles. Table I. shows the results obtained with the serum and corpuscles of the same blood.

TABLE I.

Blood of	Control. Fresh Serum 0.5 c.c. + Corpuscles. + 0.9 % NaCl 1.5 c.c. } 0.1 c.c. of 0.1 % Cobra Venom Solution.	$CaCl_2$ Addition. Fresh Serum 0.5 c.c. + Corpuscles. + N/10 $CaCl_2$ 0.5 c.c. + 0.9 % NaCl 1.0 c.c. } 0.1 c.c. of 0.1% Cobra Venom Solution.
Man	complete hæmolysis	no hæmolysis
Horse	"	"
Pig	"	"
Cat	"	"
Guinea-pig	"	"
Rabbit	"	"
Pigeon	"	"
Hen	"	"
Goose	"	"
Goat	no hæmolysis	"
Ox	"	"

The above experiment shows that the majority of the varieties of blood employed undergo complete hæmolysis by venom and that their activating substances are completely inhibited by calcium chloride. The absence of hæmolysis in the presence of $CaCl_2$ is not due to destruction of the venom, because the addition of a small quantity of free lecithin to such mixture produces complete hæmolysis. Or, the presence of a small amount of lecithin, which may be added purposely at the same time as the $CaCl_2$, leads to complete hæmolysis. There is still another way to prove that the corpuscles are sensitized by venom in the $CaCl_2$ mixture. The corpuscles may

be washed with 0.9 per cent. salt solution and finally suspended in a fresh lot of the latter. If now a small amount of lecithin or 0.5 c.c. of any activating serum is added complete hæmolysis occurs.

Whether or not the venom activators of the serums are identical with compounds contained in them cannot be determined from this experiment, as both are inactivated by $CaCl_2$. At all events, lecithin is excluded by this test as the activating agent of these serums.

The serum of the dog is extremely rich in venom activating substances and differs from other serums in its relation to inactivation with calcium chloride. Of twelve different samples of normal dog serum complete inactivation by $CaCl_2$ was obtained only in three, marked delay, in five, and slight retardation, in the rest. In this series homologous and heterogeneous corpuscles were tested and gave the same result. Thus dog's serum is an example of a fresh serum, in which lecithin exists in an available form for venom activation. The simultaneous presence of certain $CaCl_2$ inhibiting venom activators was also demonstrable; and in a few instances this class of activators alone was present in the dog serum.

VENOM ACTIVATORS OF BLOOD CORPUSCLES.

A wider variation is exhibited by the corpuscles of different species of animals in their action toward venom. Some kinds of corpuscles are promptly, while other not at all dissolved by venom. The latter class of corpuscles remains undissolved because of the absence of suitable venom activators although sensitization still occurs. The corpuscles of all bloods undergo hæmolysis when suitable activators are present. The most susceptible corpuscles are those of dog and guinea-pig, and entirely refractory are those of ox, goat and sheep. The corpuscles of horse, rabbit, rat, pig and man occupy an intermediary position. Those which approach the limit of the series of non-susceptibility require a longer incubation for hæmolysis than those which stand near the opposite end. This incubation time can be greatly shortened by adding certain activating serums. The original assertion of Flexner and Noguchi[16] that venom requires complement for its activation was based on the fact that certain washed corpuscles remained intact

[16] Flexner and Noguchi, *Jour. of Exper. Med.*, 1902, vii, 277.

during a period of experimentation in which the same corpuscles underwent complete hæmolysis when fresh serum was added. In a later paper Flexner and Noguchi[17] described the presence of complementary substances in various kinds of organic cells.

The cause of destruction of certain corpuscles by venom alone is ascribed by Kyes[18] and Kyes and Sachs[19] to the presence of endocomplement. The thermolabile nature and the inactivity at o° C. of endocomplement led them first to classify it with complements. But later they showed that the thermolability is caused by the simultaneous presence of hæmoglobin and hence concluded that it was the lecithin of the stroma. To this particular point I will return a little later.

In my experiment regarding the nature of intracellular venom activators of the integral corpuscles, I employed calcium chloride to determine whether this salt can suppress hæmolytic action of venom upon susceptible corpuscles. The result was rather remarkable. The washed corpuscles of horse, rat, rabbit, cat, guinea-pig, pig, pigeon, goose, hen and man remained undissolved in $\frac{1}{10}$ N. to $\frac{1}{100}$ N. $CaCl_2$ medium in the presence of corbra venom. The controls dissolved completely in from fifteen minutes to twenty-four hours. The protection of the susceptible corpuscles is not due to the desctruction of venom by $CaCl_2$, because by a later addition as well as by the simultaneous introduction of a small amount of lecithin, or even by the addition of old dog serum (inactive with age) complete hæmolysis can be induced. The least susceptible corpuscles require the smallest amount of $CaCl_2$ for protection. On the other hand, a trace of hæmolysis is often observed with the corpuscles of guinea-pig or man after twenty-four hours. Different samples of dog corpuscles behave differently. I employed several samples and with some there is perfect protection, with others partial protection. Dog corpuscles are liable to spontaneous hæmolysis within twenty-four hours, which fact may be responsible for irregular results.

The removal of intracellular activators by means of digestion

[17] Flexner and Noguchi, *Jour. of Path. and Bact.*, 1905, x, 111.
[18] Kyes, *loc. cit.*
[19] Kyes and Sachs, *loc. cit.*

in a calcium chloride solution gave variable results. The corpuscles of horse or pig are protected from venom after digestion in $\frac{1}{60}$ N. $CaCl_2$ for half an hour, but those of guinea-pig and dog are not. If we use $\frac{1}{10}$ N. solution these corpuscles are rendered even more susceptible to venom than the undigested samples. The mechanical injuries brought about during the procedure of washing away the calcium salt with sodium chloride solution may have some influence. Perhaps a sudden change of tonicity and breaking up of agglutinated masses of corpuscles hasten the destruction. Hence it would appear that in the majority of the corpuscles lecithin seems not to be able to play a part as activator. Before deciding this matter finally other considerations should be taken up. The presence of hæmoglobin in lecithin containing fluid, or the addition of lecithin to the corpuscular solution does not permit of the inactivation by calcium chloride. Ovovitellin of hen's egg contains a high percentage of lecithin. This lecithin-proteid is not split up with ether, but by hot alcohol. I prepared a saline suspension of ovovitellin and examined it for venom activation. It was found to be an excellent activator. Hence the question arose whether this proteid compound could not be easily inactivated by calcium chloride. This proved not to be possible. Thus lecithin, either in a mechanical mixture with hæmoglobin or corpuscular contents, or in a chemical combination with proteid, is not inactivated by $CaCl_2$. That lecithin does not exist in an available form for venom hæmolysis in susceptible as well as insusceptible corpuscles can be shown by the following experiments.

The washed corpuscles of the guinea-pig were broken up with about three times their volume of water. The stroma was separated out by adding 0.9 per cent. sodium chloride and the precipitated stroma was collected by centrifugation. The clear, intensely dark-red supernatant fluid was pipetted off and used for some tests, while the stroma was twice washed in a 0.9 per cent. sodium chloride solution.

The pink-colored stroma was tested for its venom activating property, for which the corpuscles of ox and goat were used. The activation was not rapid, but progressed rather slowly. The deep-red supernatant fluid was not activating. The test with calcium chloride showed that the activating property of the stroma is completely removed by this salt. The shaking of the stroma with a large quantity of ether also removed this property. On the other hand, the ethereal extract upon evaporation left a small amount of fatty substances, which contained chiefly aceton-soluble fats, but almost no lecithin to be detected even with venom.

I redissolved the oily mass in 0.9 per cent. salt solution, in which it showed turbidity and an acid reaction to litmus. This suspension is activating.

We have thus succeeded in locating the site and identifying the probable nature of the phosphorus-free fat acting as an intracellular venom activator. It may be added that the activating property of this suspension is completely set aside by calcium chloride. As Kyes and Sachs first showed, lecithin could be isolated from the stroma with hot alcohol. The lecithin obtained from it is activating, but entirely different from the activators of the integral corpuscles, corpuscular solution and ethereal extract of corpuscles in its relation to $CaCl_2$. While lecithin can be extracted with alcohol from any of the corpuscles, no matter whether they belong to the susceptible or the insusceptible class, in nearly equal quantities, the ether-soluble venom activators are found only in the corpuscles susceptible to venom. I have tried many times to extract an ether-soluble venom activator from the corpuscles of the ox and goat, but without success. These corpuscles are naturally venom-resistant, and their corpuscular solutions do not contain a venom activator. A later experiment with the corpuscles of the dog gave practically the same result as those of guinea-pig.

Direct evidence that certain fatty acids, soaps and neutral fats are capable of acting as venom activators can easily be given. The venom-resistant corpuscles are washed and freed from the serum. Then the minimal hæmolytic quantities of oleinic acid, sodium oleate, ammonium oleate, neurin oleate and triolein are ascertained. The subminimal hæmolytic dose of any of these chemicals is taken and mixed with the corpuscular suspension of any of the insusceptible bloods. No hæmolysis occurs after twenty-four hours. But if an adequate quantity of cobra venom is added at the same time, complete hæmolysis occurs. The addition of these chemicals to the corpuscular solution (not suspension) of an insusceptible blood renders it venom-activating. This artificially prepared activating solution of blood corpuscles behaves in the manner of the solution of susceptible corpuscles, and becomes inactive upon ethereal extraction in case of oleinic acid, organic soaps, and triolein. With the solutions whose activating property is conferred by the addition of alkaline oleate soaps, ether fails to remove it. Calcium

chloride is very effective in depriving these solutions of their acquired venom-activating property. The result with triolein is not satisfactory, as two preparations from Merck and Kahlbaum gave entirely different results, as the latter was almost entirely inactive. The activators of susceptible corpuscles were completely and permanently inactivated by boiling.

From these results it seems justifiable to conclude that lecithin does not exist in the corpuscles, irrespective of their susceptibility to venom hæmolysis, in an available form for venom. The degree of susceptibility of blood corpuscles depends chiefly upon the amount of ether-soluble activators contained in the cells. These ether-soluble activators are, doubtless, fatty acids, and especially oleinic acid. The absence of fatty acids in the insusceptible corpuscles is in perfect harmony with the reaction to venom. If we compare the amount of fatty acids[20] and the degree of venom susceptibility of different corpuscles we discover that a definite and undeniable parallelism between these two factors exists. If any amount of venom activators are present in the stroma after ethereal extraction it does not necessarily follow that they are lecithin, because the ether-insoluble soaps may be preëxistent or be formed during the manipulation of the corpuscles with water, and they would certainly take on a good share in the activation.

VENOM ACTIVATORS OF HEATED SERUM.

Calmette discovered that any serum acquires venom activating property or has its property increased by heat above 65° C. Kyes found that the maximum effect is reached at 100° C. and coincides with the maximum liberation of lecithin. That the activating property of such heated sera is due to lecithin can be further established by means of chloride of calcium, as the next table shows.

An attempt was made to ascertain whether lecithin exists in a free state or as a proteid compound in heated serum. That a non-coagulable proteid exists in serum heated to 80° to 100° C. in a neutral or slightly acidified reaction has long been known. Chabrié,[21] who first described this proteid, called it albumon. Howell's[22]

[20] Abderhalden, *Zeit. f. Physiol. Chem.*, 1898, xxv, 65.
[21] Chabrié. *Compt. rend. de l'Acad. de Sciences,* 1891, cxiii, 557.
[22] Howell, *Amer. Jour. of Physiol.,* 1906–7, xvii, 280.

TABLE II.

	Serum 0.2 c.c. Corpuscles 5 per cent. Cobra Venom 0.1 per cent., Solution 0.1 c.c. (added later). Total Volume made to 2 c.c. with 0.9 per cent. NaCl Solution.									
	Fresh.		56° C.		65° C.		85° C.		100° C.	
	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5	Saline.	$CaCl_2$ N/10 0.5
Man	C. H.	None	Slight H.	None	Much H.	Moderate	C. H.	C. H.	C. H.	C. H.
Horse	"	"	"	"	"	Much H.	"	"	"	"
Guinea pig	"	"	C. H.*	"	C. H.	C. H.	"	"	"	"
Ox	None	"	None	"	Slight H.	Slight H.	"	"	"	"
Goat	"	"	"	"	"	"	"	"	"	"

recent investigations of this proteid added many new facts. Albumon is characterized as soluble in water, indiffusible through collodium membrane, non-coagulable in presence of neutral or acid reaction upon boiling, but easily precipitable by half saturation with ammonium sulphate. It contains a large percentage of phosphorus and iron. The phosphorus represents the lecithin which exists in its molecule as a component extractable only by warm alcohol. Howell considers albumon an artificial product formed through the action of the heat, while some previous investigators considered it a normal constituent, or were uncertain on this point.

In my experiments I employed ox serum. The serum was diluted with three times its volume of water and gradually boiled at neutral reaction (neutralization by means of acetic acid). The non-coagulable portion was separated from the coagulum by filtration. The clear fluid gave the required characteristics for albumon, and upon examination it proved to be highly active for venom and not to be inactivated by calcium chloride. Ether failed to extract any activator from the fluid medium or after drying. On the other hand, warm alcohol yielded much lecithin and perhaps some other venom activating substances. The proteid precipitated out by the process of extraction was inactive. Goat serum yielded a similar albumon.

Thus the conclusion seems warranted that the lecithin of heated serum does not exist in a free state, but in a proteid compound, which is capable of reacting with venom. In this case the pre-existence of this proteid in unheated serum is conclusively excluded by the fact that neither the fresh serum, nor the coagulum of these bloods contained any available lecithin compounds.

* Guinea-pig corpuscles contain endocomplement, which accounts for complete hæmolysis. If ox or goat corpuscles are used no hæmolysis results, while the same corpuscles are readily dissolved by the addition of over-heated serum, and cannot be reduced by $CaCl_2$.

LOCATION OF AVAILABLE LECITHIN IN VARIOUS PROTEID FRACTIONS OF SERUM.

The existence of venom activators of lecithin nature in normal dog serum has been mentioned. But the question whether the availability of lecithin in this particular serum is due to looseness of a lecithin-proteid compound, or to the presence of a definite lecithin compound peculiar to this serum was left undecided.

Two different samples of dog serum were dialyzed in collodium sacs against running water and after seventy-two hours the whitish precipitates were collected by filtration and dissolved in 0.9 per cent. sodium chloride solution. The filtrate was made isotonic with the same salt. The first represents the serum globulin and the second the serum albumin. These were now tested for venom activating property. The result shows that the globulin fraction was inactive and the albumin highly active. The same serums were then fractionated with ammonium sulphate. The precipitate produced by half saturation was separated from the serum by filtration. The filtrate was then completely saturated by further addition of this salt. The precipitate was obtained by filtration. The first represents the globulin and the second the albumin fraction of the serum. Both were dialyzed in collodium sacs for seventy-two hours. In the first sac, which contained the globulin fraction, there was a large amount of whitish precipitate. This was filtered and collected; the filtrate was also preserved for testing. The content of the second sac was perfectly clear, and was used for the tests. All three were made isotonic with sodium chloride. The globulin precipitate dissolved in this medium with slight opalescence. The tests for venom activation gave the following result: the solution of the globulin precipitate and the albumin fraction were unable to activate venom, while the clear portion of the first sac was extremely active. This last fraction was non-coagulable in saline solution upon boiling, but precipitable by ammonium sulphate and heat and coagulable by alcohol. It gave a blue-violet Biuret reaction. The venom activating property of this portion was unaffected by $CaCl_2$ or ethereal extraction.

Similar tests with ox and pig serums (both non-activating) gave results differing from those with dog serum, as the globulin lacked the venom activating property.

The next question was whether the globulin and albumin fractions of these serums contain any lecithin. The examination was made by extracting the serums with hot alcohol (70° C.).

The globulin fraction of the serums of dog, horse, ox and pig yielded upon hot alcoholic extraction comparatively small amounts of lecithin. On the other hand, the albumin fraction of these serums yielded much more lecithin than the globulin. The non-coagulable portion obtained from the first sac (containing the pre-

cipitates by half saturation with ammonium sulphate) contained a large amount of lecithin.

The globulin as well as albumin fractions did not produce upon boiling, either with or without sodium chloride, as much lecithin (in available form for venom) as the whole serums from which they were isolated. In some instances even considerable reductions were observed with the globulin fraction plus the non-coagulable lecithin proteid of dog serum. I am inclined to ascribe this reduction in activity to the simultaneous presence of cholesterin in the medium. The problem, which was set at the beginning of this topic, has been resolved experimentally: the venom activating substance of lecithin nature in dog serum exists as a definite proteid compound, and does not depend upon a loose combination between lecithin and globulin or albumin. The non-venom-activating serums do not contain a similar lecithin proteid to that found in dog serum.

PREPARATION OF ARTIFICIAL VENOM ACTIVATING SERUM.

Normal serum may contain two different sets of venom activators, namely, one resembling complement and the other a proteid compound of lecithin resembling albumon. The latter is occasionally present in normal dog serum, while the former constitute the venom activating substances contained in all venom activating serums. Calcium chloride renders the first class of activators ineffective. The venom activating property of these serums, which are subject to the calcium inactivation, is completely or in part removable by ethereal extraction. The ethereal extract contains fatty acids and neutral fats, but not lecithin. The inactivation of these serums by the temperature of 56° C. is rather uncertain, although more or less reduction in activity is observed. Sometimes complete loss is obtained. The activating function of this class of venom activators is suspended at 0° C. Except in certain herbivorous mammalian bloods (ox, goat and sheep) the majority of mammalian serums and certain avian serums contain the complement-like venom activators in varying quantities, and no lecithin-like activators are to be found in these serums. Even in dog serum, which contains an available compound of lecithin, there is

a large amount of the other present. I have already mentioned that in the ethereal extract of these serums varying quantities of fatty acids and fats are contained. But more convincing is the fact that none of the non-activating serums yields more than a minute quantity of activating fatty acids.

By the term " venom activating fatty acids " oleic acid is chiefly meant. Palmitic and stearic acid are far less effective in this respect. The more oleic acid is present in the ethereal extract, the stronger is the venom activating property.

Apart from the varieties of the fatty acids, the whole amount of these acids extractable from dog serum is nearly twice as much as from the serum of ox, goat or sheep.[23] I have already mentioned that two samples of triolein which I used acted very differently, so that I have been put in doubt regarding my earlier experiments.[24]

The facts above enumerated clearly indicate that fatty acids have a direct relation to the venom activating property of blood serum.

Leaving aside the question whether fatty acids represent the entire venom activators or only part of them, I will consider next whether or not certain fatty acids and soaps can confer the venom activating property upon non-activating serums.

For this experiment two sets of normal ox serum were used: the one without any modification, and the other after being shaken with a large volume of ether. To both sets oleinic acid in amounts of 0.12[25] to 100 grams of the serum was added. In still other series, and with ox serum, sodium oleate was used instead of oleinic acid. Oleinic acid and sodium oleate are highly hæmolytic if these are dissolved in this concentration (or emulsified in case of oleinic acid) in a 0.9 per cent. sodium chloride solution; but in ox serum they remain completely inactive. When these mixtures are used as venom activators they display very powerful hæmolytic action upon insusceptible corpuscles (ox and goat). Their action is not so prompt as that of dog serum or any heated serum, but more like that of guinea-pig serum, except that it is prompter than that. By

[23] Abderhalden, *loc. cit.*

[24] Noguchi, *Jour. of Exper. Med.*, 1906, viii, 87.

[25] This proportion was taken from the data given by Abderlhalden for dog serum.

reducing the quantity of the mixture a descending scale of activation can be secured until no effect whatever is obtained. There is no marked difference between the acid and soap in the mode of action. Calcium chloride completely inactivates these mixtures. The temperature of 56° C. has a marked reducing power upon the soap and serum mixture, but hardly any upon the acid and serum mixture. Ethereal extraction removes the activating property of the latter, but not of the former. At 0° C. the latter is still slightly active, but not the former.[26]

These artificially prepared venom activating serums are not easily distinguishable from certain natural serums.

The second type of venom activating serum, in which lecithin is present in an available form, can be artificially prepared, by adding pure lecithin, or the non-coagulable proteid of heated serum, to non-activating serum. When the mixture is made its activating property cannot be stopped by calcium chloride or the temperature of 0° C. Cholesterin inhibits the effect of this mixture. Ether cannot remove much of the lecithin when once mixed with the serum; this perhaps may be due to the fact that lecithin can enter into combination with some of the serum components.[27]

THE PROTECTIVE ACTION OF CALCIUM CHLORIDE AGAINST VENOM CYTOLYSIS.

The powerful cytolytic property of various kinds of venoms upon the cells of liver, kidney, nerve, testis, and ova of different animals has been demonstrated by Flexner and Noguchi.[28] The mechanism of the cytolysis was found to be essentially the same as that of hæmolysis and that intracellular complements played an important part. Knowing no other means to eliminate the intracellular complement at that time, Flexner and Noguchi employed heat (temperature of 55° C. maintained for thirty minutes). They found that this temperature rendered the cells insusceptible to venom, unless fresh serum was added to the mixture. In this way the similarity of venom cytolysis and serum cytolysis was established.

[26] No suppression of action without serum constituents.

[27] Mayer and Terroine, *Compt. rend. d. l. Soc. d. Biol.*, 1907, lxii, 398.

[28] Flexner and Noguchi, *Jour. of Path. and Bact.*, 1905, x, 111.

Since I found in calcium chloride a powerful anti-complementary hæmolytic substance it was natural to expose the somatic cells to the influence of this chemical to establish the nature of the complementary body present in them. In this series of experiments the cells of liver, kidney, testis and brain of the guinea-pig and rat were employed. It was difficult to obtain many ganglion cells in good condition, while the testicular cells were easily obtained intact. Uniform emulsions (5 per cent.) in salt solution of these cells were measured into small test-tubes to which daboia venom was added in proportion of 1 : 100. The mixture was placed in a water thermostat at 37° C. for five hours and examined microscopically. To test the action of calcium chloride the salt was added to the suspensions of these cells in ratio of $\frac{1}{10}$ N., and half an hour later the venom was introduced.

The results are briefly as follows: the cells of the liver, kidney and testis are well preserved in the saline solution after five hours. The ganglion cells are less distinct in outline, but apparently have not disintegrated (controls). In the venom solution the testicular cells are nearly all dissolved, but the spermatozoa minus heads remain. The clearing up of the cellular elements is distinctly visible *in vitro*. The liver cells become more or less swollen and the outlines indistinct. The granules disappear and the nuclei become more distinct. Agglutination of free cells is marked. The kidney cells and tubules become gradually indistinct and a general disintegration of the former occurs. The number of cells is less than in the controls; agglutination occurs. The ganglion cells have disappeared wholly, and a general solution of granular elements has taken place. These alterations are much more pronounced in the emulsions of guinea-pigs' than of rats' brains. Not only the microscopical, but also the macroscopical appearances of the venomized emulsions is at once recognized by a marked clearing up of the original turbid suspensions. The addition of calcium chloride completely prevents the destructive action of venom upon the cells. The protection afforded by this salt is greater than is obtained by heating the cells to 55° C.

From these facts the complement-like nature of the venom activators contained in the somatic cells is once more established.

SUMMARY.

In normal serums of the majority of mammalian and avian blood there exists certain substances capable of activating venom hæmolysin. They are extractable from serum by means of ether, and are capable of conferring upon the originally non-activating serum a power to activate venom, when mixed with the latter. The ethereal extract consists of fatty acids, neutral fats and possibly also some ether soluble organic soaps. The fatty acids and soaps, especially of the oleinic series, acquire certain characteristics of complements in general, when they are mixed with serum. They are inactive without the venom in the mixture; they are inactivable with calcium chloride; they exhibit a tendency to go off in activity with age; they are inactive or only weakly active at 0° C., and they are extractable by ether. In testing the serum from which the ether soluble substances are removed, it is found that no venom activating property is left. Warm alcoholic extraction of such serum yields, however, a large quantity of lecithin. In the case of non-activating serums no venom activating fats appear in the ethereal extract. Lecithin exists in such serum in no less quantity than in the activating kind.

The addition of oleinic acid or its soluble soaps to a non-activating serum, in a ratio which corresponds to the percentage of fatty acids or soaps contained in some of the easily activating serums, will make the serum highly active in regard to venom.

In normal serum of dog there exists, besides the group of activators already mentioned, another kind of venom activators which has been identified as a lecithin compound acting in the manner of free lecithin.

A very sharp differentiation of the hæmolysis produced by this activator and by the other groups of activators is obtained by means of calcium chloride, which is powerless against lecithin or lecithin compounds, but effective in removing the action of the latter. This lecithin containing proteid can be precipitated by half saturation with ammonium sulphate, but is perfectly soluble in water, and is not coagulated in neutral alkaline salt solutions upon boiling. Alcohol precipitates a proteid-like coagulum and extracts lecithin from it; ether does not extract lecithin from this compound.

Non-activating serums do not contain any such lecithin compound.

Lecithin contained in other serum proteids, mainly as lecithalbumin, and perhaps as contained in globulin, is not able to activate venom. This is true of all the serums with which I worked; it matters not whether these fractions (obtained with ammonium sulphate) belong to the most activating serum (dog) or to the non-activating serum (ox).

The non-coagulable portion of all heated serum contains a venom activator of the nature of lecithin. This activator is contained in a non-coagulable proteid described by Howell which is identical with Chabrié's albumon. As there is no ether-extractable lecithin in this portion of the serum, the activating property of heated serum must be due to this proteid compound of lecithin. That this lecithin proteid does not pre-exist in normal serum but is produced by the action of high temperature is true of all serums except that of the dog. In venom activation we know now that lecithin becomes reactive with venom when it is transformed from other proteid compounds into the non-coagulable form, the albumon. Howell's view of the non-existence of the non-coagulable proteid in normal serum seems to receive a biological support from venom hæmolysis.

Ovovitellin derived from hen's egg is one of the best venom activators of the lecithin proteid type.

The cause of venom susceptibility of various kinds of blood corpuscles does not depend upon the existence of lecithin in the corpuscles, but solely upon the amount of fatty acids, and perhaps, also, soaps and fats, contained in the corpuscles. The protection which calcium chloride gives against venom hæmolysis is proof of the absence of lecithin activation. From the stroma of susceptible corpuscles fatty acids or some fats can be extracted with ether. After ethereal extraction the stroma becomes non-activating, while the extract contains fatty acids and some soaps or fats, which when added to venom-resistant corpuscles render the latter vulnerable to venom. The corpuscular solution of non-activating corpuscles does not contain enough fatty acids. The larger the amount of fatty acids and soaps in the corpuscles, the easier the cells undergo

venom hæmolysis. Lecithin exists in the stroma of all kinds of corpuscles, but in a form unavailable for venom activation.

The somatic cytolytic processes caused by venom requires intracellular complements. The experiments performed on the cells of liver, kidney, testis and brain of the guinea-pig and rat indicate that the substances which act as complements are inactivable by calcium chloride.