ON HIRUDIN AND HIRUDIN IMMUNITY.*

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In 1884 while pursuing studies on the coagulation of the blood, John B. Haycraft, professor of physiology in the Mason and Queen's Colleges, became interested in explaining why the blood flowing from a leech bite is not easily stopped. On March twenty-fourth of the same year he communicated to the Royal Society of London the results of the researches he had made, "On the action of a secretion, obtained from the medicinal leech, on the coagulation of the blood." It had been common knowledge that the blood flowing from a leech wound remained fluid longer than that flowing from an ordinary wound, that the blood in the alimentary canal of the leech remained fluid, and for some time after its disgorgement. Up to this time no explanation of this phenomenon had been given. It was known that when certain substances like peptone solution and solutions of other products of digestion were injected into dogs coagulation of the blood was delayed. Haycraft had been endeavoring to solve the very difficult problem, "Why does not the blood coagulate in the living blood vessels?"

Assuming that some chemical substance was injected into the wound by the leech he made a salt solution extract of the gullets and buccal cavities and another extract from the remaining portion of the alimentary canal. He first cut the tissue of two leeches into small bits and then placed them in five cubic centimeters of six per cent salt solution. The resulting extracts were of a greenish color. That made from the head portion prevented coagulation for over twenty-four hours while that made from the remainder of the alimentary tract did not appreciably prevent coagulation, faint coagulation occurring in four minutes, complete setting in thirty

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minutes; untreated blood coagulated in four minutes. Haycraft concluded from this observation that the substance retarding coagulation is present in the gullets and buccal cavities of the leech and that the faint anti-coagulating effect of the second extract is due to the secretion diffusing downwards. He failed in his attempt to isolate the pure active principle. He determined its insolubility in chloroform, ether, benzole, and alcohol. By treating the tissue first with absolute alcohol and then extracting with water he succeeded in producing an active extract in clear solution, better suited to laboratory experiments. This extract was free from albumen and gave a very small residue.

Evidently believing the anti-coagulating substance was a glandular secretion, Haycraft made serial (frozen) sections of the gullets and buccal portions of the sucker. These he stained with picrocarmine but did not succeed in demonstrating glandular structures. The skin lining the sucker he found to contain active substance. In well conducted experiments he next endeavored to determine the exact nature of the leech secretion. I have arranged what may be called the conclusions of his work as follows:

The anti-coagulating substance is not a ferment because it remains active after boiling.

The anti-coagulating substance prevents coagulation by "killing" the blood ferment. This conclusion was derived from the following experiment. The blood coagulating ferment is not soluble in distilled water. It is freely soluble in eight per cēnt salt solution. The active principle of leech is soluble in water. Haycraft dried the "washed, finely divided clot from bullock's blood" and divided it into two portions by weight. One portion was placed in a watery solution of leech extract, the other in a similar amount of distilled water. After twelve hours the clots were dried by pressing them between blotting paper and then washed thoroughly with distilled water. This treatment would remove all uncombined leech extract. They were now separately extracted with eight per cent salt solution, in which the blood coagulating ferment is soluble. The action

of the extracts was tried on hydrocele fluid (fibrinogen), which is readily coagulated by the fibrin ferment. The blood clot not treated with leech extract coagulated the hydrocele fluid. No coagulation occurred with the extract of clot treated with leech extract.

The anti-coagulating substance has no effect upon the formed elements of the blood. Rouleaux form normally and phagocytosis in the white cells is undisturbed. The formation of rouleaux is not necessarily a coagulation phenomenon.

Leech extract when injected into dogs and rabbits causes the blood to remain fluid for hours. Blood removed from three to thirty minutes after the injection remained fluid over night. That removed after thirty-eight minutes coagulated in twenty-five minutes, while that removed after sixty-three minutes coagulated in five minutes.

Leech extract and peptone solution act differently when injected into animals. The leech extract prevents coagulation of both dog's and rabbit's blood and produces no lowering in blood pressure. Peptone solutions do not prevent coagulation of rabbit's blood, and they lower blood pressure. The leech extract is excreted in the urine. Leech extract does not prevent coagulation of milk by rennet or the coagulation of myosin. If anything it hastens the latter.

When leech extract is added to blood the corpuscles settle to the bottom of the vessel leaving, in the case of a salt extract, a clear plasma, in the case of the watery extract, a plasma colored with hemoglobin which is partially dissolved.

Some time after Haycraft's work a number of observers attempted to produce a purer leech extract and isolate the active principle. Bock, Kobert, Krueger, Dickenson, Erich Schultze, and Blobel all failed to make any substantial advance on Haycraft's method. It was left for Franz and Sznbenski in 1902 to isolate the active principle, confirm the belief of Haycraft that the anti-coagulating substance was a glandular secretion, and demonstrate the little sacs between the muscle bundles of the intestinal canal recognized as early

as 1833 by Brandt. Franz and Sznbenski, however, determined more clearly the identity of these bodies. They describe them as sometimes occurring in the form of pyriform bodies giving the appearance of glands, with ducts situated at the apices, from which the active principle of leech extract is poured out into the mouth by pressure of the muscle bundles. The glands are especially numerous around the lips of the leech and are always found anterior to the so-called "constriction." Like Haycraft, Franz and Sznbenski failed in staining these glands.

Franz has prepared a substance called Hirudin which has a constant action and which is regarded as the pure active principle. Hirudin consists of minute glistening plates and scales, grayish to brownish red in color, readily soluble in water, but insoluble in alcohol or ether. It is completely dissolved in a physiological salt solution, forming an opaque liquid.

Since its introduction Hirudin has been used quite extensively in experimental medicine and physiology. One of the advantages in the use of leech extract over peptone solutions as advanced by Haycraft is that its injection is not attended by lowering of the blood pressure. Some conflicting statements have arisen because of the transitory lowering of the blood pressure after Hirudin injection. Bodong in 1905 took up the question. He found practically no changes in the pressure after Hirudin injection (two experiments). "No elevating effect and no change in pulse frequency." Von Hertzen and Ohmar recognizing in Bodong's work the lack of detailed account of the blood pressure immediately after Hirudin injections cleared up this point in sixteen experiments (with the exception of two, the animals were all curarized). The Hirudin was injected rapidly, the time varying between 8.5 seconds and one hundred and six seconds. A solution of one milligram of Hirudin to 1.5 cubic centimeters of .9 per cent salt solution was used. They figured the blood content of the animal at five per cent of its body weight and accordingly injected seven milligrams per kilogram body weight through a

burette connected in the vein. There was always an immediate fall of pressure. In from thirty to eighty seconds the pressure was the lowest and in a very short time, two to three minutes, had reached its height. The pulse decreased more or less with the fall in pressure, to increase again with its rise. They concluded with Bodong that Hirudin has no more disturbing effect upon the heart and blood vessels than the injection of a similar amount of salt solution, and for this reason Hirudin is particularly useful in experiments on the blood and circulation.

Barcroft and Mines, 1908, attempted to determine the effect of Hirudin on the gases in the blood. They maintain that the intravenous injection of Hirudin causes the oxygen of the arterial blood to rise to a greater or less extent while the carbonic acid falls, the latter being more pronounced. In the process of this investigation they observed augmented respiration which seemed to follow a fall in blood pressure incident to the Hirudin injection (.11 gram of Hirudin in five cubic centimeters Ringer's solution). In an exaggerated instance the pressure fell forty millimeters of mercury and efforts to restore the animal failed. This fall in pressure they state is only an exaggerated degree of what normally happens after Hirudin injection. The fall in pressure always preceded the quickening of respiration.

Hirudin immunity.—Immunity to the action of leech extract was first attempted by Ledux in 1894. The same year Contejean obtained only slight indications of immunity. Wendelstadt in 1901, arguing that leech extract belonged to a class of plant and animal poisons from which immunity can be obtained, succeeded not only in inducing immunity of the first but of the second and third order. He used fresh extract prepared by macerating the heads of fifteen leeches in twenty cubic centimeters of .85 per cent salt solution. The extract was injected subcutaneously or intraperitoneally in ascending doses of from one to five cubic centimeters. For fear that high temperatures might alter the extract it was not sterilized, consequently he lost many animals. In

his last experiments the leeches were first washed with four per cent formalin solution before cutting off the heads. A great many of Wendelstadt's animals, however, did not die from sepsis. This seemed to indicate some poisonous effect from the extract. The blood of these animals was usually thin, dark, and coagulated much more slowly than normally. Wendelstadt concludes: Rabbits treated with leech extract resist the power of leech extract to prevent coagulation of their blood. This decrease in the activity of leech extract upon the treated rabbit's blood is due to the formation of an antibody in the animal body. This is proved by the manner of formation and by the possibility of demonstrating an anti-antibody. The antibodies are formed in greater quantities in the pancreas, liver, and kidney in the order named.

The following investigation was begun in Professor Krehl's laboratory, Heidelberg, in the summer of 1908 under the direction of Professor Paul Morawitz now of Freiburg, and has been concluded in the medical clinic of the University of Michigan hospital. The work differs from that of Wendelstadt in that Hirudin (Sachsse and Co.) instead of freshly prepared leech extract was used, and that, for the greater part, the injections were made intravenously, and the coagulation experiments done with freshly prepared fibrinogen solution, or hydrocele fluid. At first the Hirudin was sterilized by prolonged low temperature. This was soon found to be unnecessary; only one animal died and this one showed no evidences of sepsis but did show the same characteristics as those reported by Wendelstadt. (Rabbit No. 4 died December 8 after seventeen injections. It had grown very thin and seemed to stand the injections poorly, toward the end, often lying very still after the injection, in a state of semi-collapse with rapid respiration. The maximum dose of .014 gram had been reached. Autopsy showed no signs of sepsis.) Hydrocele fluid is by all means the most satisfactory solution of fibrinogen to work with. That made from ox blood requires much time and patience. I

succeeded in demonstrating immunity by this method as well as by the direct blood coagulation method.

EXPERIMENT No. Ia.

Rabbit No. 1. Treated thirteen days.

Fibrinogen solution.

5 cc.	+ 0.0 cc.	Normal rabbit serum.				No coagulation in 24 hours.
5 "	+ 1.0 "	"	"	"	"	Coagulation in 4 hours.
5 "	+ 0.5 "	"	"	"	+ Hirudin solution.	No coagulation in 14 hours.
5 "	+ 1.0 "	Treated	"	"		Coagulation in 18 hours.
5 "	+ 1.0 "	"	"	"	+ " "	" " 18 "
5 "	+ 0.5 "	"	"	"	+ " "	" " 18 "

EXPERIMENT No. Ib.

Blood coagulation experiment.

Minutes	2	4	6	8	9	10	12	13	14	16	18	20	22	24	25	26	28	30	34	38	40	45
Normal blood . .	O	−	−	+	+ +																	
Normal blood and Hirudin	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	+	solid.
Immune blood . .	O	O	O	O	O	O	O	−	−	+	+	+	+	+	+ +							
Immune blood and Hirudin	O	O	O	O	O	O	O	?	−	−	+	+ +	+	+ +	+ +							

This rabbit (No. 1) received twelve .01 gram Hirudin injections, making a total of .12 gram. Remained in good condition.

EXPERIMENT No. IIa.

Rabbit No. 2. Treated for twenty-three days.

Fibrinogen solution.

5 cc.	+ 0.0 cc.	Normal rabbit serum.				No coagulation, 24 hours.
5 "	+ 0.75 "	"	"	"	"	Coagulation, 2 hours.
5 "	+ 0.75 "	"	"	serum + 0.1 cc.	Hirudin solution.	No coagulation, 24 hours.
5 "	+ 0.75 "	"	"	" + .04 "	" "	" " 24 "
5 "	+ 0.75 "	Treated rabbit serum				Coagulation, 2 hours.
5 "	+ 0.75 "	"	"	serum + 0.4 cc.	" "	" 6 "

EXPERIMENT IIb.

Blood coagulation experiment.

Minutes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Normal blood . .	O	O	—	+	+	+ +																	
Normal blood and Hirudin	O	O	O	O	O	O	O	O	O	O	O	O	O	—	—	—	—	—	+	+ +			
Immune blood . .	O	O	O	—	+	+ +																	
Immune blood and Hirudin . .	O	O	O	O	O	O	O	—	+	+	+	+ +											

This rabbit (No 2) received twenty-two injections of .01 gram Hirudin each, making a total of .22 gram.

These two experiments prove conclusively that immunity to Hirudin can easily be induced in rabbits; and confirms the work of Wendelstadt.

EXPERIMENT No. III.

Rabbit No. 3 was treated from Nov. 17, 1909, to Jan. 10, 1910, fifty-four days, with intravenous, and a few subcutaneous, injections of Hirudin, beginning with .01 gram and gradually increasing to the maximum dose of .022 gram, which was reached December 12 and maintained until the last injection. This animal received in all thirty-five injections, gained in weight, and was in every respect perfectly well.

On inserting the canula into the carotid as soon as the artery was opened a clot quickly formed for one-sixteenth of an inch back into the vessel and no blood would flow. The other carotid was similarly treated with the same result. The vessels were then severed and I endeavored to collect the blood as it flowed from the vessel. Only a few drops could be collected because of the rapid clotting back into the severed vessels. The large vessels of the abdomen were then opened. Only in this way was I able to collect enough blood for the experiments which follow. Twenty-five cubic centimeters in all were secured. The quick clotting occurred in the severed veins as well as in the arteries.

EXPERIMENT IIIa. (Control.)

Tube No.	Normal Serum.	Hydrocele Fluid.	Coagulation Time in Incubator.			
			30 M.	40 M.	50 M.	60 M.
1	0 drops.	2 cc.	O	O	O	O
2	3 "	2 "	—	+	+	+
3	4 "	2 "	+	+ +	+ +	+ +
4	5 "	2 "	+ +	+ + +	+ + +	+ + +
5	0 "	2 "	O	O	O	O

EXPERIMENT IIIb. (Immune blood.)

Rabbit No. 3. Blood for experiment taken January 13, three days after last injection of Hirudin.

Tube No.	Immune Serum.	Hydrocele Fluid.	Coagulation Time.			
			30 M.	40 M.	50 M.	60 M.
1	1 drop.	2 cc.	O	O	O	O
2	2 drops.	2 "	O	O	—	
3	3 "	2 "	—	+	+	++
4	4 "	2 "	—	+	++	++
5	5 "	2 "	+	++	+++	+++
6	10 "	2 "	++	+++	++++	++++

EXPERIMENT IIIc. (Immune blood.)

Tube No.	Immune Serum.	Hirudin Solution Diluted ¾.	Hydrocele Fluid.	Time in Incubator.															
				M. 30	M. 40	M. 50	M. 60	H.M. 1-10	H.M. 1-20	H.M. 1-40	H. 2	H. 2½	H. 3	H. 3½	H. 7	H. 8	H. 19½	H. 21½	H. 22
1 . . .	0.5 cc.	1 drop.	2 cc.	O	O	O	O	O	O	O	O	O	O	O	+	+	+	+	++
2 . . .	0.5 + "	2 drops.	2 "	O	O	O	O	O	O	O	O	O	O	O	−	−	−	−	+
3 . . .	0.5 "	3 "	2 "	O	O	O	O	O	O	O	O	O	O	+	++	++	− ++	− ++	+++
4 . . .	0.5 + "	4 "	2 "	O	O	O	O	O	O	O	O	O	O	O	O	O	−	−	+
5 . . .	0.5 "	5 "	2 "	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
6 . . .	0.5 "	6 "	2 "	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

EXPERIMENT IIId. (Control.)

Tube No.	Normal Serum.	Hirudin Solution Diluted ½.	Hydrocele Fluid.	Coagulation Time.											
				1	2	3	4	5	6	7	8	9	10	12	18	24
1	0.5 cc.	1 drop.	2 cc.	O	O	O	O	O	O	O	O	O	O	O	O	O
2 . . .	0.5 "	1 "	2 "	O	O	O	O	O	O	O	O	O	O	O	O	O
3	0.5 "	2 drops.	2 "	O	O	O	O	O	O	O	O	O	O	O	O	O
4	0.5 "	3 "	2 "	O	O	O	O	O	O	O	O	O	O	O	O	O

The blood of this animal (Exp. III.) coagulated almost immediately and resisted the action of a strong solution of Hirudin: five cubic centimeters blood plus three drops of Hirudin solution (one-tenth gram dissolved in ten cubic centimeters of salt solution) coagulated to the point of complete setting in five minutes. Normal blood similarly treated remained fluid for twenty-four hours.

In this rabbit we have induced a pronounced immunity to the action of Hirudin. The experiment was in all respects the most satisfactory of the series owing to the long period of immunization and to my being able to substitute hydrocele fluid for the fibrinogen solution. (I am indebted to Dr. Kolig of the surgical staff (Prof. de Nancrede, Chief) for supplying my needs in this respect.)

A peculiar phenomenon occurred in all of the experiments where fibrinogen solution was used, Experiment IV., which did not occur in the hydrocele fluid series, and which I am at a loss to explain. When coagulation takes place it is almost, if not always, more marked in the tube containing the smallest proportion of serum. This suggested the possibility of fibrinolysis being very early induced with rabbit serum in those tubes containing an amount above .1 cubic centimeter per five cubic centimeters of the fibrinogen solution. Dr. Morawitz suggested trying to coagulate the uncoagulated tubes with activated serum. If coagulation should occur it would point to or prove that fibrinolysis had occurred. Activated rabbit's serum produced no change in these tubes. It was found a number of times that when no

coagulation occurred in the fibrinogen and serum tubes in several hours frequently they were all coagulated inside of twenty-four hours. This would seem to contraindicate fibrinolysis. The phenomenon may suggest a peculiarity of ox fibrinogen to the action of rabbit serum. With the hydrocele fluid just the opposite condition occurred; as one would expect, coagulation took place in those tubes containing the greater portion of serum.

EXPERIMENT IV.

July 31, 1908.

Tube.	Normal Serum.	Fibrinogen Solution.	Coagulation Time in Hours.						
			1	1½	2	3	4	24	
1	0.00	5 cc.	O	O	O	O	O	+	Clear.
2	0.75	5 "	O	O	O	O	O	+	"
3	0.50	5 "	O	O	O	O	−+	+	"
4	0.25	5 "	−	−	−	−+	−+	+	Cloudy.
5	0.10	5 "	−+	−++	−++	+	++	++	"+

July 31, 1908.

Tube.	Immune Serum.	Fibrinogen Solution.	Coagulation Time in Hours.						
			1	1½	2	3	4	24	
1.	0.00	5 cc.	O	O	O	O	O	O	
2.	0.75	5 "	−+	−++	−++	−++	−++	+	Clear.
3.	0.50	5 "	−+	−++	−++	−++	−++	+	"
4.	0.25	5 "	−+	−+++	−+++	−+++	−+++	+	Cloudy.
5.	0.10	5 "	−++	+	+	+	+	+	"

The comparisons here are accurately made. The conditions were the same in each set. The physical changes are represented by — meaning beginning coagulation, a distinct viscosity. + first sign of solidity. −+++ being less than +.

As demonstrated by Haycraft, the formed elements in leech extract blood remain practically intact and ameboid movement of the white cells is maintained for a considerable time. Because of this fact one might reason that at least the cytases are not set free early. If, however, these formed

elements are broken up, as for instance by the addition of water, the zymogen goes over into the plasma and coagulation takes place (Pickelharing). On the other hand, Hirudin plasma, that separated from the formed elements, does not coagulate either on the addition of water or dilute acetic acid. This is strong argument in support of the belief that Hirudin and leech extract act by preserving the formed elements of the blood from destruction. In this respect Hirudin plasma differs from peptone plasma. Sooner or later in Hirudinized blood the cells undergo degeneration. If blood is treated with weak Hirudin solution, coagulation may be delayed for a few hours only. If larger amounts of Hirudin are added, the blood may be caused to remain fluid indefinitely. The hemoglobin of such blood becomes reduced, and according to Bosc and Delezenne the blood develops a high resistance to the influence of putrefactive bacteria. This resistance must come either from the cytases set free, or from an antibacterial action of the Hirudin itself. The latter point remains unproved. Bosc and Delezenne believe the germicidal action is due to alexines set free from the leucocytes.

"Hirudin plasma is coagulated by the addition of thrombokinase, tissue juice, depending upon the amount of Hirudin present. If only a little Hirudin is present the thrombokinase may form so much ferment with the thrombogen that the antithrombin is entirely neutralized. If on the other hand there is much Hirudin in the plasma, even on the addition of very large amounts of tissue juice, there is not sufficient thrombin formed to influence coagulation, because the thrombogen content is not unlimited in amount. It is apparent from this that a neutralization does not exist between the thrombokinase and the Hirudin. The Hirudin neutralizes, as do also the antibodies of the peptone plasma, only the thrombin, perhaps only the thrombogen" (Morawitz).

If normal rabbits receive an injection of Hirudin, blood withdrawn in five or ten minutes will remain fluid for many hours, while that removed in an hour's time coagulates more

quickly. Hirudin immune rabbit's blood removed a few minutes after injection, while it does not always coagulate with the same rapidity as normal blood, depending upon the degree of immunity gained, will coagulate in minutes where the normal injected rabbit's blood requires hours. The immunity induced by leech extract as has been shown by Wendelstadt lasts for many days after the injections have ceased but is ultimately lost.

The plasma of Hirudin immune blood does not coagulate solutions of fibrinogen. If the cells centrifugalized out of the citrated immune plasma are not separated but allowed to remain in the tube for a certain time (over night) coagulation occurs, while no coagulation occurs in the plasma separated from the cells. Normal serum added to immune plasma produces coagulation of hydrocele fluid. These experiments support the idea that the immune body is not in the plasma, but seems to be in the fibrin ferment itself or the pro-ferment. In Hirudin immune blood anti antithrombin bodies are formed in such quantities that correspondingly large quantities of Hirudin are necessary for their neutralization. In other words, the cytase of leucocytes has been enhanced in amount, consequently more active leech extract principle is required to neutralize it than was necessary for normal blood.

Immune and normal blood are inactivated by heating to 57° C. and by treating with dilute hydrochloric acid. These sera cannot be reactivated (Wendelstadt).

It is believed that all blood-sucking animals are supplied with an anti blood-coagulating substance. In addition to the leech, it has been demonstrated in certain snakes, the earth worm, and in the ankylostoma caninum. I failed in an attempt to extract an anticoagulating substance from the macerated bodies of several hundred hookworms. These were brought from the Island of Guam and had been preserved in alcohol for many months.

[The writer wishes to thank Professor Krehl for placing his laboratory at his disposal, and Professor Morawitz for helpful suggestions and numerous courtesies extended throughout the entire work.]

CONCLUSIONS.

1. Rabbits treated by intravenous and subcutaneous injections of Hirudin in gradually increasing doses develop a marked resistance to the power of Hirudin to prevent the coagulation of their blood in vitro.

2. This increased resistance to the anticoagulating property of Hirudin is due to the development of anti antithrombin bodies in the animal body which are in all probability elaborated by the leucocytes of the blood in the form of thrombin or prothrombin.

BIBLIOGRAPHY.

Barcroft and Mines. The effect of Hirudin on the gases in the arterial blood. Jr. Physiol., xxxvi, 275, 1907, 1908.

Bock. Untersuchung uber die Wirkung verschiedener Gifte. Arch. f. exper. Path. u. Pharm., xli, 160.

Blobel. Versuche uber transfusion mit dem von Blutgeln gasogenen Blut. Inag. Dissert., Greifswald, 1892. Quoted by Franz.

Bodong, von Andreas. Uber Hiruden. Arch. f. exper. Path. u. Pharm., lii, 254, 1905.

Bosc u. Delezenne. Faulinsunfahigkeit des durch Blutegelextrakt gemachten Blutes. Comp. r. Soc. Biol., cxxiii, 465. Quoted by Morawitz.

Brandt. Quoted by Franz. Arch. f. Exp. Path. u. Pharm., xl, 1902, 343.

Camus u. Lequeux. Wirkung des Regenwurmextraktes auf die Blutgerinnung. Comp. r. Soc. Biol., lii, 1900, 690. Cited by Morawitz.

Contejean. Sur Differents Procedes Proposes Pour Rendre le Sang Incoagulable. Comp. r. Soc. Biol., Dixieme Serie 1, 1894, 833, 834.

Dickenson. Journal of Physiology. M. Foster, xi, 566.

Fano. Das Verhalten des Peptons u. Tryptons gegen Blut u. Lymphe. Arch. f. Anat. u. Physiol., 1881, 276.

Franz von Fredrich. Ueber den die Blutgerinnung aufhebenden Bestandteil des medizineschen Blutegels. Arch. für. Path. u. Pharmakol., xl, 1902, 342.

Fubini. Ueber das von Blutegeln gesogene Blut. Moleschotts Unters. z. Naturlehre., xiv, 520. Cit. Morawitz.

Haycraft, John B. On the action of a secretion obtained from the medicinal leech on the coagulation of the blood. Proc. Royal Soc., London, xxxvi, 1884, 478.

von Hertzen und Ohman. Ueber die Einwirkung des Hirudins auf den Kreislauf. Skandinavisches Archiv. fur Physiologie, xx, 1, 1908.

Jacobi. Uber Hirudin. Deutsche Med. Wochr., 1904, xxx, 1786.

Kobert. Arbeiten des Pharmocologische Instituts zu Dorpat, xiii, 1896, 64. Cit. Franz.

Ledoux. Vergleichende Untersuchungen über die hauptsächlichsten

Substanzen welche die Gerinnung des Blutes aufheben. Arch. de Biol., 1895, xiv, 63–103.

Loeb und Smith. Uber eine die Blutgerinnung hemmende Substanz im Anchylostomum caninum. Centrl. f. Bakteriol., xxxvii, 1.

Millanby, John. The coagulation of blood. The action of snake venoms, peptone, and leech extract. Jour. Phys., xxxviii, 1908, 1909.

Morawitz, Paul. Beitrage zur Kenntnis der Blutgerinnung. Archive. f. Klin. Med., lxxix, 432.

Morawitz, Paul. Die Chemie der Blutgerinnung. Ergebnesse der Physiologie, Asher u. Spiro, iv, 306, 1905. (This article gives an exhaustive study of the subject with bibliography.)

Schmidt. Mühlheim, Zur Kenntnis des Peptons und seiner physiolog. Bedutung. Arch. f. Anat. u. Physiol., 1880, 33. Cit. Haycraft and Morawitz.

Wendelstadt, Dr. H. Ueber einer Antikörper gegen Blutegelextract. Archives Internat. de Pharmacodynamie, ix, 407, 1901.

Schultze Erich. Uber die Verwendung von Blutegelextrakt bei der Transfusion des Butes. Inaug. Dissertat., Greifswald, 1892. Cit. Franz.

Sahli. Uber die Verwendung von Blutegelextrakts auf die Thrombenbildung. Zentralbl. f. innere Med., xv, 497.