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ON CERTAIN THERMOSTABILE VENOM ACTIVATORS.

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The nature of the cytolytic processes involved in venom cytolysis was made clear through the researches of Flexner and Noguchi, Calmette, and Kyes and Sachs. Flexner and Noguchi [1] first demonstrated that venom contains an intermediary body (amboceptor) for blood corpuscles and, later, they proved that venom also possesses intermediary bodies which are capable of uniting with the somatic cells of a wide series of animals and of bringing them under the solvent influence of extracellular and intracellular complements. The complements which they described are thermolabile bodies; while the researches of Calmette [2] pointed to the existence in serum of a thermostabile body which united with the venom hæmolytic principle and caused hæmolysis, and this body was discovered by Kyes and Sachs [3] to be lecithin. Recently Kyes has produced a crystalline substance which he calls cobra lecithid, which retains the property of bringing about solution of red blood corpuscles.

In order to study further the manner of action of lecithin in respect to its complementing property and to compare it with substances of related nature, Kyes tested other phosphorized fats such as kephalin, protagon, and cerebrin. He found that kephalin[4] was also capable of activating venom, although in less

[1] Flexner and Noguchi, *Journal of Experimental Medicine*, 1902, vi, 277; *Journal of Pathology and Bacteriology*, 1902, viii, 379; *University of Pennsylvania Medical Bulletin*, 1903, xvi, 163.

[2] Calmette, *Compt. rend. Acad. d. Soc.*, Paris, 1902, cxxxiv, 1446.

[3] Kyes, *Berliner klin. Wochenschrift*, 1902, 886, 918; 1903, 956, 982. Kyes and Sachs, *op. cit.*, 1903, 21, 57, 82.

[4] I had also found that kephalin is complementary to venom, but I did not think it worth while to publish the observation after Kyes's paper appeared.

degree than lecithin, while the other two substances were devoid of this power. This fact is of theoretical interest, since kephalin stands closely related to lecithin chemically. On the other hand, Kyes found that cholin, glycerino-phosphoric acid, and some fats were inactive in this respect.

The nature of the case of venom activation calls for a study of the inherent hæmolytic powers of the substances employed as activators, and this should be carried out in a strictly quantitative manner. The great difficulty in the study of the hæmolytic power of lecithin is that this substance is very unstable, and that it seems almost impossible to get a well-defined chemical body. Most of the commercial preparations of lecithin comprise a series of rather different bodies. The impression exists that lecithin is an almost indifferent substance, while as a matter of fact some preparations of lecithin are powerful hæmolyzing agents. The source of misapprehension is to be found, on the one hand, in the variation of the substance called lecithin, and on the other, in the velocity of reaction, which is often overlooked. If we compare the hæmolysis caused by cobra venom plus lecithin with that of lecithin[5] alone the final effect may be quite the same in both cases, but it is obtained much more quickly in the first case. The difference may in some cases be so great that the reaction is finished almost instantaneously with cobra plus lecithin, while it requires six hours or more for its completion with lecithin alone. I can point to other examples of great difference in the velocity of reaction, but not in the degree of hæmolysis. Equivalent amounts of most acids produce the same final hæmolysis, but the rapidity is much greater for the strongly dissociated acids.

Kyes studied the hæmolytic value of lecithin, and he found that, as compared with lecithin in the presence of venom, it was 200 to 300 times less active in the pure state. This estimation must be considered as conditional upon the manner of experi-

[5] Madsen and Noguchi, "Toxines et antitoxines," *L'influence de la temperature sur la vitesse de réaction*, ii; *Académie royale des sciences et des lettres de Danemark; Bulletin de l'année 1904*, 447; and *Centralbl. f. Bakt. und Parasitenk.* 1905, xxxvii, Abt. i., Referate, 356.

mentation. Venom-lecithin hæmolysis is complete within fifteen minutes, while simple lecithin hæmolysis is, as I have pointed out, complete only after about six hours. Hence, an absolute comparison of activity should be made not at once but after the lapse of at least this period. That it is essential to study the end reaction will be conceded when it is considered that the proportion at an early stage between lecithin hæmolysis and venom-lecithin hæmolysis may be as 1 to 1000, while at the end of the process it may be only as 1 to 20. A quantitative study of the reactions has indeed shown me that, as measured by the constant of the end of the reaction, the difference in hæmolytic activity of the pure lecithin as compared with the lecithin and cobra venom mixture is as 1 to 16. This proportion indicates the activating strength of the lecithin used by me; and a determination of the activating strength of other substances may equally be made by bearing in mind and observing the reaction velocity. It is in this way alone that any knowledge of absolute strength can be obtained. The present investigation had for its purpose the study of other substances than those already studied by Kyes with the view of ascertaining whether similar activating properties would be exhibited by any of them.

METHODS.

Materials.—All the chemicals except lecithin were procured directly from Merck at Darmstadt immediately before being used. The lecithin was obtained, first, from hens' eggs, and was prepared by Dr. Madsen at this Institute; and, second, from the cardiac muscle of the ox, this sample having been prepared for me by Professor Bock at the Pharmacological Laboratory of the Copenhagen University.

The solutions were made normal, and employed as such with the few exceptions in which percentage solutions were used. All water-soluble chemicals were dissolved in distilled water, while the insoluble ones were emulsified in water by means of an equal weight of gum arabic. Lecithin, while insoluble in water, can be emulsified readily without the use of the suspending gum. Triolein, tripalmitin, and tristearin are not emulsified

by these means; but, by first dissolving them in a small amount of ether and then shaking them with a larger quantity of methyl alcohol, they can be rendered emulsifiable. At least triolein can be suspended in this way, while tripalmitin and tristearin were never rendered wholly satisfactory for experiment. The solutions of natrium oleinicum, natrium stearinicum, and natrium palmitinicum, on account of the liability to dissociation, were always freshly prepared before being used.

The venoms employed were cobra, water moccasin, habu (Trimeresurus), and daboia. They were dissolved in 0.9 per cent. salt solution.

The blood corpuscles were those of the horse washed twice in salt solution and suspended in the proportion of 1 part of the condensed corpuscles to 99 parts of the 0.9 per cent. salt solution. These washed corpuscles are not hæmolyzed by any of the venoms alone. Hence, they are suitable for such an investigation as the present.

Test of Activation.—Activation of venom can be accomplished by means of two kinds of substances: First, those which have no inherent capacity to cause hæmolysis, and, second, those which, while causing independent hæmolysis, act in weaker concentrations in the presence of venom. In the latter case the velocity of the reaction is, as a rule, and especially with lecithin, accelerated. The thermolabile serum complements belong to the first and lecithin and some other chemical substances to the second class of substances.

Technique.—Each tube contained 8 cubic centimeters of the 1 per cent suspension of condensed horse's corpuscles. To each tube varying amounts of the substance to be tested for activation were added, and, at the same time, in the main series, 0.2 cubic centimeters of a 0.3 per cent. solution of cobra or other venom. The mixtures were first thoroughly shaken, then placed at 37° C. for two hours, and kept fifteen hours longer at 15° C. The reading was made at the end of this time, i. e., 17 hours. In testing suspended, insoluble substances, several shakings were given during this period.

The estimation of the hæmolytic power was made on the basis

of the extremes of action, namely, the quantity which sufficed to produce a trace and the quantity required just to produce complete hæmolysis. Control tubes, minus the activator and venom, were exposed in every series in duplicate exactly to the same conditions of shaking, temperature, etc.

The degree of dilution of the normal solutions was adjusted according to the activity of the substance. The stronger the native activity of the substance the lower the degree of normal strength, and *vice versa*. In this way the quantity of normal solution added was made to vary between 0.2 cubic centimeter and 1 cubic centimeter. In spite of their feeble power as activators, the higher fatty acids could not be used in stronger concentrations than $\frac{1}{10}$ N. on account of their high molecular weights; on the other hand, all the higher acrylic acids and their salts and lecithin[6] displayed such high power that they were employed in concentrations varying from $\frac{1}{100}$ N. to $\frac{1}{1000}$ N.

Table I gives the results with each substance tested, the calculation having been made on the basis of 1 N. solution, unless special mention to the contrary is made. The sign — indicates no hæmolysis with the highest concentration employed.

An examination of this table will reveal certain new facts. Many acids produce hæmolysis, but a striking difference is apparent between the action of the normal fatty acids and the monocarbonic acrylic acids. The hæmolytic power of the normal fatty acids seems to become weaker with the higher members until, with some of the higher acids, no hæmolyzing effect is produced. The reverse is true of the acrylic monocarbonic acids, since with them the larger the number of carbon atoms the stronger is the hæmolytic power exerted by them.

The disappearance of the hæmolytic power in the higher

[6] The molecular weight of lecithin is rather indefinite, but in this case I have taken it for a dioleinic lecithin ($C_{44}H_{86}O_9NP = 807$), and it was made into a 0.807 per cent. emulsion corresponding to $\frac{1}{100}$N. From this concentration any desirable quantity was made by means of addition of a certain quantity of 0.9 per cent. NaCl solution. The figures given in the tables are calculated on the basis of 1 N. in order to make possible the comparison of the results with those obtained with more weakly acting chemicals.

TABLE I.

Name.	Native hæmolytic power.		Hæmolytic power upon the venomized corpuscles.	
	Dose for C.H.	Dose for Trace H.	Dose for C.H.	Dose for C.H.
Acid. formicum	0.018	0.01	0.018	0.01
" aceticum	0.018	0.01	0.018	0.01
" propionicum	0.018	0.01	0.018	0.01
" butyricum	0.018	0.01	0.018	0.01
" palmitinicum	0.05	0.015	0.007	0.002
" stearinicum	—	0.08	—	0.018
" arachinicum	—	—	—	—
" ceratinicum	—	—	—	—
Acid. crotonolicum	0.025	0.015	0.005	0.001
" nonylicum	0.025	0.01	0.01	0.005
" undecylenicum	0.018	0.01	0.006	0.0004
" oleinicum	0.002	0.0008	0.0002	0.00005
" elaidenicum	0.02	0.0025	0.003	0.0002
Acid. ricinolicum	0.015	0.002	0.003	0.00015
Acid. maleinicum	0.013	0.008	0.013	0.008
" fumaricum	0.011	0.008	0.011	0.008
" citraconicum	0.015	0.008	0.015	0.008
" itaconicum	0.017	0.01	0.017	0.01
Acid. sorbinicum	0.017	0.01	0.017	0.01
Acid. oxalicum	0.014	0.006	0.014	0.006
" succinicum	0.012	0.005	0.012	0.005
" tartaricum	0.012	0.005	0.012	0.005
" citricum	0.008	0.0035	0.008	0.0035
Acid. glycerino-phosphoricum	1.	0.05	1.	0.05
Acid. hydrochloricum	0.018	0.01	0.018	0.01
" sulphuricum	0.009	0.006	0.009	0.006
" phosphoricum	0.009	0.007	0.009	0.007
Natrium stearinicum	0.03	0.015	0.015	0.005
" oleinicum	0.0008	0.0005	0.0002	0.00005
" crotonolicum 1 %	0.4	0.1	0.15	0.02
Tripalmitin	—	—	—	—
Tristearin	—	—	—	—
Triolein	0.0018	0.0006	0.00008	0.000015
Lecithin (egg)	0.002	0.00065	0.00012	0.00004
" (ox's heart), unknown concentration	2.	1.2	0.2	0.08
Neurin 1 %	1.5	1.	1.5	1.
Cholin 1 %	1.5	1.	1.5	1.
Alcohol methylenicus 10 N	2.	1.5	2.	1.5
" ethylenicus 10 N	1.6	1.	1.6	1.
" allylicus 10 N	0.5	0.3	0.07	0.03

normal acids may be due in part to their insolubility in water and in part to the reduction of the reaction velocity. That insolubility in water is not the only factor is shown by the behavior of the acrylic acids, which, while also insoluble in water, are, nevertheless, strongly hæmolytic. It is interesting to note that an isomer of oleinic acid, namely elaidenic acid, possesses much weaker action than the former acid, and, further, that ricinolic acid, the oxydate of oleinic acid, exhibits about the same power as elaidenic acid. It may be pointed out in this place that oleinic acid, sodium oleate, triolein, and lecithin possess the strongest hæmolytic action of all the chemicals studied, sodium oleate causing $2\frac{1}{2}$ to 3 times the degree of hæmolysis produced by oleinic acid or lecithin, twice as much as triolein, and $22\frac{1}{2}$ times as much as hydrochloric acid or the lower normal fatty acids.

With tripalmitin and tristearin no hæmolytic action was noted, although they were used in concentrated (1 N.) ethereal solutions, the volume of ethereal solution being, of course, limited to subhæmolytic amounts (to 8 c.c. less than 0.15 c.c.). However, the results with these substances are inconclusive.

The hæmolytic action of cholin and neurin is about the same, and the degree is about that of most monovalent acids. On the other hand, the power of the alcohols is weak, methyl alcohol standing below ethyl, and ethyl below allyl alcohol in hæmolytic power. It may be pointed out that the saturated alcohols tested were weak agents as compared with the far more active unsaturated allyl alcohol.

With this preliminary study of the hæmolytic activity of different chemicals we can proceed to a similar study of the same bodies in the presence of venom. Hitherto, there have been carefully studied, in this respect, only lecithin and kephalin and certain overheated serums in which the activating substances are probably of lecithin-like nature. Kyes, however, noted that a few neutral fats and certain salts of fatty acids activate venom in a minor degree.

Since I have shown that a relation or constant exists between the inherent and the venom activating hæmolytic power of certain chemicals, I felt the need of a standard with which any

of the active chemicals can be compared. For the purpose of this standard, I chose lecithin. According to my own experiments, lecithin possesses the following power: In 1 N. suspension the "complete" hæmolytic dose is 0.002; the "trace" hæmolytic dose, 0.00065. In the presence of cobra venom the "complete" dose is 0.00012, the "trace" dose, 0.00004. Hence, by the addition of cobra venom, lecithin is raised in power for complete hæmolysis according to the equation $\frac{0.002}{0.00012}$, or 16.6 times, and for traces of hæmolysis, $\frac{0.00065}{0.00004}$, or 16.25 times. And since lecithin has been considered to be an activator in the true sense, as it combines with the venom hæmolytic amboceptor to form a compound,[7] we may, for the present, call any substance, which is raised in power by venom in similar degree, a venom activator. Certain chemicals in the following table fulfill this requirement.

While the substances enumerated in Table II would appear to be activating in an unmistakable way, they certainly fulfill the requirements of my definition, although in different degrees. The strongest body in this respect, of those tested, is triolein, and as compared with the weakest body, nonylic acid, it is twenty times as powerful an activator. It is interesting to recall that the three strongest activators—triolein, oleinic acid, and lecithin—are all related chemically to each other. That they do not act in a peculiar manner in the presence of venom and are not alone raised to multiples of their former hæmolytic strength through its presence, is shown by the conduct of such weak activators as ricinolic, undecylenic, crotonolic, and perhaps also stearinic acid, which, while showing comparatively small inherent activity, are raised in about the same proportion when tested in the presence of venom. On the other hand, the body, natrium oleate, which possesses the strongest inherent hæmolytic powers, is one of the poorer activators. In the presence of venom, oleinic acid is made as strong a hæmolyzing agent as natrium oleate; in acting without venom the latter is two and a half times as strong as oleinic acid.

The activity limits, for the production of traces and com-

[7] *Cf.* Kyes, loc. cit.

TABLE II.

	Complete hæmolysis.				Trace of hæmolysis.				Through venomization the hæmolytic power is increased by so many times.	
	Without venom.		With venom.		Without venom.		With venom.			
	Dose in c.c. 1 N. solution.	Hæmolytic units per 1 c.c.	Dose in c.c. 1 N. solution.	Hæmolytic units per 1 c.c.	Dose in c.c. 1 N. solution.	Hæmolytic units per 1 c.c.	Dose in c.c. 1 N. solution.	Hæmolytic units per 1 c.c.	For complete H.	For trace of H.
Lecithin	0.002	500.0	0.00012	8333.3	0.00065	1538.4	0.00004	25000.0	16.6	16.25
Triolein	0.0018	555.5	0.00008	12500.0	0.0006	1666.6	0.000015	66666.6	22.5	40.
Acidum oleinicum	0.002	500.0	0.0002	5000.0	0.0008	1250.0	0.00005	20000.0	10.	16.
" ricinolicum	0.015	66.6	0.003	333.3	0.002	500.0	0.00015	6666.6	5.	13.3
" elaidenicum	0.02	50.0	0.003	333.3	0.0025	400.0	0.0002	5000 0	6.6	12.5
" undecylenicum	0.018	55.5	0.006	166.6	0.01	100.0	0.0004	2500.0	3.	25.
" nonylicum	0.025	40.0	0.01	100.0	0.01	100.0	0.005	200.0	2.5	2.
" crotonolicum	0.025	40.0	0.005	200.0	0.015	66.6	0.001	1000.0	5.	15.
" palmitinicum	0.05	20.0	0.007	142.6	0.015	66.6	0.002	500.0	7.1	7.5
" stearinicum	0.2	5.0	0.08	12.5	0.2	5.0	0.018	55.5	2.5	11.1
Natrium "	0.03	33.3	0.015	66.6	0.015	66.6	0.005	200.0	2.	3.
" crotonolicum	0.04	25.00	0.015	66.6	0.01	100.0	0.002	500.0	2.6	5.
" oleinicum	0.0008	1250.0	0.0002	5000.0	0.0005	2000.0	0 00005	20000.0	4.	10.
Alcohol allylicus	5.0	0.2	c.7	1.4	3.0	0.3	0.3	3.3	1.5	10.

plete hæmolysis, are quite widely separated by venomization, the increase being greater at the lower limits. The reason for this difference has not been established, but it may to some extent be connected with the manner of distribution of the insoluble bodies in the fluid. In the presence of the smaller quantities of insoluble substance a more minute distribution is possibly obtained, thus favoring union with the venom and action upon the corpuscles. What this difference depends on is at present merely speculation; a special study would be required for its determination. The rate of hæmolysis in the presence of venom differs markedly for the different chemicals. With lecithin the reaction is rapidly produced, while with the rest of the activators it proceeds much more slowly. All the other hæmolytic substances tested, whether organic or inorganic, acted indifferently upon the normal and the venomized corpuscles. Possible exceptions are methyl and ethyl alcohols, in which a slight increase of hæmolysis was observed in the presence of venom.

In the course of my work I have used other venoms than cobra poison. I have not tested all the chemicals anew with them, but have confined my studies to lecithin, triolein, and oleinic acid. The manner of carrying out the tests was slightly modified. Twice-washed horse corpuscles (1 per cent in 0.9 per cent NaCl sol.) were mixed with uniform quantities of the three activators mentioned. The quantities employed were insufficient to produce inherent hæmolysis, but sufficient to bring about this change in the presence of venom, which was added in varying amounts. The mixtures were kept at 37° C. for two hours and at 15° C. for fifteen hours subsequently. The blood suspension was 8 cubic centimeters, the activator 0.0002 of a 1 N. solution, and the venom in 1 per cent solution of the dried poison, varying quantities per tube. Table III gives the detailed results.

It will be observed that all the venoms are activated by the three chemicals employed, and, excluding a few irregularities, these range themselves in the following order of activity: triolein, lecithin, oleinic acid. On the other hand, the reaction velocity is greatest with lecithin. One special fact may be men-

TABLE III.

Kind of venom.	Cobra 1 %.		Water Moccasin 1 %.		Trimeresurus. 1 %		Daboia 1 %.		Crotalus 1 %.	
Kind of activator.	Dose for C. H.	Dose for trace H.	Dose for C. H.	Dose for trace H.	Dose for C. H.	Dose for trace H.	Dose for C. H.	Dose for trace H.	Dose for C. H.	Dose for trace H.
Lecithin 1 N. 0.0002.	0.04	0.01	0.06	0.02	0.012	0.004	0.04	0.014	0.2	0.4
Triolein 1 N. 0.0002	0.03	0.01	0.03	0.01	0.006	0.002	0.03	0.01	1.5	0.5
Oleinic Acid 1 N. 0.0002	0.05	0.016	0.05	0.016	0.02	0.01	0.04	0.01	2.5	1.

tioned about Crotalus venom. According to the results of these experiments, it appears to be a poor hæmolyzer, being 50 times weaker than cobra, water moccasin, and daboia venoms, and 100 to 150 times weaker than Trimeresurus venom. If, however, a more appropriate activator is employed, this difference disappears to a large extent. In the presence of dog's serum, whether fresh or after heating, the relative strength rises as compared with Trimeresurus as 1 to 10, and at the same time the reaction velocity is much accelerated. It is very probable that dog's serum, and perhaps other serums, contain thermostabile activators especially adapted to this venom. I might mention that several years ago I isolated from dog's serum, by ether extraction, a grayish hygroscopic powder, which was highly activating for Crotalus venom. I was unable at the time to identify this substance and I have not been able to return to its study.

As regards the quantitative relations of venom and activator, it may be said that the degree of hæmolysis depends on the relative quantities of each substance used. If to a given quantity of blood suspension more venom is added, then less activator is needed, and *vice versa*. Morgenroth and Sachs [8] studied the quantitative relations of serum hæmolysis, and Kyes has made observations on cobra venom and lecithin regarding the degree of hæmolysis, from which he concluded that the greater the quantity of venom employed the less the amount of lecithin needed. No study having been made of the more delicate quantitative relations of thermostabile activators and venom, I have tried, in

[8] Morgenroth and Sachs, *Ehrlich's Gesammelte Arbeiten zur Immunitätsforschung*, 1904, 359.

this paper, to supply that deficiency. I have studied from this point of view cobra venom and three activators, namely, lecithin, triolein, and oleinic acid.

Series A. Venom Uniform, Activator Variable.—Each tube contained 8 cubic centimeters of 1 per cent. horse's washed corpuscles in 0.9 per cent. NaCl. The tubes, after receiving the venom and activators were kept at 37° C. for two hours and at 15° C. for four hours. Table IV gives the details of the experiment.

TABLE IV.

Cobra venom 1 %.		1 N. Lecithin.		1 N. Triolein.		1 N. Oleinic acid.		
Uniform dose for each set of tubes.	Proportion.	Amount for C. H.	Proportion.	Amount for C. H.	Proportion.	Amount for C. H.	Proportion.	Square root of proportion of venom.
i. 0.0025	1	0.0005	1	0.0003	1	0.0009	1	1.
ii. 0.005	2	0.00035	1:1.4	0.00025	1:1.2	0.0006	1:1.5	1.41
iii. 0.01	4	0.00025	1:2.0	0.00016	1:1.9	0.0004	1:2.25	2.
iv. 0.02	8	0.00018	1:2.5	0.00012	1:2.5	0.0003	1:3.0	2.83
v. 0.04	16	0.00012	1:4.1	0.00008	1:3.75	0.0002	1:4.25	4.
vi. 0.08	32	0.0001	1:5.0	0.00005	1:6.0	0.0001215	1:9.0	5.66
vii. 0.16	64	0.000075	1:6.6	0.00004	1:7.5	0.0001	1:9.0	8.

From this table the conclusion can be drawn that an increase in the quantity of venom does not reduce markedly the requirement in activator, although it reduces the requirements systematically. The definiteness of the relation between venom and activator is striking in spite of a few irregularities, for which technical errors may easily account, and it may be expressed in the form of a rule which might be framed as follows: *The requirement in activator for producing an equal degree of hæmolysis of horse's corpuscles is in proportion to the square root of the amount of venom present in the mixture.*

Series B. Activator Uniform, Venom Variable.—This experiment is the reverse of the preceding. All conditions were the same as in Series A, except that with a constant amount of activator, varying quantities of venom were employed. The results are given in Table V.

A study of this table suffices to show that the degree of action of the activator is different from that of the intermediary body, and that as the former is increased the latter may be diminished

TABLE V.

1. LECITHIN + COBRA VENOM.

1 N. Lecithin.			Cobra venom 1 %.		Square of proportion of the amount of lecithin.
	Uniform dose for each set.	Proportion.	Dose for C.H.	Proportion.	
i.	0.00005	1	0.3	1	1
ii.	0.0001	2	0.08	1: 3.8	4
iii.	0.0002	4	0.02	1:15.0	16
iv.	0.0004	8	0.005	1:60.0	64
v.	0.0008	16	0.0015	1:200.0	256

2. TRIOLEIN + COBRA VENOM.

1 N. Triolein			Cobra Venom 1 %.		Square of proportion of the amount of triolein.
	Uniform dose for each set.	Proportion.	Dose for C.H.	Proportion.	
i.	0.00005	1	0.275	1	1
ii.	0.0001	2	0.08	1:3.4	4
iii.	0.0002	4	0.025	1:11.0	16
iv.	0.0004	8	0.0045	1:55.5	64
v.	0.0008	16	0.0012	1:230.0	256

3. OLEINIC ACID + COBRA VENOM.

1 N. Oleinic Acid.			Cobra Venom 1 %.		Square of proportion of the amount of oleinic acid.
	Uniform dose for each set.	Proportion.	Dose for C.H.	Proportion.	
i.	0.0001	1	0.225	1	1
ii.	0.0002	2	0.06	1:3.8	4
iii.	0.0004	4	0.0125	1.18.0	16
iv.	0.0008	8	0.003	1:75.0	64

without failure to cause hæmolysis. In this instance a rule may also be formulated, which might read: *An increase in activator permits of a reduction in venom approximately in proportion of the square of the amount of activator employed.*

Inhibition of Lecithin-Venom Hæmolysis by Cholesterin.—While we[9] failed to observe definite protective action of cholesterin against venom-complement hæmolysis, Kyes [10] found that cholesterin is capable of preventing venom-lecithin hæmolysis. I have experienced no difficulty in confirming this observation of Kyes, and I have extended my study of the subject to the question as to whether cholesterin acts upon the venom hæmolysin, or upon the activator.

The manner of study was as follows: A series of tubes was prepared, containing venom in increasing and lecithin in decreasing amounts. According to this plan and in keeping with the observations made in an earlier part of this paper, several different hæmolytic mixtures will be obtained. To these mixtures cholesterin is added in exactly the quantity needed to prevent hæmolysis. If the amount of cholesterin required is proportional to the venom used, the presumption would naturally be that it reacted with that substance, while if it is proportional to the lecithin, then the presumption would be in favor of reaction with that body. Should the amount of cholesterin remain about constant throughout the experiments, the probability would be that it acted upon the blood corpuscles and protected them directly from hæmolysis.

Five series of tests were made. The orders were as follows: i. Venom + lecithin + cholesterin + blood; ii. venom + cholesterin + lecithin + blood; iii. lecithin + cholesterin + venom + blood; iv. blood + lecithin + cholesterin + venom; v. blood + venom + cholesterin + lecithin. The results of the tests are given in Table VI which follows.

Table VI shows that a relation exists between the cholesterin and lecithin in the mixtures and not between cholesterin and venom or corpuscles, and this fact seems to prove conclusively that the antihæmolytic action of cholesterin depends on its reaction with lecithin. In order that lecithin-venom hæmolysis may be prevented by cholesterin, a certain interval of action between the lecithin and cholesterin is required with minimal

[9] Flexner and Noguchi, loc. cit.

[10] Kyes, loc. cit.

TABLE VI.

	Combination of venom, lecithin, cholesterin,* and blood suspension.	Cobra venom 0.2 % plus lecithin 1 N.	Correlative amounts of venom and lecithin, representing units of hæmolytic power.			
			0.1 0.0004	0.2 0.0003	0.4 0.0002	1.6 0.0001
i.	Venom + lecithin, 6 hours—36° C. + *cholesterin*, 15 minutes—15° C. + blood. " + " " " + " 3 " —15° C. + " " + " 3 min. —36° C. + " " " + "	Takes 1 N. cholesterin. " " " " " "	0.0008 0.0012 0.0012	0.0006 0.0010 0.0010	0.0004 0.0007 0.0008	0.00016 0.00036 0.0004
ii.	Venom + *cholesterin*, 6 hours—36° C. + lecithin, 15 minutes—15° C. + blood. " + " " " + " 5 " " + " " + " 3 min. — 36° C. + " " " + "	" " " " " " " " "	0.0008 0.0012 0.0012	0.0006 0.0010 0.0010	0.0004 0.0008 0.0007	0.00016 0.00032 0.00036
iii.	Lecithin + *cholesterin*, 6 hours—36° C. + venom, 15 minutes—15° C. + blood. " + " " " + " 3 " " + " " + " 3 min. —36° C. + " " " + "	" " " " " " " " "	0.00076 0.0008 0.0012	0.00058 0.0006 0.0010	0.00036 0.00036 0.0006	0.00016 0.00016 0.00036
iv.	Blood + lecithin, 60 min.—36° C. + *cholesterin*, 15 minutes—15° C. + venom.	" " "	0.0008	0.0006	0.0004	0.0002
v.	Blood + venom, 30 min.—36° C. + *cholesterin*, 15 minutes—15° C. + lecithin.	" " "	0.0018	0.0012	0.0008	0.0004

* The amount of cholesterin used was contained in 0.2 c.c. of methyl alcohol.

quantities of cholesterin, while with larger amounts of this substance the time interval is greatly diminished.

It is probable that the reaction between cholesterin and lecithin depends on their solubilities in each other, and, hence, if the conditions are so modified as to alter essentially their solubilities, the result may be quite or wholly changed. That this is true can be shown by the action of methyl alcohol, which is capable of setting aside the antihæmolytic action of cholesterin. Thus, if 0.4 cubic centimeter of methyl alcohol is added to 8 cubic centimeters of the mixture of horses' corpuscles, venom, and lecithin, the quantity of cholesterin required to prevent hæmolysis will be greatly increased, while a quantity of the alcohol over 0.8 cubic centimeter so changes the conditions as to render cholesterin impotent in preventing, although it may still delay, hæmolysis as Table VII exhibits.

TABLE VII.

Hæmolytic mixture. Amount of methyl alcohol.	Cobra venom 0.2 % Lecithin 1 N.	0.1 0.0004.	0.2. 0.0003.	0.4. 0.0002.	1.6. 0.0001.
0.1	Cholesterin 1 N.	0.0007	0.00056	0.0004	0.00016
0.2	" "	0.0008	0.0006	0.0004	0.00018
0.3	" "	0.0010	0.0008	0.0005	0.00022
0.4	" "	0.0025	0.0020	0.0015	0.0008
0.5	" "	0.0060	0.0040	0.0028	0.0015
0.6	" "	0.0100	0.0080	0.0050	0.0040
0.7	" "	0.0400	0.0300	0.0200	0.0150
0.8	" "	Hæmolysis retarded, but no protection.			
1.0	" "	"	"	" "	"

This study was carried out while I was being maintained abroad by a grant from the Carnegie Institution of Washington, D.C., and it is a continuation of the studies on venom which I began at the University of Pennsylvania with Professor Flexner, in which I had the support and advice of Dr. S. Weir Mitchell.

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