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THE SEPARATION, NATURE AND METHOD OF ACTION OF A CONSTITUENT OF TISSUE EXTRACTS WHICH INCREASES THE COAGUABILITY OF BLOOD ON INTERNAL ADMINISTRATION.

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THE SEPARATION, NATURE AND METHOD OF ACTION OF A CONSTITUENT OF TISSUE EXTRACTS WHICH INCREASES THE COAGULABILITY OF BLOOD ON INTERNAL ADMINISTRATION.

The great mass of literature on the subject of blood clotting is very confusing. The fact that damaged tissue normally plays a role in initiating blood clotting has been long known, but the mechanisms by which it carried out its part have only recently come to light. DeBlainville (1834) applied this knowledge of the action of damaged tissue to his studies and was able to induce intravascular clotting by the intravenous injection of brain extracts. It was not until the work of Wooldridge (1885) that we find any definite statement of the nature of the active constituent of these tissue materials. This active material he called "Tissue Fibrinogen" and believed it to be a protein-lipoid complex. The lipoid he called a lecithin-like material, but he found egg lecithin inactive. At an earlier date (1861) Alexander Schmidt had studied a protein material, obtained from the clot, which caused the clotting of "salted" blood. He believed it to be an enzyme and called it "Fibrin Ferment" or Thrombin. This does not preexist in the plasma but is formed from a precursor called prothrombin by Schmidt. Morawitz (1904) assumed that the accelerative substances from the tissues were activating this precursor of thrombin and

for this reason called them "thrombokinases". Bordet (1920) called these active materials "cytozyme" to indicate their source. The material obtained from the serum (the precursor of thrombin) he called serozyme. Bordet and his coworkers, Gengou and Delange, established the mechanism of thrombin formation by a combination of cytozyme and serozyme in the presence of calcium salts. Cytozyme Bordet and Delange (1913) showed to be a thermostable substance of a lipoid nature which they called lecithin. Bordet also noted, however, that there is another active material from tissue, a thermolabile substance, "Unquestionably of a protein nature". Nolf (1906) substantiated the lipoid nature of the materials called by him "Thromboplastic Substances". Finally Howell (1912) definitely established cephalin as the lipoid substance acting as a thrombokinase.

It is well to note here that cephalin does not cause clotting on intravascular injection as did the "Tissue Fibrinogen" of Wooldridge. It is then apparent that cephalin is not the whole story of the part played by tissues in blood clotting. Bordet had indicated an action other than that of cephalin, but it was not until the work of Mills that we find any clear explanation of this mechanism. Following the work of Wherry and Irwin

(1918) on death caused by intravenous injection of tissue materials, Mills (1919) revived and expanded many of the views of Wooldridge. He separated and purified a definite substance of a protein-lipoid nature as Wooldridge had suggested and retained the name for it of tissue fibrinogen. This substance he found to be a most potent accelerant of coagulation in the test tube. It also caused rapid death by thrombosis when injected intravenously. Unlike thrombin it required calcium salts in order to clot oxalated plasma. The active material in tissues and included in thrombokinase have now been established by Mills to be two, tissue fibrinogen and free cephalin.

Recently some most interesting observations have been made by Wadsworth, Maltaner and Maltaner (1927) on the nature of the lipoidal tissue thrombokinase. They separated from tissue an active thrombokinase of lipoid nature free from nitrogen, evidently neither tissue fibrinogen nor cephalin. They showed it to have the common structure of lecithin and cephalin with the nitrogen base removed. They were able partially to decompose pure lecithin with calcium chloride in a slightly alkaline medium and obtained a substance which hastened the clotting of shed blood

while the original pure lecithin was inactive. They did not test its activity in vivo.

The mass of experimental data and numerous theories founded thereon has been well outlined by Pickering (1928). It is sufficient to note here that it was much clarified by Mills and Mathews (1924) who showed that there were two mechanisms of clotting, the thrombin mechanism as outlined by Bordet and others, and the tissue fibrinogen mechanism outlined by Mills. By the latter mechanism tissue fibrinogen with calcium and blood fibrinogen initiates the formation of fibrin.

Mills (1923) obtained most surprising results with his tissue fibrinogen preparation when administered in vivo. Not only was it a powerful coagulant as Wooldridge had already observed, both in the test tube and when injected into the circulating blood, but also on intraperitoneal or subcutaneous injection in animals or on oral or subcutaneous administration in man it accelerated the coagulability of the blood. On the basis of the work of Wooldridge and of Mills the Wm. S. Merrell Company put this preparation on the market under the name of "Fibrogen". As first manufactured "Fibrogen" on numerous tests both animal and clinical was found to shorten the coagu-

lation time of the blood when internally administered. But in the course of manufacture various changes were made in the process in order to shorten the time required for its manufacture and give what was believed to be an improved product. As it was then thought that the action in vivo was due to the same substance as that acting in vitro, i.e. the tissue fibrinogen, the effect of these changes in manufacture was tested only in vitro owing to the greater ease and accuracy of this method. Some of these preparations, when tested in vivo by Dr. Gerwe of this laboratory, were found to have lost their power of augmenting coagulability although having an improved power of shortening the clotting time of shed blood. This observation very clearly indicated that the substance shortening clotting time on intraperitoneal or subcutaneous injection or oral administration was not the tissue fibrinogen itself.

Mills (1923) had first considered that the in vivo activity was due to the direct absorption of tissue fibrinogen as such, for he found in the urine of dogs after administration of tissue fibrinogen a substance behaving like tissue fibrinogen in hastening blood clotting. The loss of the power to augment the coagulability of blood

with full retention of the power to accelerate clotting in the test tube indicated that the in vivo action could not be due to the tissue fibrinogen as such, but must be due to some other substance present in Mills' earlier preparations and in the substance as first manufactured.

That there were two substances in the preparations, one of which (tissue fibrinogen) acted directly either in vitro or on intravenous injection, and the other on intraperitoneal injection or oral administration, was indicated also by clinical evidence. Cases were reported of persistent bleeding due to non-coagulability of the blood in which prompt coagulation within one hour occured on the injection subcutaneously or taking orally of a few cubic centimeters of the commercial preparation; and this preparation, although so active in the body, was found either inactive in the test tube or even strongly anti-coagulant on shed blood. This observation indicated that the substance in these preparations acting internally might be a decomposition product of tissue fibrinogen. It was in fact observed many years ago by Conradi that tissue fibrinogen after undergoing autolysis or bacteriolysis was changed into an active anti-coagulant so far as its in vitro action was concerned.

The proof that there were indeed two active substances in the original preparations, one of which had been greatly weakened or removed by improved methods of manufacture, was obtained by some observations of Billing (1930). Knowing that the venom of Crotalus adamanteus had a powerful anti-coagulant action in the test tube and that the blood of animals dead of snake bite was reported often to be incoagulable, Billing thought that perhaps "Fibrogen" might act as an antidote to some snake venoms. He accordingly injected fibrogen intraperitoneally into rats and followed it after an interval by a minimum lethal dose of crotalus venom. He expected to find that it would take a greater dose of crotalus venom to kill the rat; but he actually found to his great astonishment, that it only took from one-half to one-third the minimum lethal dose of venom to kill a rat if the fibrogen were injected about one quarter of an hour before the venom. This result was at the time quite inexplicable. Some two or three years later, when it was found that the "Fibrogen" manufactured by the new process was of reduced activity when given by mouth or intraperitoneally, these experiments were repeated by Billing and Mathews (1931). They found that the new fibrogen, if it were inactive intraperitoneally,

had no effect at all on the toxicity of the venom, although it was more active as a coagulant in the test tube and intravenously than the earlier products. A series of tests were then made of various preparations of tissue fibrinogen manufactured in various ways; and it was found that whenever the preparation shortened clotting time after intraperitoneal injection, it also increased the toxicity of crotalus venom.

These facts appeared at first very difficult to understand, owing to the belief that venom was an anticoagulant both in and outside the body. Autopsy of the rats immediately after death showed, however, that in many of those cases where fibrogen had been injected as well as the venom, and also occasionally when only venom had been injected, the blood was found clotted in the heart and great veins. It has since been found that both venom and thrombin change fibrinogen in a similar manner, but the venom causes also a destruction of the fibrinogen. In any case the observations showed that the power of the fibrogen to increase the coagulability of the blood on intraperitoneal administration as well as on a subsequent venom injection was not due to the tissue fibrinogen but to some other substance. These facts were in complete accord with the clinical findings already reported.

The present work was started at the suggestion of Professor Mathews, with the purpose of ascertaining the nature and mode of action of this substance which augments the coagulability of blood on internal administration. The early work was done through the courtesy of Dr. Mills in the laboratory of Experimental Medicine at The Cincinnati General Hospital. The later part of the work was done in the Biochemistry Department under the guidance of Professor Mathews.

I. SUBSTANCES AFFECTING THE COAGULABILITY OF THE BLOOD WHEN TAKEN INTERNALLY.

HISTORICAL

That the coagulability of the blood is not a fixed thing but may be altered in either direction, namely, towards a shortened clotting time, or a more prolonged period of inductance, is a well established fact. Clinically such variations of coagulability are frequently met with, usually to the great distress of both physician and patient. Following an operation the blood may become, for reasons quite unknown, hyper-coagulable and the appearance of intravascular clots may cause serious trouble in the circulation, or even sudden death. A clot in the coronary artery, for example, is one of the important causes of death from heart disease. Milk-leg is not an infrequent complication of child birth and of appendicitis.

And, on the other hand, failure of the blood to clot when it should is almost equally distressing. Hemophilia or delayed clotting is by no means infrequent, and any operation on a 'bleeder' is a terrifying experience for the surgeon. That it is possible by physiological means to temporarily shorten the clotting period in such cases has been shown by Vines (1920) by producing an anaphylactic shock. And recently it has been found by (1931) that the injection of the female hormone "Theelin" greatly

reduces clotting time in some cases, at least of hemophilia.

These facts leave no doubt in the mind that a physiological mechanism exists in the body for the control of the clotting power of the blood. While the details of that mechanism are not yet well known, it raises the hope that by the use of the proper medicines the mechanism may be brought into activity or reduced in its activity just as are the other physiological mechanisms.

As a matter of fact there are in the literature observations indicating that in addition to 'fibrogen' other drugs may also affect this mechanism. A number of plant extracts have been reported to have anti-hemorrhagic powers. The use of ergot in parturition has long been known; and while this presumably owes its power chiefly to its action on the uterine musculature or blood vessels, it may also have a direct action on the physiological mechanism which controls the coagulability of the blood. Hydrastis has been used for a similar action (Solus-Cohen & Githens, 1928). The common smart weed (polygonum hydropiper) has been used as a household remedy by the Russian peasants. Muszynski (1921) reported that a fluid extract of this plant acted as an excellent hemostatic in all cases of internal hemorrhage, but it is uncertain just how it acted. 'Ceanothyn', an extract of the plant Ceanothus

americanus, is claimed to be a potent hemostatic for internal hemorrhage when taken internally. Takahashi (1928) (1928) reported 'illicin', which is a constituent of the Japanese plant Illicum anisatum, to be an excellent hemostatic acting on intravenous or subcutaneous injections or oral administration.

The anesthetics are reported as having different effects on coagulability. Mendenhall (1915) reported chloroform and chloral hydrate as prolonging coagulation time, while ether definitely shortened it. This action he attributes to adrenalin. Adrenalin has been used a good deal as a styptic, that is, to cause the contraction of the blood vessels. This is not the only action of adrenalin, as will be seen in a later part of this paper. Of more immediate importance are the materials of animal origin, namely, tissue extracts.

Numerous preparations have appeared that were designed to act when injected or taken orally, as thrombokinases or activators of prothrombin. These materials were usually tissue extracts containing cephalin. They act as accelerators of clotting in the test tube, but as a rule show no demonstrable action on oral administration or intraperitoneal injection. Especially noteworthy is the fact that cephalin does not cause intravascular clotting even

when injected directly into the blood stream. Outstanding among the active principles of tissue preparations is tissue fibrinogen which is not a true thrombokinase in the sense that it activates prothrombin. It acts directly on the fibrinogen of the blood. Unlike cephalin it causes intravascular clotting when injected directly into the blood stream. This brief summary shows how meager is our information concerning substances which act on the mechanism which controls the coagulability of the blood.

METHOD OF TESTING

The first question which arose was as to the method of determining the clotting time of the blood. That finally adopted was the common test tube method, essentially that suggested by Lee and White (1913), with modifications. Rabbits were used as experimental animals. Each animal was fasted for at least 24 hours before determining the clotting time as it has been shown that ingestion of food shortens the clotting time of blood (Necheles and Mills (1928). Great care was taken that the animals did not become excited, as this also affects the coagulability of the blood. The animal to be tested was handled gently. It was found wise to be "on good terms" with him before attempting to use him for a test animal. Tests on new animals often showed relatively short clotting times. The

rabbit was placed under the left arm with the ventral side up. The hind quarters and legs were held firmly but gently between the left arm and the body of the operator. The ears and forelegs of the animal were held in the left hand. After considerable handling the animals lie in this position with little or no struggling and apparently contented. This method of holding allows the operator full control of the animal and complete knowledge of his movements. It was found to be far more preferable than having an assistant hold the animal or than using an animal board, which is out of the question, as it causes too much excitement. This position also gives a clear field to work. A swab was wet with alcohol and used to lay the hair away from the field of action, as well as to partially sterilize the field. The syringes used were standard 2 cubic centimeter 'Vim, Emerald'. The needles were 'Vim' stainless steel hypodermic needles 1-1/2 inches long and 22 guage. The syringe and needle were cleaned and dried with alcohol and ether, and freshly oiled with paraffin oil. The syringe was manipulated with the right hand, and a heart puncture made. It is essential that the puncture be made quickly and accurately. If the heart is not punctured on the first entrance the needle should be withdrawn and a fresh one substituted, as prodding around in search of the heart is not only bad for the animal but contami-

nates the needle with tissue juice. If too much trouble was encountered, it was found wise to give both the rabbit and the operator a rest. The puncture was made into the left ventricle when possible, thus obtaining arterial blood. If venous blood is drawn it is readily detected by its color. An extra cubic centimeter of blood, over the necessary 2 cubic centimeters, was drawn each time into the syringe so that a small part at the first and at the last of withdrawal may be discarded. This was done in order to avoid as much as possible the presence in the final sample of tissue juice which might be washed in at the time of puncture or withdrawal of the needle. The blood was placed at once in two tubes, one cubic centimeter in each tube. The tubes used were uniform pyrex tubes 1 cm. in diameter by 10 cm. in height. They had been carefully cleaned with water and acid dichromate, rinsed with distilled water, thoroughly dried and placed in the constant temperature bath at 40°C. before the blood was added to them. They remained in the bath throughout the experiment. Each sample was observed at the end of the first five minutes, and every one minute thereafter, by gently raising and tilting it slightly until it showed a sign of increased viscosity; then it was observed every one-half minute. The end point was taken as that

time when the tube could be inverted without loss of blood. The time from the withdrawal from the heart to this end point was taken as the clotting time.

In order to test materials to be studied, they were injected intraperitoneally into the rabbits after the normal clotting time had been determined by the above method. The injection was made immediately after the normal was determined. In each case one hour was allowed after injection in order that the material may take effect. Then the clotting time was again determined as nearly as possible in the same manner as the normal. The change in clotting time observed divided by the normal clotting time was recorded as effect.

Rabbits were used as experimental animals for all tests unless otherwise stated. Although Pickering states that these animals have a very variable clotting time, it was observed that after the technic of handling them and of testing the blood had been thoroughly mastered, quite consistent duplicates could usually be obtained on the same animal and at different times. (See Table I). But if the necessary precautions are not taken, a wide variability will be encountered. Even under the best circumstances occasionally a wide variation from the normal

TABLE 1.

Date	Animal	Clotting Time in Min. Normal	Clotting Time in Min. After	Effect % of Normal
2/12/30	211	6.0	7.0	00 ?
2/24/30	298	19.0	18.0	00
11/12/31	570	18.0	17.0	00
11/12/31	569	12.5	13.5	00
11/12/31	571	13.5	19.5	00
11/12/31	572	26.0	10.0	61
12/33/31	629	17.5	21.0	00
12/3/31	635	17.5	28.5	00
12/3/31	42	29.5	35.0	00
12/3/31	640	25.0	23.5	00
12/3/31	643	32.5	33.0	00
10/17/31	593	21.5	19.0	11
11/27/31	1	26.0	22.5	13
12/16/31	1	31.5	27.5	11
10/16/31	1576	22.5	20.5	00
10/16/31	577	23.0	24.5	00
12/20/31	106	32.5	29.5	00
12/20/31	107	47.0	31.5	30
11/12/31	573	22.0	22.5	00
11/12/31	586	20.0	15.0	25
11/12/31	574	25.5	20.5	18

(Table 1 continued on page 17a)

TABLE 1 (Continued)

Date	Animal	Clotting Time in Min.		Effect
		Normal	After	
12/3/31	626	30.5	30.5	00
12/3/31	45	24.0	25.0	00
Saline Injection.				
2/25/30	299	20.0	21.0	00
4/25/30	472	24.0	23.5	00
4/25/30	473	18.0	19.0	00
4/25/30	475	30.0	31.0	00
6/11/30	1001	18.5	23.0	00
Water Injection				
3/25/30	393	20.0	20.0	00
3/27/30	309	22.0	20.0	00

clotting time will be observed. The cause or causes of this will be discussed later; but the occurence of such variations necessitates a long series of observations in order that reliable conclusions can be drawn.

EXPERIMENTAL OBSERVATIONS

The first tests made were on 'Fibrogen', the commercial preparation of Mills' tissue fibrinogen. These tests were essentially for orientation and practice. They are of interest to observe, however. The very short normals at the first of the table evidently are due to poor technic, and such short clotting times very naturally make it difficult for any substance even if active to produce any further effect. However, it is clear that these fresh preparations immediately after manufacture were relatively inactive. (See Table II). It is interesting to note that some of these same fresh preparations were quite active when given orally, even though no action was observed on intraperitoneal injection. (See Table 2 B). (Prep. 22).

Some old preparations which had been in an ordinary ice box for over a year were also tested. Although the preparations were presumably sterile, some change had taken place on standing. There was no bad odor or other sign of putrifaction. The change, whatever its nature, was evidently autolytic. These materials were found to be definitely active both intraperitoneally and orally. (See Table 3).

From these observations as well as the clinical cases already cited it would appear that the active material for which we were searching was probably set free from the

TABLE 2.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Tissue Fibrinogen - Fresh A. Intraperitoneal Injection. Dose, 2c.c.					
10/29/29	1	11.0	11.5	00	21
10/29/29	2	8.5	9.5	00	21
12/12/29	148	10.0	8.5	15	22
12/12/29	161	8.0	4.0	50	22
12/12/29	150	9.5	10.0	00	23
12/12/29	151	6.0	6.0	00	23
12/12/29	156	13.0	3.5	70	24
12/12/29	153	9.0	5.0	35	24
10/19/31	599	25.0	25.0	00	25
10/19/31	600	25.0	24.5	00	25
11/7/31	644	25.5	18.5	27	26
11/7/31	648	27.5	28.0	00	26
11/7/31	650	24.5	20.0	14	26
B. Oral administration to rabbits. Dose 3c.c.					
10/19/31	1597	28.5	18.0	37	25
10/19/31	626	21.0	17.5	17	25
10/19/31	629	23.0	16.5	29	25
10/19/31	630	20.0	14.0	30	25

TABLE 3.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Tissue Fibrinogen - Old A. Intraperitoneal Injection. Dose, 2c.c.					
10/29/29	3	11.0	5.0	55	3A1
10/29/29	4	12.0	7.0	41	3A1
B. Oral Administration. Dose, 3c.c.					
10/17/31	1588	29.0	14.5	50	3B1
10/17/31	1595	22.5	17.0	25	3B1
10/17/31	1591	24.0	12.0	50	3B1

tissue fibrinogen or formed by a partial decomposition of this, or some other constituent of the fresh material.

An attempt was now made to produce this partial decomposition by weak acid treatment of fresh tissue material. Mills hadobtained activity in materials so treated. Fresh calf lung was obtained at the butcher's and ground in a meat chopper after freeing from the larger tubes. Suspensions were made of this ground tissue in N/10 HCl (20gm. tissue per 100 cc.). This mixture was allowed to stand for one hour at 40°C. Then it was neutralized by adding a quantity of NaOH equivalent to the acid used. This material was tested as to its activity by intraperitoneal injection of 2cc. of the supernatant, unfiltered liquid. This liquid had finely divided material in suspension. It gave consistent and strong reductions in clotting time. (See Table 4.). A portion of this material was boiled for ten minutes and filtered. Some coagulable protein and solid suspension were removed in this manner. This filtrate was also found very active in the two tests made. (See Table 4B). One of these tests, however, is so abnormal in the clotting time as to suggest a possible contamination of the blood by some tissue juice. When this material was evaporated to dryness on the steam bath and the residue suspended in water, very irregular results were obtained. A few tests (3) showed sharp reductions, but the major-

TABLE 4.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % Of Normal	Prep.
A. Lung Tissue - Treated with N/10 HCl at 40° and Neutralized.					
2/4/30	211	17.0	11.5	31	4A1
2/4/30	212	15.0	16.5	00	4A1
2/26/30	136	30.0	15.0	50	4A2
2/26/30	214	30.5	16.5	45	4A2
2/27/30	211	16.5	6.0	62	4A2
3/10/30	213	25.5	9.0	62	4A3
3/10/30	214	37.5	8.5	77	4A3
3/10/30	225	38.5	19.0	50	4A3
3/10/30	293	28.0	2010	30	4A3
B. (4A3) Boiled Ten minutes and Filtered					
3/10/30	136	10.0	2.0	80?	4B1
3/10/30	211	31.0	12.0	62	4B1

ity of tests (5) were negative. (See Table 4C). This same material was dialyzed for 12 hours against running water and tested. No activity was detected. (See Table 4D). These results indicated that there was in lung tissue a substance which was set free by treatment with N/10 HCl and which acted on the physiological mechanism which controls coagulability; that this substance was destroyed by evaporation to dryness on the steam bath. An attempt was also made to extract it by buffer solution.

EXTRACTION BY ACID BUFFER

Mills suggested the use of acid buffers in extracting tissues, as he had also obtained active materials by this method. An acid buffer was made from a mixture of M/15 Na_2HPO_4 and M/15 KH_2PO_4 which gave a pH of 5.6. A heavy suspension was made with ground and dried lung tissue in this acid buffer and allowed to stand for two hours. (20gm. tissue to 33cc. acid buffer). Then the quantity of M/15 Na_2HPO_4 which would be necessary to bring the acid buffer to a pH of 7.45 was added (169cc.) and the suspension filtered. The filtrates from several preparations made in this manner were tested by intraperitoneal injection and gave, as a rule, very sharp reductions in clotting time. (See Table 5). The only exception, #284, already had a short clotting time before injection.

TABLE 4 (Continued)

C. (4A3) Evaporated Dry and Suspended in H_2O.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
3/10/30	210	21.5	10.0	40	4C1
3/10/30	216	24.5	17.5	30	4C1
3/11/30	299	31.0	15.0	50	4C1
3/13/30	306	12.0	15.0	00	4C1
3/13/30	307	17.5	18.0	00	4C1
3/13/30	308	12.0	11.0	00	4C1
3/14/30	316	19.5	20.0	00	4C1
3/14/30	318	24.0	20.0	16	4C1
3/14/30	322	19.0	18.0	00	4C1
D. (4C1) Dialyzed for 12 Hrs. Against Running H_2O					
3/13/30	312	15.5	18.0	00	4D1
3/13/30	313	20.5	21.0	00	4D1
3/13/30	314	11.0	18.0	00	4D1
3/13/30	309	19.5	18.5	00	4D1
3/13/30	310	20.5	21.5	00	4D1

TABLE 5.

Lung Tissue Extracted by Phosphate Buffers. Dose, 2cc.					
Date	Rabbit	Clotting Time in Min.		Effect	Prep.
		Normal	After Inj.	% of Normal	"
2/19/30	278	18.0	7.5	60	51
2/19/30	284	12.0	11.5	00	51
2/19/30	285	18.5	5.5	70	51
2/19/30	277	20.0	2.5	87	51
2/26/30	216	30.0	11.5	61	52
2/26/30	225	15.5	10.0	35	52
3/4/30	277	19.5	4.0	80	53
3/4/30	284	13.5	10.5	23	53
3/4/30	285	16.5	9.5	46	53

DIGESTION BY STRONG ACID.

It was considered probable that the active principle was broken away from the more complex tissue material by acid treatment. The problem was then, "How could this principle be separated from the extraneous material?" Lloyds reagent was tried without success. The material had been found to be stable to boiling and to weak acid treatment. If it would stand strong acid digestion it could not be of a complex nature, i.e. proteins and lipids of the tissue, as these materials are broken down by such treatment. Acid digestion experiments were tried. Lung tissue was treated with 20% HCl (200gm. tissue per 600 cc. acid). It was refluxed on the hot plate at boiling temperature for six hours. The resultant mixture gave a negative biuret reaction. It was then neutralized with NaOH and the salt dialyzed away. From time to time samples were taken from the solution undergoing dialysis and tested. The sample taken after 16 hours dialysis was very active (see Table 6A). Subsequent samples were taken at 48 hours (see Table 6B), 84 hours (see Table 6C), 90 hours (see Table 6D), and 120 hours (see Table 6E). These samples gave variable results, but most of them when injected gave sharp reductions in clotting time. The inactivity of the 48 hour sample is unexplained. A portion of the 90 hour

TABLE 6

Lung Tissue Boiled 6 Hrs. in 20% HCl; Neutralized and Filtered. Dose, 2cc.

A. Dialyzed 16 Hours.

Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
3/14/30	323	18.5	5.5	70	6A1
3/14/30	324	17.0	11.5	32	6A1
3/14/30	325	18.0	12.0	30	6A1
3/15/30	206	34.0	21.0	39	6A1
3/15/30	289	19.0	7.0	63	6A1
3/15/30	290	14.5	9.5	33	6A1
3/15/30	291	39.0	13.0	66	6A1
3/15/30	319	25.0	5.5	78	6A1
3/15/30	320	16.0	6.5	60	6A1
3/15/30	321	15.5	5.5	63	6A1
3/28/30	322	23.5	6.5	73	6A1
3/28/30	323	19.0	9.5	50	6A1
3/29/30	321	21.5	12.5	42	6A1
B. Dialyzed 48 Hrs.					
3/19/30	302	26.0	20.5	21	6B1
3/19/30	301	25.5	29.0	00	6B1
3/19/30	283	25.0	30.0	00	6B1
3/19/30	278	14.5	13.0	10	6B1

TABLE 6 (Continued)

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
C. Dialyzed 84 Hours. Dose, 2cc.					
3/20/30	136	21.5	7.5	69	6C1
3/20/30	214	16.5	10.5	36	6C1
3/20/30	216	16.5	13.0	21	6C1
3/20/30	225	34.0	20.0	41	6C1
D. Dialyzed 90 Hours.					
3/24/30	294	16.0	12.0	26	6D1
3/24/30	298	18.0	15.0	16	6D1
3/24/30	313	20.0	11.0	45	6D1
E. Dialyzed 120 Hours.					
3/28/30	317	22.0	15.5	32	6E1
3/28/30	318	17.5	18.0	00	6E1
3/28/30	319	36.0	11.0	69	6E1
3/28/30	320	27.0	8.5	68	6E1

dialyzate was subjected to distillation until about half of the liquid had distilled over. The remaining liquid was tested, and its activity found comparable with the original preparation (see Table 7A). There was, however no increase due to concentration. The distillate was examined. It gave some very sharp reductions and as many negative results. These results were obtained early in the investigation before certain sources of error connected with handling the rabbits had been recognized. The presence of the active material in any amount was certainly doubtful (see Table 7B).

Another batch of hashed lung tissue was boiled with 20% HCl (6cc. per gm. of tissue). This time it was boiled for 48 hours and dialyzed for 48 hours without neutralizing. The resultant dialyzate gave a pH of 4.3 when determined by the potentiometer using a quinhydrone electrode. When tested by intraperitoneal injection in rabbits it was found very active as was the previous preparation (see Table 8A). As observed above this material was definitely acid, pH of 4.3. It is very interesting to note that when it was buffered by adding M/15 Na_2HPO_4 until it reached a pH of 7.4 it was ineffective in reducing the clotting time or at least very weak in its activity since any change in clotting time less than 20% was considered questionable.

TABLE 7.

Date	Rabbit	Clotting in Min. Normal	Clotting in Min. After Inj.	Effect % of Normal	Prep.
A. (6D) Distilled - Residue, Dose, 2cc.					
3/27/30	316	18.0	14.0	22	7A1
3/27/30	297	17.0	17.0	00	7A1
3/27/30	306	22.0	22.5	00	7A1
3/27/30	307	31.5	20.0	36	7A1
3/27/30	308	32.0	7.0	77	7A1
B. (6D) Distilled - Distillate					
3/27/31	292	18.0	8.0	55	7B1
3/27/31	309	22.0	20.0	00	7B1
3/27/31	293	20.0	20.0	00	7B1
3/27/31	310	25.5	17.0	30	7B1

TABLE 8

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Lung Tissue Boiled 48 Hours in 20% HCl A. Dialyzed 48 Hours. pH, 4.3					
7/27/30	545	26.0	16.0	43	8A1
7/27/30	586	24.5	7.0	70	8A1
8/8/30	14	20.5	15.0	26	8A1
8/8/30	16	23.5	19.5	17	8A1
8/8/30	18	13.0	8.0	38	8A1
8/8/30	460	20.0	5.5	70	8A1
B. (8A) Buffered with Phosphate to pH = 7.4					
8/2/30	306	22.0	15.5	29	8B1
8/2/30	466	22.5	19.0	15	8B1
8/8/30	296	16.5	14.0	15	8B1
8/8/30	320	11.5	12.5	00	8B1

Two preparations of brain tissue were digested with 20% HCl as in the case of the lung tissue. One was digested for 2-1/2 hours and dialyzed for 24 hours (see Table 9A); the other was digested 36 hours and dialyzed for 106 hours (see Table 9B). As may be seen, part of the tests on these materials were definitely positive while an equal number were definitely negative. Of the six experiments, half negative and half positive, two of the positive have such short clotting times, namely 3.5 and 4.5 minutes, that in the light of information obtained later in the investigation I believe they are probably due to a fault of technic. I believe them to be due to the inclusion of some tissue juice at the time of drawing the blood. At the time I accordingly interpreted these results as negative in spite of the single quite positive experiment. All that can be safely concluded is that in this short series of tests brain tissue did not yield so consistently active preparations as lung tissue. Therefore, I returned to the study of lung tissue.

BENZENE EXTRACTS OF TISSUE

The active material for which I was searching seemed from the above data to be resistant to boiling and digesting with strong acid. The complex proteins and lipids of the tissue were broken down by such treatment into

TABLE 9.

Brain Tissue Boiled with 20% HCl A. Digested 2.5 Hours and Dialyzed 24 Hours					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	% Effect of Normal	Prep.
7/6/30	14	18.0	3.5	80	9A1
7/6/30	16	22.5	23.0	00	9A1
7/6/30	5	26.0	26.0	00	9A1
B. Digested 36 Hours and Dialyzed 106 Hours					
7/6/30	299	18.5	21.0	00	9B1
7/6/30	306	24.5	14.5	40	9B1
7/6/30	320	20.0	4.5	67	9B1

simpler components. From which of these two main classes, if from either, was the active material derived? To settle this question the lipids were extracted and they and the proteins digested separately.

Dry hashed calf lung was refluxed with five consecutive fractions of benzene. The benzene solution was evaporated to dryness under a vacuum. A portion of the lipid residue was suspended in .9% NaCl solution and tested for its in vivo power of increasing coagulability (see Table 10). Some activity was observed, but it was relatively slight and doubtfully significant since it was barely 20%. No. 225, however, is clearcut, but its interpretation is difficult. A portion of the protein residue was suspended in weak HCl and neutralized with NaOH. No appreciable activity was detected since in #292, the only positive result, the possibility of error in drawing the blood was suggested by the very short clotting time of 7 minutes. The normal was also shorter than usual.

The benzene soluble material was now suspended in 10% HCl and digested at a boiling temperature for 48 hours. The acid was removed by dialyzing for 48 hours against running water. This material showed a very sharp reduction in clotting time on intraperitoneal injection. A number of similar preparations were made and found to be

TABLE 10.

Lung Tissue - Benzene Extract Suspended in Saline.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
4/5/30	296	25.0	20.0	20	101
4/5/30	297	23.5	25.0	00	101
4/5/30	298	29.0	23.0	20	101
4/5/30	299	35.0	24.0	25	101
4/3/30	225	31.5	15.5	50	101

TABLE 11.

Lung Tissue - Protein Residue after Benzene Extraction.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
4/3/30	136	15.0	16.0	00	111
4/3/30	206	29.0	30.0	00	111
4/5/30	292	14.0	7.0	50	112
4/5/30	293	32.0	28.0	12	112
4/5/30	294	25.0	29.0	00	112

similarly active. The results of these tests are collected in table 12A. Two rabbits were injected intravenously with the same material. In both experiments the blood coagulated more rapidly after injection (see Table 12B).

An attempt was now made to concentrate the active material by boiling off the water. This concentrated material showed a marked decrease in activity when tested (see Table 12C&D). Either the active principle was volatile, or it was rendered inert by the heating at a reaction near the neutral point. The benzene soluble material from brain treated in the same way as the lung tissue above also gave good reductions in clotting time but more variable. (See Table 13A). On intravenous injection it was found active (see Table 13B). As with the lung tissue preparations, when concentrated by boiling these materials from brain tissue lost their activity. It is very confusing to observe that the digested lung lipid when evaporated to dryness and redissolved, gave sharp reductions of clotting time in three tests (see Table 12E). The sample evaporated to dryness was from a different batch of material than the concentrates tested above. This may explain the apparent discrepancy. Also the three results were too

TABLE 12.

A. Lung Tissue - Benzene Extract (8) with 10% HCl. Digested 48 Hours and Dialyzed 48 Hours.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
4/10/30	470	27.0	19.0	30	121
4/10/30	471	25.5	12.0	54	121
4/10/30	472	18.0	4.0	70	121
4/10/30	473	21.5	5.5	74	121
4/10/30	474	20.0	4.0	80	121
4/11/30	576	21.0	17.5	17	122
4/11/30	577	27.0	13.0	52	122
4/11/30	580	22.0	7.5	66	122
4/11/30	581	34.0	5.5	87	122
4/11/30	582	16.0	6.0	62	122
4/11/30	587	22.0	11.0	50	122
4/16/30	313	35.0	17.0	50	123
4/16/30	316	26.0	8.0	70	123
4/16/30	317	27.0	15.5	43	123
4/16/30	318	25.0	13.5	43	123
4/16/30	320	25.0	5.0	80	123
4/16/30	321	26.0	16.0	46	123
4/16/30	323	20.0	9.5	50	123
4/17/30	453	28.5	4.5	84	124
4/17/30	290	25.0	14.5	41	124
4/19/30	292	17.0	14.0	20	125
4/19/30	306	16.0	16.5	00	125
4/19/30	307	18.0	11.0	38	125

(Continued on Page 39)

TABLE 12 (Continued)

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
6/6/30	292	27.5	21.0	24	126
6/6/30	316	15.0	7.5	50	126
6/6/30	320	24.0	6.5	72	126
6/6/30	290	30.0	20.0	30	126
6/6/30	206	25.0	22.0	12	126
B. Intravenous Injection of (126)					
6/6/30	467	22.5	19.0	16	126
6/6/30	370	21.0	5.0	67	126
C. (126) Concentrated by boiling to 3/4 vol.					
6/9/30	590	11.5	35.0	00	12C1
6/9/30	591	37.0	34.5	00	12C1
6/9/30	592	18.0	20.0	00	12C1
D. (126) Concentrated by Boiling to 1/3 vol.					
6/12/30	225	25.5	24.5	00	12D1
6/12/30	475	34.0	13.0	61	12D1
6/12/30	577	18.0	19.5	00	12D1
6/12/30	5	30.5	19.0	37	12D1
6/12/30	6	22.0	21.0	00	12D1
6/12/30	7	26.5	19.5	27	12D1

(Continued on Page 40)

TABLE 12. (Continued)

E. (122) Preparation - Boiled to Dryness and Redissolved.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
4/18/30	210	9.0	3.0	66 ?	12E1
4/18/30	225	36.5	18.5	48	12E1
4/18/30	206	18.0	9.5	47 ?	12E1

TABLE 13

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
A. Brain Tissue (dry) -Benzene Extract Boiled with 10% HCl. Digested 48 Hours & Dialyzed 48 Hours.					
6/6/30	373	25.5	24.0	00	13A1
6/6/30	460	20.5	12.0	41	13A1
6/6/30	588	27.5	14.5	47	13A1
6/6/30	586	23.5	20.0	14	13A1
B. Intravenous Injection of (131)					
6/3/30	242	25.0	2.5	90 ?	13A1
6/3/30	52	26.0	11.0	58	13A1
C. (131) Concentrated to 1/2 Vol.					
6/9/30	595	15.5	14.0	00	13C1
6/9/30	596	23.5	23.0	00	13C1
6/9/30	597	15.0	8.0	46	13C1
6/9/30	598	46.0	43.0	00	13C1

few considering the variability generally observed in these tests to permit a positive conclusion.

The benzene insoluble residue, which was inactive, was digested with 10% HCl in the same manner as the benzene soluble material above. The dialyzate from this preparation was found to be very active (see Table 14). However it appeared less active than the benzene soluble material. It is evident that not all the activity went into the benzene extract. It is well known that it is impossible to extract all the lipid from the complex proteins of the tissue by such benzene treatment. Since the benzene insoluble material still contained an appreciable quantity of lipid, the source of the active material, whether lipid or protein, is doubtful. But as it was found in the lipid fraction which contains no protein it is more likely that this is its source. Of course there might be more than one active material.

It was observed that the material extracted by cold benzene when digested was much less active than the subsequent extract with hot benzene, similarly digested. This was found to be true with both brain and lung tissues (see Tables 15 & 16).

TABLE 14.

Lung Tissue - Insoluble Residue (9) with 10% HCl Digested 20 Hours - Dialyzed 48 Hours.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
4/10/30	463	18.0	12.0	33	141
"	464	18.5	16.5	10	"
"	467	18.0	12.5	30	"
"	468	28.0	14.0	50	"

TABLE 15.

Lung Tissue (dry) - Benzene Extract with 10% HCl A. Cold Extract - Digest 4 Hours - Dialyzed 12 Hours.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
6/21/30	9	26.0	24.5	00	15A1
"	11	21.0	19.5	00	15A1
"	13	7.5	6.5	13?	15A1
B. Hot Extract - Digested 4 Hours - Dialyzed 12 Hours					
6/21/30	8	26.0	18.0	30	15B1
"	10	27.0	15.5	41	15B1
"	12	24.0	17.5	27	15B1
8/15/30	15	31.0	26.0	16	15B2
"	292	25.0	20.0	20	15B2
"	582	20.0	16.0	20	15B2

TABLE 16.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Brain Tissue - Benzene Extract with 10% HCl. A. Cold Extract - Digested 4 Hours - Dialyzed 12 Hrs.					
7/15/30	17	24.5	23.5	00	16A1
"	525	21.0	16.5	21	"
"	460	6.5	12.0	00	"
"	545	22.5	19.0	15	"
B. Hot Extract - Digested 4 Hours - Dialyzed 12 Hrs.					
7/6/30	466	30.0	15.5	48	16B1
"	52	29.0	13.0	55	"
"	595	19.5	24.0	00	"
7/15/30	1002	36.0	32.0	00	"
"	7	31.5	22.0	27	"
"	8	26.5	19.5	27	"
C. (16B1) Concentrated to 1/2 volume					
7/16/30	19	24.0	11.0	54	16C1
"	296	22.0	14.5	40	"
"	467	28.0	24.0	14	"
"	596	24.0	20.0	17	"

CEPHALIN AND ITS DECOMPOSITION PRODUCTS.

The very pronounced activity obtained with the digestion products of the benzene soluble material of tissues indicated that the active principle might be a decomposition product of the phospholipids which are associated with all tissues. Crude preparations of brain cephalin were made by precipitating it from the ether extract of dry brain tissue with absolute ethanol. Samples of this material were digested with 10% HCl and dialyzed for 12 hours to get rid of the acid. Such preparations gave pronounced reductions of clotting time when injected (see Table 17A). Another sample of crude brain cephalin was digested with strong alcoholic HCl (10% HCl in 80% Ethanol). The resultant mixture was evaporated to dryness and suspended in water. It was found weakly active (see Table 17C).

The active substance might have been either a partial decomposition product of cephalin or one of the simpler products of total hydrolysis by acid, namely Aminoethanol, glycerolphosphoric acid or the fatty acids. If it were a partial hydrolysis product, it would be broken down by extended boiling with strong acid. A sample of cephalin was suspended in strong HCl (10%) in 85% alcohol and placed on the hot plate. Samples were taken at 8, 17 and 37

TABLE 17.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Crude Cephalin (Brain) A. Digested with 10% HCl and Dialyzed 12 Hours					
8/16/30	6	17.0	12.5	18	17A1
"	16	25.5	17.0	30	"
"	18	19.5	8.0	47	"
"	460	25.0	12.5	50	"
"	525	28.0	10.5	60	"
"	298	22.0	14.0	36	"
"	466	25.0	17.0	32	"
B. Preparation (17A1) Autoclaved.					
9/22/30	54	20.0	9.5	52	17B1
"	55	20.5	13.0	37	"
9/23/30	57	23.0	13.0	43	"
"	60	21.0	13.0	38	"
"	61	24.0	9.0	62	"
9/25/30	64	20.0	11.0	45	"
"	65	21.0	12.5	40	"
C. Crude Cephalin (Brain) Digested 24 Hrs. in Alcoholic HCl.					
3/28/31	38	26.0	18.0	30	17C1
"	99	18.0	15.0	16	17C1

days. These were evaporated to dryness and redissolved in water, (see Table 18). It will be seen that some activity was retained even after 17 days on the steam bath. However, the last sample (37 days) tested inactive. Cephalin and any but the very simplest of its hydrolysis products should have been broken down long before this preparation lost its activity.

Samples of aminoethanol (commercial preparation) were tested in amounts which might have been present in the amounts of hydrolysate injected, and found inactive (see Table 19A). The solution was definitely basic. A portion of it was therefore neutralized with HCl. When tested, this preparation was also found to be inert (see Table 19B). The aminoethanol reacts strongly with oleic acid evolving heat and resulting in a wax-like soap. This material was tested and found to be inactive or nearly so (see Table 20).

Among other preparations I tested lyso-cephalin. Lyso-cephalin was prepared by Levene (1923) in a manner analagous to that first used by Delezenne in preparing lyso-lecithin. An enzyme (cephalinase) is contained in cobra venom which splits out the unsaturated fatty acid leaving the rest of the molecule intact. Billing (1930) has shown that Crotalus venom also splits off the unsaturated fatty

TABLE 18.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
Pure Cephalin (Brain) in Alcoholic HCl. A. Digested 8 days.					
2/10/31	40	25.5	21.5	15	18A1
"	43	30.0	9.5	66	"
"	44	20.0	19.0	00	"
"	45	25.0	16.0	36	"
"	49	22.0	16.0	27	"
B. Digested 17 Days.					
2/18/31	32	24.5	17.0	30	18B1
"	34	29.5	15.5	49	"
"	39	18.0	22.0	00	"
"	47	21.0	18.0	14	"
C. Digested 37 Days					
3/10/31	67	27.0	26.0	00	18C1
"	65	24.5	25.5	00	"

TABLE 19.

A. Aminoethanol

Date	Rabbit	Dose	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
10/13/30	394	2mg.	20.0	15.0	25	19A1
"	64	2mg.	23.5	25.5	00	"
"	65	2mg.	14.5	16.0	00	"
B. (19A1) Neutralized with HCl						
10/13/30	54		25.5	4.5	80 ?	19B1
"	55		22.0	22.0	00	"
"	544		19.0	33.0	00	"

TABLE 20.

Aminoethyl Oleate

Date	Rabbit	Dose	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
5/8/31	65	20mg.	26.5	21.5	19	201
"	28	20mg.	20.5	19.5	00	"
"	38	10mg.	27.0	16.5	37	"
"	00	10mg.	23.0	22.5	00	"

acid. He did not attempt, however, to separate lyso-cephalin.

A crude preparation of brain cephalin was treated with crotalus venom. Five grams of cephalin were emulsified in 200 cc. of 1% saline and digested with .05gm. of venom at 40 C. for 2 hours. It was boiled to kill the action of the venom, and without filtration the resultant mixture was precipitated with acetone (5 vols.). The acetone precipitate gave sharp reductions in clotting time when injected (see Table 21A). This precipitate was washed with ether, and the ether insoluble residue tested. It was found very active (see table 21B). The part of the acetone precipitate which dissolved in ether was found inactive (see Table 21C). The part of the original digest which remained in solution in acetone was also found to be without action (see Table 21D).

A preparation of pure brain cephalin was tried. Brain tissue was dried and exhaustively extracted with acetone. The residue was thoroughly extracted with absolute ether, and the extract concentrated at room temperature in vacuo. The cephalin was precipitated from the ether solution by pouring it into about 4 volumes of absolute ethanol. The preparation was purified by repeated reprecipitation from ether solution by ethanol, and finally precipitated from hot methanol. (N = 1.75%, P = 3.88%). Fifty grams of this pure pre-

TABLE 21.

Crude Cephalin (Brain) Digested with Crotalus Venom.					
A. Precipitated with Acetone.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
1/30/31	79	16.0	9.0	43	21A1
"	57	23.5	8.0	65	"
"	67	25.0	19.0	24	"
"	77	18.0	14.5	20	"
B. Ether Insoluble Portion of Acetone Ppt.					
2/9/31	26	18.0	10.0	41	21B1
"	64	26.0	10.0	63	21B1
"	65	28.0	19.0	32	21B1
"	75	27.0	14.0	48	"
"	53	24.5	12.5	49	"
C. Ether Soluble Portion of Acetone Ppt.					
2/3/31	78	17.0	22.0	00	21C1
"	00	20.0	23.0	00	21C1
D. Acetone Soluble Portion of Original Digest					
2/6/31	99	26.0	26.5	00	21D1
2/6/31	394	30.0	28.5	00	21D1

paration were suspended and emulsified in 500 cc. of .85% NaCl solution. Two grams of crotalus venom was added, and the mixture digested at 40°C. for 25 hours. The digested mass was boiled to kill the venom action. This material did not prove to be very active when 2cc. of the mixture were injected (see Table 22A). The mixture was then precipitated by 3 or 4 volumes of acetone. The resultant precipitate was washed with ether, dissolved in ethanol, and precipitated by being poured into 5 volumes of ether. Lysocephalin is insoluble in ether, whereas cephalin if any remained was soluble. The precipitate was dried and suspended in water. Very active reductions in clotting time were obtained with this material in doses of 1/2 to 2mg. injected into 2 kilo rabbits (see Table 22B).

The material remaining unprecipitated by acetone was extracted with ether. It yielded an ether soluble oil which appeared to be the unsaturated fatty acid split from the cephalin. This material was also found to be somewhat active when injected in the same dosage (see Table 23).

TABLE 22.

Pure Cephalin (Brain) Digested with Crotalus Venom						
A. Boiled to Kill the Venom Action.						
Date	Rabbit	Dose	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
3/17/31	28	2cc.	16.5	15.0	00	22A1
"	99	2cc.	16.0	11.0	27	"
B. Ppt. with Acetone, Dissolved in Alcohol and Ppt by Ether.						
3/24/31	75	2. mg	24.0	5.0	79	22B1
"	36	1. mg	21.5	8.5	62	"
"	65	0.5mg	26.5	7.0	73	"
3/28/31	28	2. mg	20.5	11.0	46	"
"	36	0.8mg	25.0	16.0	36	"
5/27/31	548	1. mg	21.5	15.5	30	22B2
"	549	1. mg	16.5	13.0	21	"
"	550	2. mg	33.5	14.5	56	"
"	551	2. mg	23.5	18.0	23	"

TABLE 23

Ether Soluble Portion From Venom Action. (Unsat. Fatty Acid)					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
7/10/31	00	24.0	19.0	21	231
"	248	25.0	16.5	34	"
"	250	26.5	22.0	17	"

ALCOHOL INSOLUBLE FRACTION OF "LEXIN".

"Lexin" is the commercial preparation of the phospholipid obtained from soy bean. The alcohol insoluble fraction of this phospholipid mixture (cephalin-like material when digested with 10% HCl and neutralized gave sharp reductions in clotting time (see Table 24A). However, when it was dialyzed for 12 hours against running water, its activity was greatly reduced (see Table 24B). The same fraction of the phospholipid was also digested with 10% alcoholic HCl. When evaporated to dryness and taken up again in water it gave sharp reductions in clotting time on injection. (See Table 25). Digestion of this same soy bean phospholipid with H_2SO_4 and precipitation of the excess acid with $Ba(OH)_2$ gave no active preparations (see Table 26). Analysis of the cephalin-like phospholipid from soy bean "Lexin" gave variable values, N = .9 to 1.%, P = about 3%, showing that it was not pure cephalin since this required N = 1.88% P = 4.17%. Also pure cephalin is soluble in glacial acetic acid and over 90% of this material was found insoluble in glacial acetic.

TABLE 24.

Alcohol Insoluble Fraction of Soy Bean Phospholipid					
A. Digested with 10% HCl and Neutralized.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
10/4/30	69	24.5	22.0	17	24A1
"	70	13.5	12.0	11	"
"	71	21.0	13.0	24	"
"	72	29.5	12.0	59	"
10/6/30	58	18.5	11.0	40	24A2
10/19/30	53	22.0	12.0	45	"
"	57	16.0	10.5	34	"
B. Dialyzed 12 Hours					
10/19/30	67	21.5	22.5	00	24B1
"	68	23.0	20.0	13	"

TABLE 25.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Alcohol Insoluble Fraction of Soy Bean Phospholipid.					
A. Digested with Alcoholic HCl.					
10/24/30	76	33.0	17.0	49	25A1
11/4/30	57	34.0	21.0	35	"
"	67	49.0	32.0	30	"
"	78	10.0	14.0	00	"
11/11/30	55	25.0	18.0	32	25B1
"	394	27.0	20.00	26	"
"	57	18.0	14.0	22	"
B. Preparation (25A1) Mixed with Fresh Fibrogen.					
11/14/30	75	20.0	22.0	00	25B1
"	65	21.0	20.5	00	"
11/19/30	67	20.0	13.5	25	25B2
"	79	18.5	10.5	43	"
C. Ether Soluble Portion from Dry Material (25A1).					
10/28/30	68	35.0	21.0	40	25C1
"	544	20.0	6.0	70	"
D. Alcohol Soluble Portion from Residue of (25C1).					
10/28/30	53	24.0	15.0	40	25D1
"	55	29.0	19.0	34	"

TABLE 26.

Alcohol Insoluble Fraction of Soy Bean Phospholipid.					
A. Digested with H_2SO_4 and Acid Ppt. with $Ba(OH)_2$					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
10/24/30	73	19.5	18.0	00	26A1
"	74	25.5	21.0	16	"
B. $BaSO_4$ Ppt. Washed with Acid Alcohol.					
11/1/30	54	13.0	13.5	00	26B1
"	65	12.0	11.5	00	"
"	75	6.0	7.5	00	"

MATERIALS COMMONLY ASSOCIATED WITH CEPHALIN.

It was possible that even in apparently pure preparations of cephalin someof the materials commonly associated with cephalin might be present. Crude brain cephalin which has stood for some time contains appreciable quantities of ether insoluble material, possible decomposition products of cephalin. Such ether insoluble material gave some very sharp reductions of clotting time as seen in table 27A. The ether insoluble, alcohol soluble, material (probably principally phrenosin and kerasin) from dry brain tissue when digested with alcoholic HCl on the steam bath showed no effect on clotting time when injected (see Table 27C).

The foregoing results pointed toward lyso-cephalin as the active material.

PRODUCTS OF VENOM ACTION ON BRAIN LECITHIN.

Lecithin was prepared by concentrating the alcohol solution from the cephalin preparation and reprecipitating it several times from ether solution with dry acetone. The analysis ran N = 1.58%, P = 3.33% against the theoretical values of N = 1.74%, P = 3.87%. This lecithin-like cephalin yielded active materials when treated with crotalus venom. The digest precipitated with acetone, dissolved in alcohol, and precipitated with 5 volumes of ether yielded a product which should have contained the lysolecithin but

TABLE 27.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
Materials Associated with Cephalin or Lecithin					
A. Ether Insoluble Material in Crude Cephalin					
2/13/31	73	27.0	16.0	40	27A1
"	77	28.5	18.0	36	"
"	100	21.0	11.0	47	"
2/18/31	42	26.0	14.0	46	"
"	28	20.5	19.5	00	"
B. Alcohol Soluble Material from Residue of (27A1)					
3/2/31	99	20.5	13.0	36	27B1
"	29	21.0	12.0	43	"
"	50	19.5	17.0	00	"
"	78	18.5	15.5	00	"
C. Glyco-Lipins Digested with Alcoholic HCl					
11/24/30	53	25.0	23.0	00	27C1
"	68	29.0	28.5	00	"
"	77	18.0	18.0	00	"

which on analysis gave 4.20% P and 1.14% N whereas 5.75% P and 2.60% N were required. The product was certainly not pure lysolecithin, but may have contained some of this compound (see Table 28A). The low figures for nitrogen might indicate a partial splitting off of the nitrogen base. A water soluble soap which had a very high nitrogen content, certainly more than 3%, was separated from the digest. This would indicate that the freed nitrogen base had combined with a part of the freed fatty acid forming a soap. This soap contained not more than a trace of phosphorous. When tested it gave definite reductions in clotting time (see Table 28B). It is interesting to note that no soap was obtained from the cephalin preparation when treated with venom. Also the nitrogen content of the product (N = 2.50) was much higher than the nitrogen content of the original cephalin (N = 1.75). A small amount of ether soluble oil which appeared to be the unsaturated fatty acid was separated from the lecithin digest. This was found active (see Table 28C).

OTHER PRODUCTS FROM LECITHIN.

The nitrogen base obtained from lecithin is choline. A pure sample of choline hydrochloride was obtained from the Eastman Company. In doses of 1 1mg. it was found active, but in larger doses it was found inactive (see Table 29).

TABLE 28.

Lecithin (Brain) Digested with Crotalus Venom						
A. Ppt. with Acetone, Dissolved in Alcohol & Ppt. with Ether.						
Date	Rabbit	Dose	Clotting Time in Min Normal	After Inj.	% ofNormal Effect	Prep
6/11/30	558	1.mg.	26.0	16.5	36	28A1
"	559	1/2mg	22.0	10.0	54	"
"	548	1.mg.	16.0	13.0	20	"
B. Water Soluble Soap from Action of Venom						
6/30/31	560		18.0	12.5	30	28B1
"	555		18.0	5.0	62	"
"	559		15.0	10.0	33	"
"	548		13.0	11.5	17	"
"	557		12.0	10.0	17	"
"	550		25.0	17.0	28	"
C. Ether Soluble Oil from Venom Action (Unsat. Fatty Acid.						
7/8/31	555		27.5	16.0	42	28C1
"	562		21.5	10.5	53	"
"	561		25.0	18.0	10	"
"	557		29.5	21.5	28	"
"	559		26.0	15.5	40	"

TABLE 29.

Choline Hydrochloride						
Date	Rabbit	Dose	Clotting Time in Min. Normal	After Inj.	Effect % of Nor.	Prep
1/28/31	26	4 mg	24.5	24.5	00	291
"	27	2 mg	22.0	22.0	00	"
"	28	1 mg	20.0	12.0	40	"
"	99	1 mg	27.0	16.0	40	"
5/14/31	36	1 mg	19.0	13.0	36	"
"	75	1 mg	20.0	18.5	00	"

A derived lecithin was made according to Wadsworth, Maltaner & Maltaner (1930). A 5% lecithin emulsion was made faintly alkaline with NH_4OH and 10% CaCl solution added. On warming a precipitate formed. This precipitate was separated, washed and freed from Ca by suspending in N/10 HCl and shaking for an hour. The product was extracted with ether and dried. Two of the three tests tried showed sharp reductions in clotting time when injected (see Table 30).

ALCOHOL SOLUBLE FRACTION OF "LEXIN".

Preparations were made using the alcohol soluble (lecithin-like) fraction of the "lexin" from soy bean. Samples were digested with 10% HCl (see Table 31) and also with crotalus venom (see Table 32A&B). Of these preparations not one was found significantly active. It is interesting to note that while the clotting time was definitely longer than the normal after injection of the acetone precipitate from the venom action, the clotting time was definitely though not sharply reduced from the normal after injection of this material in dilutions of 1/50 and 1/33 (see Table 32C).

LYSOLECITHIN ?FROM RICE.

Motoe Nwata (1930) has reported separation of lysolecithin from polished rice with a .2% yield. A preparation was made according to his methods. Rice was exhaustively extracted with 95% ethanol. The concentrated extract was

TABLE 30.

Derived Lecithin (According to Wadsworth & Maltaner					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
1/20/31	94	23.0	15.5	32	301
"	95	16.0	16.0	00	"
"	97	25.0	12.0	52	"

TABLE 31.

"Lecithin" (Soy Bean) Digested with 10% HCl					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
11/11/30	53	23.0	11.0	51	311
"	77	18.0	18.5	00	"

TABLE 32.

"Lecithin" (Soy Bean) Digested with Crotalus Venom.						
A. Acetone Soluble Portion						
Date	Rabbit	Dose	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep
12/30/30	82	2mg.	24.0	24.0	00	32A1
"	83	"	24.0	22.5	00	"
"	84	"	22.5	21.0	00	"
B. Acetone Insoluble Portion						
12/30/30	86	2mg.	25.0	31.0	00	32B1
"	87	"	23.5	28.5	00	"
C. Preparation (32B1) Diluted						
12/31/30	90		23.0	20.0	13 (1/50)	32C1
"	91		21.0	16.0	24 (1/33)	32C2

precipitated by being poured into a large volume of ether. After reprecipitation only a very small quantity of material was obtained, not more than 1/20 the yield reported by the original author. The amount was too small to be analysed, and its identity was very doubtful. This material was tested and on the whole gave very sharp reductions of clotting time (see Table).

FATTY ACIDS.

It was observed above that the ether soluble oil from the action of venom on both lecithin and cephalin was active. This material appeared to be an unsaturated fatty acid. Tests were made by injection of a 4% emulsion of stearic acid with negative results (see Table 34). A 4% emulsion of oleic acid, however, was found to give very sharp and consistent reductions (see Table 35). A similar emulsion was made of oleic acid containing 4% acid and 2% cephalin. This preparation was much more stable and just as active as the emulsion without cephalin (see Table 24B). A small amount of lenolenic acid was made from raw linseed oil. The oil was saponified with NaOH. The resultant soap was dissolved in glacial acetic acid and brominated. The hexa bromide was separated by its insolubility in ether, the lower brominated acids being insoluble. This hexa bromide

TABLE 33.

Extract from Rice (Lyso-Lecithin?)						
Date	Rabbit	Dose	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep
11/24/30	73	1 mg	9.5	22.0	00	331
11/29/30	57	1 mg	9.5	6.5	31	"
"	67	2 mg	17.0	8.0	64	"
"	64	1 mg	21.0	16.0	25	"
"	75	1 mg	20.0	15.0	25	"
12/1/30	55	2 mg	20.0	15.0	25	"
"	65	2 mg	27.0	18.0	34	"
"	394	2 mg	35.5	16.0	54	"
12/8/30	53	50 mg	20.0	22.0	00	"
"	67	10 mg	25.5	19.5	23	"
"	77	5 mg	22.0	14.5	34	"

TABLE 34

Stearic Acid Emulsion. Dose, 2cc. of 4% Emulsion					
Date	Rabbit	Clotting Time In Min. Normal	After Inj.	Effect % of Normal	Prep.
4/25/30	475	30.0	31.0	00	341
"	576	13.0	14.0	00	"
"	473	18.0	19.0	00	"
"	472	24.0	23.5	00	"

TABLE 35.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
A. Oleic Acid Emulsion. Dose, 2cc. of 4% Emulsion					
7/15/31	558	21.5	11.5	47	35A1
"	556	20.5	8.5	58	"
"	563	23.5	18.0	28	"
"	564	19.0	16.0	16	"
7/21/31	557	23.5	17.5	25	35A2
"	562	19.5	13.0	33	"
"	555	22.5	17.0	24	"
"	551	21.0	12.0	43	"
B. Oleic Acid Emulsified with Cephalin					
7/30/31	564	25.0	15.0	40	35B1
"	557	21.5	13.0	40	"
"	562	18.0	13.0	28	"
"	548	31.0	6.0	84?	"
10/13/31	570	26.5	20.0	25	35B2

was freed from bromine by treating with HCl and zinc in ethanol. The resultant acid was tested and foundto give active reductions in 1 cc. doses of an approximately 2% emulsion (see Table 36). Sodium ricinoleate was tried in 2 mg. doses intraperitoneally without effect (see Table 37). This material in .05 to .1 gm. doses injected into the heart causes instant death by thrombosis. The ventricles were found solidly clotted.

FRESH TISSUE EXTRACTS.

While studying these materials, I discovered that they acted through the spleen and were ineffective in the absence of this organ. (This will be taken up in the next section of this paper.) Tests were made to see if saline extracts of splenic tissue would be effective in reducing the clotting time when injected intraperitoneally. They were found to be very active. This action lead to a study of numerous tissues to find out if other fresh tissues contained this activity. The studies were not extensive. Three grams of fresh tissue were hashed in 25cc. of .9% NaCl, boiled 3 min. and filtered. Two cubic centimeters of this preparation were used for each injection. Rabbit tissues were used, and the following ones were tested: lung, brain, stomach, liver, kidney, muscle, testicular, intestinal,

TABLE 36.

Lenolenic Acid Emulsion. Dose, 2cc. of 4% Emulsion.					
Date	Rabbit	Clotting Time in Min. Normal	After inj.	Effect % of Normal	Prep.
10/22/31	648	21.5	16.0	26	361
"	649	21.5	10.0	53	"
11/14/31	1596	28.0	14.0	50	362
"	626	23.0	19.0	17	"
"	627	22.0	19.0	14	"

TABLE 37.

Sodium Ricinoleate. Dose, 2mg.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
10/27/31	2001	18.5	20.0	00	371
"	1004	20.5	21.5	00	"
"	1007	22.0	21.0	00	"
"	1008	18.5	20.0	00	"

pancreatic, heart, and arterial blood. The results were variable on all these tissues (see Table 38). However, no consistent reductions were obtained with other than the splenic tissue.

SPLENIC TISSUE.

Splenic tissue was obtained by splenectomy of normal animals under ether anesthesia. Thistissue when treated as the above tissues by boiling 3 min. in .9% NaCl and filtering, gave consistently sharp reductions in clotting time (see Table 39). A rabbit was decapitated and the spleen removed at once. An extract of this tissue made as above was definitely less active than the normal tissue extracts. (See Table 38B). Spleens were obtained from normal rabbits whose organs had been thoroughly perfused with Ringers Solution. (Courtesy of Mr. Schmidt). Saline extracts of this tissue showed relatively inconsistent results (see Table 39C). An extract of the normal spleen made with cold .9% NaCl instead of boiling, was found to be active. (See Table 40). An attempt was made to augment the activity of the splenic tissue by treating for one hour at 40°C. with 10% H Cl and subsequently neutralizing with NaOH, a method which proved effective in the early experiments. The results were negative (see Table 41).

TABLE 38.

Extracts of Fresh Tissue in 0.9% NaCl Boiled 3 Min.					
A. Rabbit Lung Tissue. Dose, 2cc.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
1/21/30	190	23.0	18.5	18	38A1
11/2/31	587	25.0	24.5	00	38A2
"	1588	22.0	22.0	00	"
B. Rabbit Brain Tissue					
1/21/30	153	20.5	18.0	11	38B1
11/2/31	589	27.0	28.0	00	38B2
"	1590	22.5	25.0	00	"
C. Rabbit Stomach Tissue					
11/2/31	593	25.0	20.0	20	38C1
"	1594	23.0	20.0	15	"
D. Rabbit Liver Tissue					
11/2/31	1595	20.5	16.0	20	38D1
"	64	23.5	25.5	00	"
E. Rabbit Kidney Tissue					
11/4/31	1596	31.0	28.0	00	38E1
"	1597	26.5	11.0	59	"
F. Rabbit Muscle Tissue					
11/6/31	638	22.5	25.5	00	38F1
"	639	23.0	13.0	40	"

TABLE 38 (Continued)

Extracts of Fresh Tissue ------ in 0.9% NaCl, Boiled 3 Minutes.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
G. Rabbit Testicular Tissue					
11/6/31	633	25.5	22.5	00	38G1
"	637	31.5	24.5	23	"
H. Rabbit Small Intestine					
11/4/31	600	28.5	30.0	00	38H1
"	626	22.0	22.5	00	"
I. Rabbit Pancreatic Tissue					
11/6/31	631	28.5	28.5	00	38I1
"	632	26.0	26.5	00	"
J. Rabbit Heart					
11/4/31	627	24.5	15.0	38	38J1
"	628	26.5	10.0	62	"
11/12/31	567	21.5	21.0	00	38J2
11/13/31	587	26.5	26.0	00	"
"	588	25.5	26.5	00	"
"	1594	37.0	30.0	00	"
"	1595	23.0	22.0	00	"
K. Rabbit Arterial Blood					
11/10/31	1006	28.5	16.0	43	38K1
"	1008	20.5	21.5	00	"
"	31	22.0	17.0	22	"
"	33	21.5	18.0	16	"
12/2/31	1568	25.5	25.5	00	38K2
"	588	29.0	29.5	00	"
"	1590	27.0	29.0	00	"
"	627	27.5	27.0	00	"

TABLE 39.

Rabbit Splenic Tissue (Fresh) in 0.9% NaCl Boiled 3 Min.

A. Normal Rabbits under Ether . Dose, 2cc.

Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep
4/7/31	532	25.0	11.5	54	39A1
"	522	28.5	13.5	52	"
"	530	33.0	9.0	75	"
4/9/31	513	25.0	12.0	52	"
4/15/31	36	20.0	6.5	70	"
10/22/31	641	30.0	14.5	51	39A2
"	643	31.0	23.0	26	"
"	644	25.0	19.0	24	"
"	650	21.5	15.0	39	"
11/2/31	1591	25.5	11.5	54	39A3
"	1592	29.0	12.0	58	"
11/7/31	642	28.5	15.0	47	"
"	649	27.0	14.0	48	"
B. Decapitated Rabbit Spleen Removed at once					
11/10/31	2001	31.5	30.0	00	39B1
"	2003	27.5	14.5	47	"
"	1004	20.0	20.0	00	"
"	1005	24.5	20.0	18	"

TABLE 39 (Continued)

Rabbit Splenic Tissue (Fresh) in 0.9% NaCl Boiled 3 Min.

C. Rabbit's Organs Perfused with Ringer's Solution Before Removal of Spleen (Courtesy of Mr. Schmidt

Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
11/10/31	1013	36.0	25.0	33	39C1
"	1017	46.5	25.5	45	"
11/11/31	1036	25.0	25.0	00	"
"	1034	22.5	19.0	15	"
"	37	25.5	25.0	00	"
"	35	23.0	20.0	11	"
11/12/31	574	20.5	12.5	39	39C2
"	573	22.5	16.5	27	"
11/23/31	1586	30.0	22.0	26	"
"	1590	27.0	26.0	00	"
"	1592	32.0	22.0	30	"
"	1593	23.0	21.0	00	"
11/30/31	570	27.0	24.0	11	39C3

TABLE 40.

Rabbit Splenic Tissue (Fresh) in 0.9% NaCl in Cold.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
4/13/31	28	22.0	9.0	59	401
"	65	28.5	8.5	26	"
4/15/31	75	20.5	21.0	00	"
"	38	27.0	16.5	37	"

TABLE 41.

Rabbit Splenic Tissue with N/10 HCl for 1 Hr. at 40° Neutralized with NaOH.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
11/18/31	1043	29.5	28.0	00	411
"	1042	25.0	25.0	00	"
"	41	31.5	16.5	47	"
"	635	28.0	26.5	00	"
"	640	25.5	29.0	00	"

Extracts of beef spleen made with .9% NaCl and boiled as was rabbit spleen proved to be less consistently active. (See Table 42). They did, however, give very active reductions in the majority of tests. Similar extracts made with acetone dried beef spleen gave sharp reductions in clotting time, although but three tests were made. (See Table 43). An alcoholic extract (in 95% ethanol) of air dried beef spleen was tested after evaporation of the alcohol and dilution with water. The extract showed sharp though inconsistent reductions (see Table 44). Using alsohol which contained 1% HCl, an extract was made from the same dried beef spleen. The extract was neutralized after filtering, the alcohol evaporated, and the residue taken up with water. The results were comparable with the previous neutral alcohol extract but were in no way better (see Table 45). Extracts were made with dog spleen in .9% NaCl and boiled as before. These were comparable in activity with the beef splenic extracts but still less consistent than the rabbit splenic extracts. (See Table 46). The greater activity of the rabbit spleen may be due to a specificity or to an intrinsically greater quantity of active material; but it is more likely due to the fact that the extracts were prepared from very fresh tissue direct from the animal, while the others were not.

TABLE 42.

Beef Splenic Tissue (Fresh) in 0.9 NaCl Boiled 3 Min.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
8/4/31	563	23.0	15.5	32	421
"	551	20.5	13.5	34	"
"	557	23.0	10.0	51	"
"	550	25.0	17.0	28	"
11/30/31	1592	26.5	21.0	20	422
"	1595	27.0	23.0	15	"
"	572	28.5	26.0	00	"
"	574	30.0	27.5	00	"
"	568	25.0	10.5	58	"
11/24/31	643	31.5	28.5	00	423
"	644	28.5	20.0	30	"

TABLE 43.

Beef Splenic Tissue Acetone Dried in 0.9% NaCl Boiled 3 Min.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
10/20/31	631	23.0	16.0	30	431
"	636	30.0	18.0	32	"
"	637	31.0	15.5	49	"

TABLE 44.

Beef Splenic Tissue Air Dried Extracted with 95% Alcohol.					
Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep.
11/14/31	1597	31.0	20.5	34	441
"	600	32.0	29.0	00	"
"	628	26.0	21.0	19	"
"	629	31.5	18.5	41	"
11/24/31	641	22.5	20.0	11	442
"	650	31.0	22.0	29	"

TABLE 45.

Beef Splenic Tissue Extracted with Acid Alcohol And Neutralized.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
11/27/31	1006	35.0	31.5	10	451
"	1015	38.0	32.0	14	"
"	2003	28.5	17.5	40	"
"	1004	30.0	30.0	00	"
"	1007	35.0	24.0	31	"
"	1014	31.5	15.5	52	"
11/27/31	1008	26.5	21.0	21	452
"	1009	29.0	10.0	65	"
"	1011	26.0	24.0	00	"

TABLE 46.

Dog Splenic Tissue in 0.9% NaCl Boiled 3 Min.					
Date	Rabbit	Clotting Time in Min. Normal	Clotting Time in Min. After Inj.	Effect % of Normal	Prep.
12/9/31	54	27.0	17.5	35	461
"	55	25.5	27.0	00	"
"	57	27.5	21.0	23	"
"	59	26.0	20.5	20	"
12/10/31	80	31.5	22.5	29	"
12/20/31	101	25.0	22.5	10	"
"	102	29.5	20.5	31	"
"	103	26.0	26.5	00	"
12/23/31	1586	34.0	30.0	11	"
"	588	38.0	12.0	66	"
"	1598	30.0	28.5	00	"

MISCELLANEOUS MATERIALS.

A few miscellaneous materials were tested which should be recorded here. Theelin (commercial preparation) was injected intraperitoneally and intravenously without effect. (See Table 47 A&B). Adrenin 1:1000 gave some very sharp reductions and also some negative ones (see Table 47C). The preparation was not fresh, but the animals showed excitement in most cases. Ceanothyn (a commercial alcoholic extract of the plant ceanothus americanus) gave very inconsistent results. (See Table 47 D&E).

TABLE 47.

Date	Rabbit	Dose	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Prep
Miscellaneous Materials Tested						
A. Theelin Intraperitoneal Injection - Dose, 1cc.						
4/3/31	505		20.0	18.0	10	47A1
"	506		22.0	21.0	00	"
"	507		22.0	21.5	00	"
B. Theelin Intravenous Injection - Dose 1cc.						
4/6/31	546		24.0	21.0	12	47B1
"	520		19.0	18.0	00	"
C. Adrenin 1:1000						
12/26/31	1058	.5cc	28.0	11.0	67	47C1
"	108	.5cc	9.0	10.0	00?	"
"	109	.5cc	19.0	20.0	00	"
"	110	.5cc	17.5	12.0	56	"
D. Ceanothyn Intraperitoneal Injection						
10/16/31	579	1.0cc	28.0	28.5	00	47D1
"	1580	1.0cc	25.0	15.5	42	"
"	1577	2.0cc	23.0	24.5	00	"
"	583	2.0cc	25.5	13.5	43	"
10/17/31	587	2.0cc	28.0	16.5	41	"
"	1590	2.0cc	28.5	12.0	26	"
"	1594	2.0cc	21.0	14.5	31	"
11/30/31	573	2.0cc	25.0	17.5	30	"
"	571	2.0cc	26.5	18.0	32	"

TABLE 47 (Continued)

Miscellaneous Materials Tested						
D. Ceanothyn Intraperitoneal Injection (Continued)						
Date	Rabbit	Dose	Clotting Time in Min Normal	After Inj.	Effect % of Normal	Prep
11/13/31	593	2.0cc	23.0	21.0	00	47D1
12/2/31	629	"	23.0	20.5	10	"
12/23/31	1594	"	32.0	31.5	00	"
"	630	"	24.5	24.0	00	"
E. Ceanothyn Oral Administration						
10/17/31	1586	3.0cc	28.5	20.0	29	47D1
"	589	"	21.5	16.0	26	"

DISCUSSION.

In spite of the apparent inconsistencies and great variability of the foregoing data, certain significant features may be observed. The first outstanding facts are the stability of the active material and its apparent production from tissue materials by weak or strong acid treatment. The next point is the apparent source of the active material found in the lipoid, that is the benzene soluble, part of the tissue materials. There followed in natural sequence the production of the material from purified phospholipins, notably brain cephalin. Some indications of activity are derived from the study of the products of cephalin decomposition. It is evident, however, that no one material has been separated which can be identified as the active principle for which I have been searching.

Outstanding among the fresh tissue preparations are the splenic extracts. Nothing of significance has yet been drawn from this observation, but it should be noted that the spleen is an active seat of destruction of tissue materials in the form of cellular constituents of the blood.

It is disconcerting to observe that even the most active materials studied could not be trusted to give consistent reductions in clotting time when tested in rabbits. It is simple to attribute these difficulties to the individual variations in the animals, especially as rabbits are so widely

considered to be variable and untrustworthy in blood coagulation studies. However, these variations must have a cause. In this particular work I believe the following sections will throw much light on the difficulty. The other factor which must be considered in viewing this variability is the technic of determining the coagulability of the blood. Mechanical agitation of the sample and contact of the blood with the raw glass surface are significant factors. Certain points in the method as first described were studied. In brief it was found unnecessary to observe the tube before 10 minutes after the sample had been taken, and unnecessary to remove the tube from the bath and tilt it in order to observe the viscosity changes.

The syringe and needle were well oiled, the blood carefully drawn from the animal's heart and placed in the tube without excess pressure or unnecessary contact with the sides of the tube. The tube was not observed for the first ten minutes, then the viscosity was observed by vibrating the rack with a gentle tap. To determine the final end point the tube was removed and tilted. In each test two tubes were used per sample, but only one of these was observed. The other was allowed to stand without being disturbed until the first had clotted. In this way it was possible to check on the agitation of handling. When these series of precautions were observed with greatest care, no

reduction in clotting time was detected after injection of materials previously found active. It is evident, then, that the change produced by injection of the materials studied was manifested as a difference in the reaction of the blood to surface contact and agitation. Such a property has been attributed to the blood platelets. These bodies I found of great significance in this connection, and I have devoted the third section of this paper to their relation to my problem.

THE SPLEEN AND COAGULABILITY.

Materials that were studied in the first section of this work appeared to act through some physiological mechanism. They did not, as a rule, cause a decrease in the clotting time of blood or recalcified plasma in the test tube. They acted no more rapidly when injected intravenously than when injected intraperitoneally. The spleen has been considered for some time to have an effect on coagulability. Yamada (1918) found that removal of the spleen caused a decrease in serum thrombin. Szenes (1920) stated that treatment with X-ray in the region of the spleen caused a decrease in the clotting time of the blood. Stephan (1920) substantiated the work of both Yamada and Szenes. He found the serum from an animal whose spleen had been exposed to X-ray to be more effective in accelerating the clotting of blood than the normal serum. He found that the spleen was the only organ which gave this reaction. Neuffer(1921) found X-ray treatment of the spleen to be very advantageous in cases of hemophilia. This action he ascribed to destruction of leucocytes and liberation of a thrombokinase in the spleen. Wolisch (1921), Fleissly (1921), and Bernhard (1924) all claim that the spleen is not the organ governing the coagulability of the blood. This conclusion is based, however, on their success in causing an increase in coagulability of the blood by X-ray in the absence of the spleen. While this

work indicates action of X-ray other than its action on the spleen, it does not disprove the fact that the spleen does have an action. Tichy (1920) substantiated the action of the spleen but claimed a more potent action for the liver. Ohara (1921) found X-ray and also diathermy of the spleen effective in reducing clotting time, but "Splenic Fluid" lengthened it. The action of the spleen was put on a firmer basis by Bruno (1929). He found the blood from the splenic vein to be more coagulable than that from the arterial system, and he showed this difference to be greatly enhanced by stimulation of the spleen with X-ray or electric current. Bruno claimed to have separated an active thrombokinase which acted in vivo. This material was lipoid in nature.

EXPERIMENTAL EVIDENCE.

If the material whose physiological action had been studied did not give the same reduction in clotting time after injection in a splenectomized animal, it would indicate a definite relation of the action to the activity of the spleen. A group of rabbits was splenectomized and given 2 or 3 weeks to recover. The actual recovery from operative effects is a matter of a few days, but for studies of coagulability the blood did not become stable for a period of two weeks at least. These animals gave long and consistent clotting times as contrasted with the erratic results obtained with the

normal rabbit. These rabbits, when injected with materials previously studies on normal animals and found active, showed no change in the clotting time of their blood (see Table 48).

A clear conception of this relation of the spleen to coagulability is dependent upon an understanding first of the mechanism or mechanisms by which injected materials control the splenic activity, and second the mechanism or mechanisms by which the spleen controls coagulability. The third section of this paper aids greatly in clearing up these problems.

Examination of the normal clotting times recorded will show that there occurs a minority of clotting times which are much shorter than the general run. It was found impractical to use animals with such short clotting times because the most active material would as a rule fail to produce any further reduction. It is reasonable to conclude that this short clotting time represented the condition which would normally follow injection, i.e. the condition following stimulation of the spleen. In order to verify this conclusion a distribution curve was plotted.

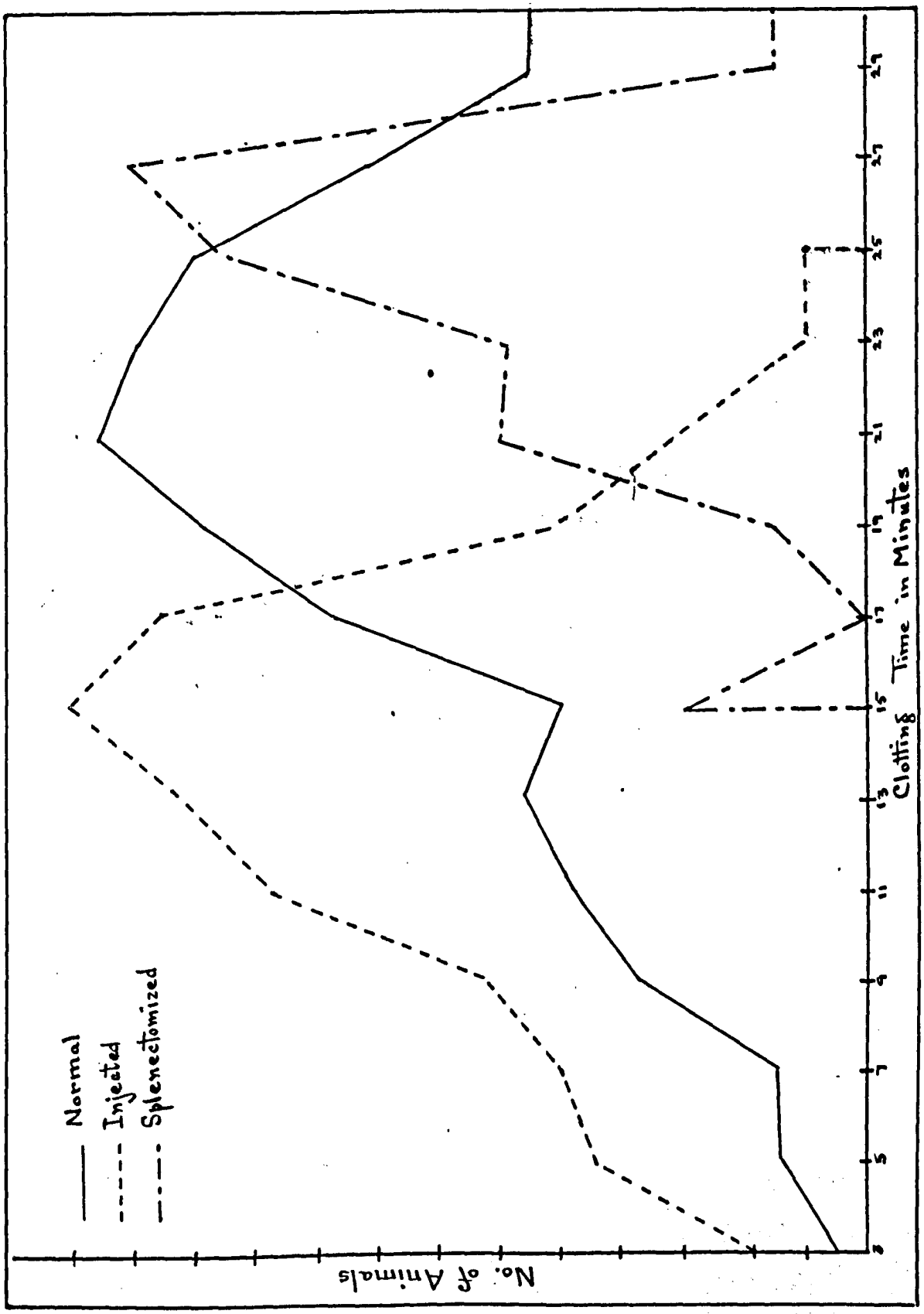
The clotting time was divided into 2 minute intervals and plotted along the abscissa. The animals occuring within each interval were calculated as % of the total number of

TABLE 48.

The Effect of Various Materials on Splenectomized Animals.

Date	Rabbit	Clotting Time in Min. Normal	After Inj.	Effect % of Normal	Material
10/20/30	20	15.0	15.5	00	24A2
"	52	22.5	22.0	00	"
"	58	24.5	23.0	00	"
11/4/30	20	28.0	27.5	00	25A1
"	52	23.0	25.0	00	"
"	58	26.0	23.5	00	"
12/1/30	52	24.0	24.0	00	321
1/24/31	20	21.0	20.5	00	301
"	52	24.0	25.0	00	"
4/8/31	20	25.5	26.0	00	38A1
"	52	26.5	26.5	00	"
11/24/31	28	30.0	33.0	00	423
10/6/30	20	21.5	26.0	00	24A1
3/17/31	394	27.0			

animals andplotted along the ordinate. Such a curve shows a grouping of animals around two peaks. The major peak represents the normal clotting time and is centered around a point corresponding to about 22 minutes. A minor peak represents the short clotting time and is centered at a point corresponding to about 12 minutes. (See Graph I). This is indeed the curve that would be expected if the spleen were to be found in two conditions of activity. In order to verify this conclusion two more graphs were superimposed on this normal. The first curve represents the distribution of normal animals in their periods of clotting time after injection of active materials. Such a curve, whose maximum occurs at about 14 min., includes the minor peak of the normal curve. The second curve represents the distribution ofsplenectomized animals in their relative periods of clotting time. As would be expected this curve comes within the range of the major peak of the normal curve. Its peak, however, occurs at a definitely higher point than the normal, namely 26 minutes. This displacement of the peak of splenectomized animals to a point farther along the abscissa than the normal is significant and will be discussed later. A small hump occurs at about 5 minutes in both the normal and injected animals. This is interprated as due to tissue



GRAPH 1.

juice or serum of blood extravasate into the pericardium which might have been admixed with the sample of blood drawn.

SUMMARY OF THIS SECTION.

We have one very distinct fact presented in this section, namely, that the spleen plays a significant role in the physiological control of blood coagulation. It is evident from the accompanying graph that in the normal, the spleen is found in two states: an inactive state in which the clotting time is comparable with the clotting time of animals whose spleen has been removed, and an active condition in which the clotting time is much shorter than the normal and is comparable with the clotting time of animals whose spleen has been stimulated to activity.

PLATELETS, BLOOD COAGULATION AND THE MECHANISM OF ACTION OF THE SPLEEN.

The bodies known as blood platelets were observed by F. Arnold (1845). They were named platelets by Bizzozero (1882). Wooldridge (1890) observed a precipitate obtained from peptone plasma and identified it as the previously observed blood platelets. He observed that these platelets took an active part in clotting and concluded that they contained his 'A. Fibrinogen'. There soon arose a dispute as to the actual existence of these bodies. Many believed them to be artifacts produced by manipulation and not preexisting in the circulating blood. There is little question left today as to their actual existence, for they have been definitely demonstrated in circulating blood (Clark, 193). In considering the source of platelets we find various theories put forward. Schmidt (1875) considered them to be debris of leucocytes set free when the blood was shed. This, however, has been disproved. They have been considered to be immature red cells and also to be separated segments of broken down erythrocytes. Wright (1906) observed platelet-like bodies separating from pseudopodia of the giant cells (megakaryocytes) of the bone marrow and spleen. He demonstrated the similarity of these to the circulating platelets and considered them to be identical. The work of Wright was widely accepted and generally substantiated by subsequent

work.

The function of platelets in the coagulation of blood as mentioned before was observed by Wooldridge and other early workers. Bordet and DeLange (1913) showed that they will produce thrombin with serum, or in other words, activate the coagulative enzyme (serozyme). Thus platelets would be expected to liberate, on disintegration, a thrombokinase (cytozyme of Bordet, or cephalin of Howell). In fact they do contain cephalin. Mills (1927) has shown that they contain tissue fibrinogen as well as cephalin. Therefore, they would constitute a most potent factor in initiation of clotting when disintegrated by contact with damaged tissue or other surface action.

There is one outstanding piece of work on the disintegration and life of platelets, namely, that of Deetjen (1909). He found that while normal platelets were rapidly disintegrated in contact with ordinary glass, they were stable in contact with quartz slides, thus leaving the platelets adhered to the slide. Now, he was able to test their resistance to reagents by flooding the slide with the solution to be tested. He found that traces of acid or alkali caused an instant disintegration while Mn salts, peptone, hirudin, heparin and H_2O_2 caused them to be more resistent to disintegration. While heparinized platelets were resistent to acid and alkali, it is interesting to note that they were disintegrated by normal serum. This

matter of destructibility may prove of great significance. Minot andLee (1916) have shown that washed platelets from hemophiliac blood were ineffective in clotting normal platelet-free plasma, while washed normal platelets were found to cause normal clotting in hemophiliac blood. Mills (1927) observed that tissue fibrinogen (subcutaneous) caused a more rapid clumping of platelets than normal and noted the same to be true after injection of adrenalin. He also observed that the passage of an electric current in vitro accelerated disintegration and, as Deetjen and others had observed, that peptone inhibited platelet disintegration.

Numerous workers have reported materials affecting the platelet count. Sawtchenko-Matzenko (1909) reported a decrease under the influence of diphtheria toxin. Pio Foa (1916) reported that pyridine destroyed platelets in the circulation. Lio Ruuth (1926) observed a thrombocytosis produced by an extract of rye ergot and concluded from the theory of Wright that it stimulated the bone marrow. Hultgren (1926) observed the same effect for benzene and drew the same conclusions. Brandberg (1927) observed a thrombocytosis produced by the ethyl ester of chaulmoogra oil. Matsumoto (1927) found that injection of secretin caused an increase in the number of platelets per cu. m.m. of blood. Claeson (1928) reported that injection of sodium cinnamate

caused an initial thrombopenia followed by a thrombocytosis and attributed this to an effect on the bone marrow.

It is only in the most recent work that the relation of the spleen to this change in platelet count has been brought out. Binet and Kaplan (1928) found that adrenaline caused a temporary increase in platelets, and that removal of the spleen eliminated the effect. They postulated that the spleen was acting as a reservoir for platelets and that its contraction would throw a large number of them into circulation. Field (1930) found that an increase in the blood platelet count of cats followed excitation, an effect which did not occur in the absence of the spleen.

METHODS USED.

The first method used in making platelet counts was the same as that used by Field. The diluting solution contained NaCl .7 gm. andSodium Oxalate .3 gm. was made up to 100cc. with H_2O. The blood was diluted in a red cell diluting pipet (1:100) with the above solution. It was shaken for 2 min., placed in a standard counting chamber, and allowed to settle for 5 min. before being counted. For convenience the method was later changed to that of Wright. The diluting fluid was made fresh from two parts of stock brilliant crysyl blue solution (1:300) and three parts of a KCN solution (1:1400). The

rest of the procedure was the same as before. The results of the two methods were comparable, but the latter method simplified the counting and was probably more accurate.

Blood was obtained from humans by puncture of the finger after it had been washed with soap and water and cleansed with 95% alcohol. The first drop was wiped off to avoid contamination with materials washed up by the blood. In rabbits, heart puncture was used. It was found that ear puncture was never as good because of the difficulty in getting clean blood. The heart puncture was made with the precautions described in the early part of this paper.

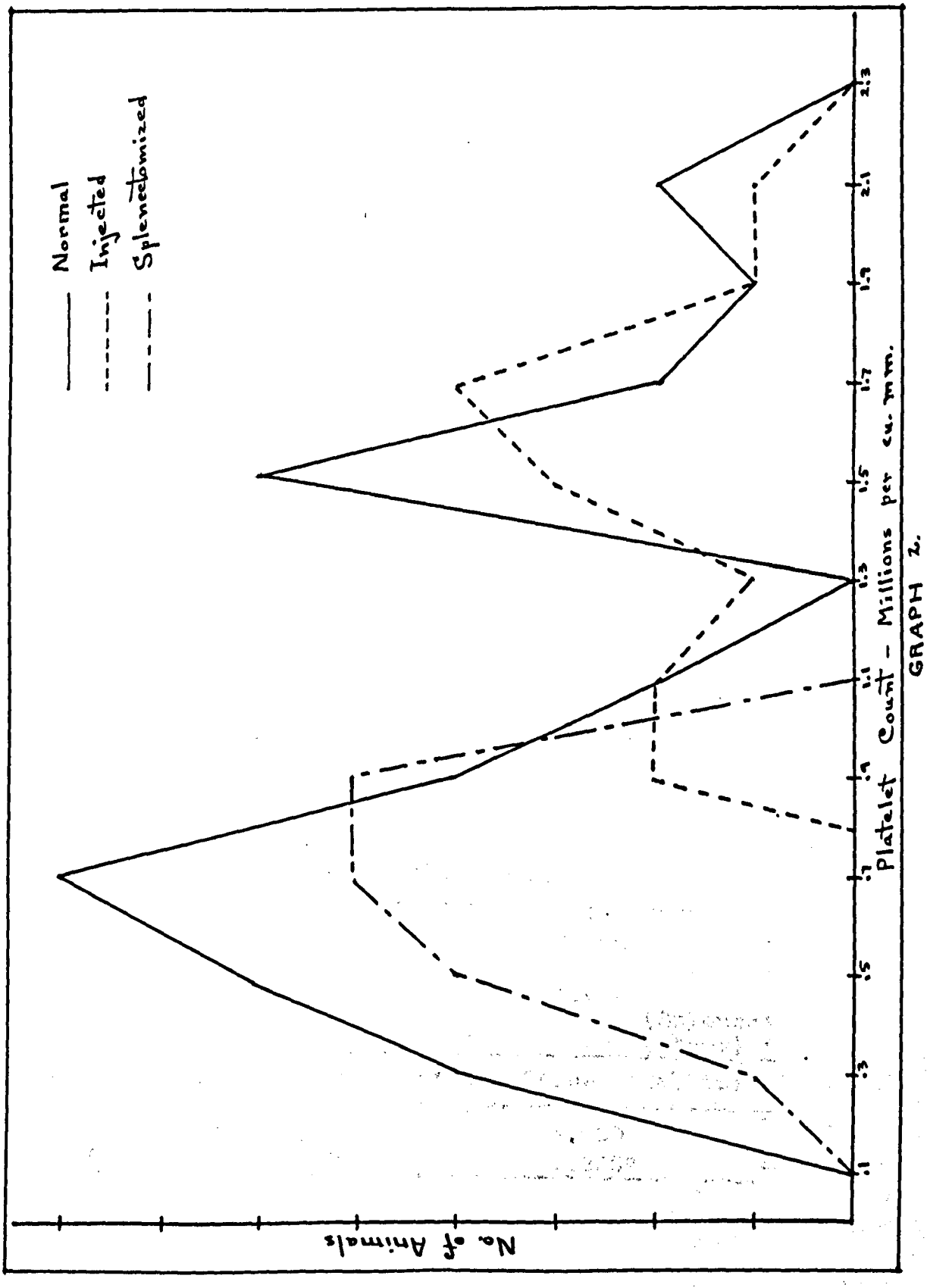
EXPERIMENTAL OBSERVATIONS.

Platelet counts made on the blood of normal rabbits are not consistent. The condition of excitement or the state of digestion both do their part in causing the count to be higher than normal. It is interesting to note that the curve of the normal platelet count gives two peaks very similar to the two peaks given by the normal clotting times as shown in the previous part of this paper. The low count was comparable with the normal long clotting time, and with the low count and long clotting time of the splenectomized animal. The high count was comparable with the short clotting time in the normal animal after injection of materials

which stimulate the spleen. (See graph 2). This is a good substantiation of the reservoir theory of splenic action.

The effect of a number of materials was studied. There are probably three classes of materials which affect a change in the spleen. First, the materials which are irritating or exciting, that is, which act through the suprarenals and adrenaline on the spleen, such as acid or alkaline substances, decomposition products of tissue, etc. Second, the materials which take effect through initiations of the digestive activity, such as food, protein, tissue materials, etc. Third, the materials which act directly on the spleen, such as products of decomposition of tissue, adrenaline, etc. The activity of various materials may be observed in the accompanying table (see Table 49).

Platelet count was determined on human subjects before and after an ordinary meal. (I was able to check the results obtained on myself through the courtesy of Mr. Boyd and Mr. Schmidt of this Department). A very sharp increase was observed. The final count was taken about one hour after a meal, while the normal was taken just before eating. Some of the counts were very high which suggested the possibility of emulsified fat globules confusing the count. To check this, a test was made using coagulated egg white, which is practically all protein. A sharp increase was observed, al-



GRAPH 2.

TABLE 49.

Effect of various Materials on Platelet Count		
A. A Normal Meal (Human Subjects)		
Administration	Normal Count x 10^3	After Injection x 10^3
Oral	700	1,020
"	340	6,405
"	450	1,515
"	595	1,630
"	600	5,020
B. Coagulated Egg White (Human Subject)		
Oral	735	1,695
C. Tissue Fibrinogen (Human Subjects)		
Oral	445	824
"	485	805
"	395	1,725
D. Tissue Fibrinogen (Rabbit Tests)		
Oral	675	2,190
"	530	1,465
"	485	350 (Splenectomy)
E. Acid Alcohol Extracts of the Spleen (Rabbit)		
Intraperitoneal	955	4,000
"	1,800	1,235?

TABLE 49 (Continued)

Effect of Various Materials on Platelet Count			
F. Ether Extract of the Spleen (Rabbits)			
Administration	Normal Count $x10^3$	After Injection $x10^3$	
Intraperitoneal	1,875	1,980 ?	
"	1,760	1,790 ?	
G. Adrenaline (Rabbit tests)			
Intraperitoneal	1,460	4,905	(Splenec-
"	450	420	(tomy)
"	485	475	"
H. Excitement (Rabbit)			
	690	1,025	
I. Ceanothyn			
Oral	1,480	1,445 ?	
J. Hot Water (Human Subject)			
Oral	970	730	

though it did reach the very high counts sometimes observed after meals.

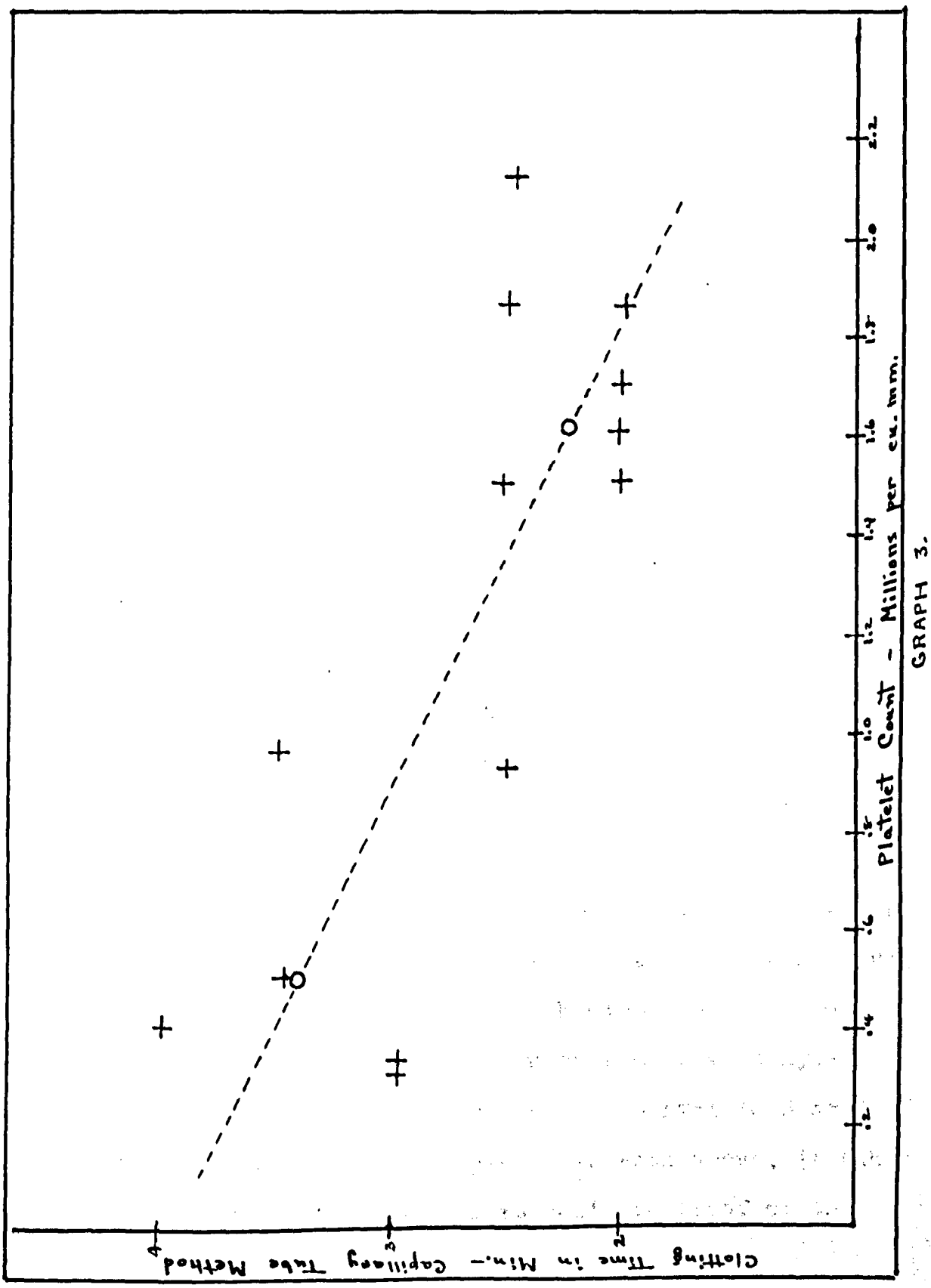
Fresh sterile tissue materials (tissue fibrinogen) showed a sharp increase in platelets when taken orally, both in humans and rabbits. In studying its effect on clotting time it was found to be, as a rule, ineffective when injected intraperitoneally while the same fresh materials showed an effect when taken orally. Old preparations, which had possibly been autolysed or otherwise changed, showed some effect on clotting time when injected intraperitoneally. The above facts may be interpreted in several ways. The fresh preparations taken orally may stimulate the digestive mechanism and through it the spleen. The old preparations through irritation of the peritoneum may act through the suprarenals. It is, however, very possible that there is an active substance which is a partial decomposition product of the tissue materials. This appears in the old preparations which have stood for some time and acts directly on the spleen when injected. The action of the digestive juices may cause the production of this same substance from the fresh tissue material taken orally. There is so far in this work insufficient evidence to prove this statement, and it must be regarded merely as a probable explanation.

PLATELETS AND CLOTTING TIME.

As previously observed, if the test tube method for determining clotting time be refined so as to eliminate as far as possible any surface contact or other factor effecting the disintegration of platelets, the injection of material previously found active has no effect on the clotting time. However, if a method be used which takes into account this surface effect, as did the test tube method first used, the platelet count shows an inverse relation to the clotting time. This is evident if we compare the normal distribution curve of clotting time (graph I)with the normal distribution curve of platelet count (graph II). The capillary tube method of Peterson and Mills (1923) also takes into account the surface effects. The clotting time is so short by this method that the accurate detection of small changes in coagulability is difficult. There are also a number of other sources of error. However, very wide differences in platelet count do give relative changes in clotting time as determined by this method. (See graph III).

PLATELET COUNT AFTER SPLENECTOMY.

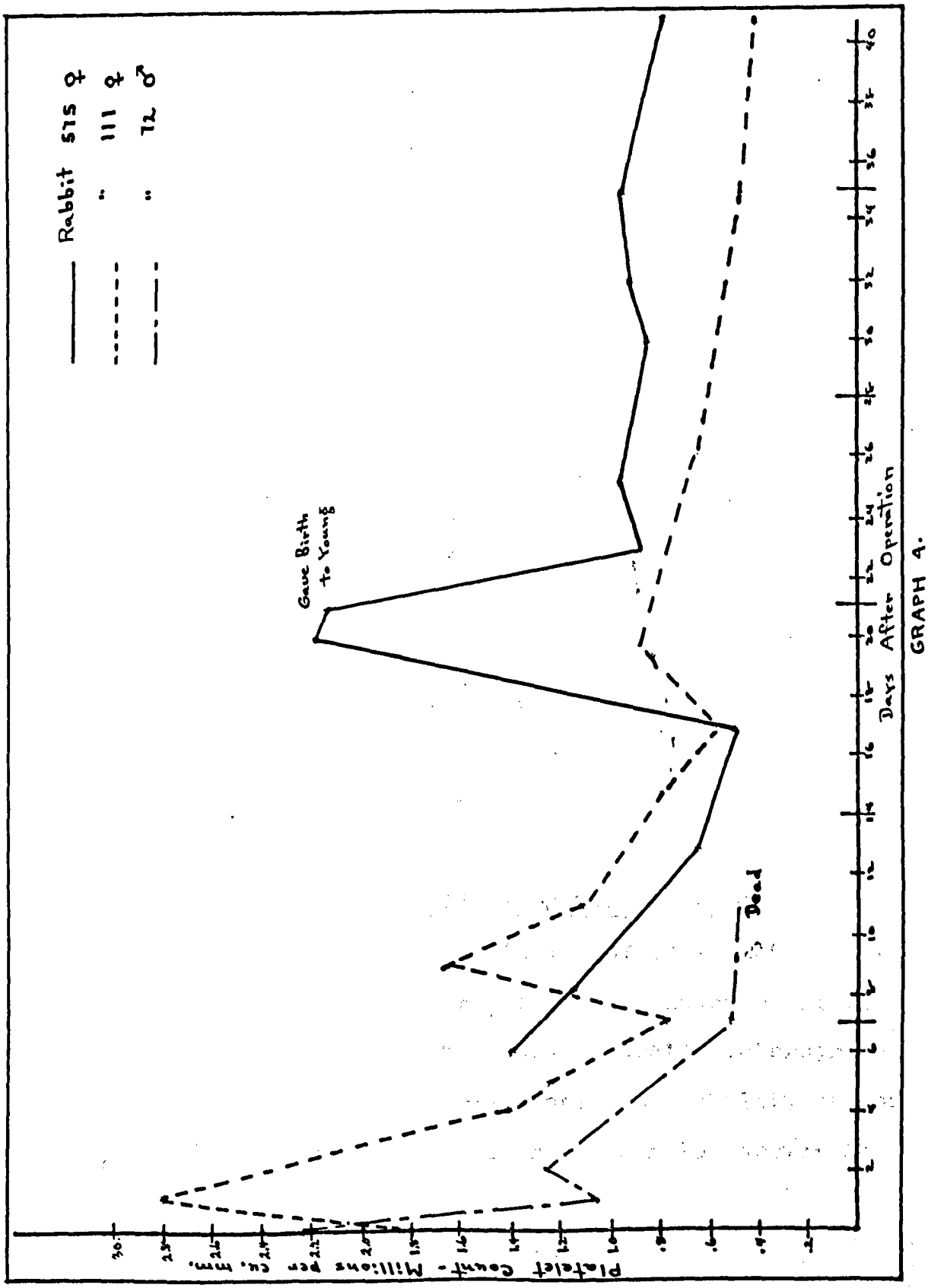
It was observed before that rabbits recovered in a few days from the operative effects of splenectomy. However, the clotting time did not reach its high level (25 to 30min.) until two or three weeks after operation, or at least was



GRAPH 3.

very inconsistent for that length of time. The same fact has been observed with the platelet count. Counts taken within the first two weeks were found, as a rule, to be high. In order to have a clear conception of what was going on, three animals (2 females and one male) were splenectomized. The changes in platelet count were followed, starting immediately after operation. The curves of platelet count for each animal were plotted against days after operation. The count in the case of the two females reached a base level; one in three weeks, the other in about two. The male died in 15 days. His platelet count had reached the base level, but it is of course uncertain whether or not his count would have remained at this level. The first female showed an abnormal jump in the curve during the first two weeks. She was pregnant at the time and gave birth to a litter of young at the peak of this jump in the curve. This may explain the abnormality. The count, however, did reach a base level soon after and remained there indefinitely with only slight variations, as didthe count for the other female. (See graph IV).

Stimulation of the digestive mechanism causes an increase in platelet count as has been stated previously. This abnormal count, however, returned to normal in 3 or 4 hours. In the absence of the spleen, as seen above, it takes 2 or 3 weeks. This is an even more decisive proof of the



GRAPH 4.

reservoir action of the spleen. In the first case the stimulation of the normal spleen caused it to eject its stored platelets into the circulation. Upon cessation of stimulation the spleen started picking up the excess platelets from the circulation. In this way it takes only 3 or 4 hours for the count to return to normal. The excitement of operation and the effect of ether cause a great increase in the platelet count, that is, stimulate the spleen to eject its platelets. In the second case, however, the spleen was removed. In the absence of the spleen it took the body 2 or 3 weeks to take care of the excess platelets. When the normal is reached, the count remains the same for an indefinite period. We can see that there is unquestionably another source of platelets which maintains the normal level. We have lost, however, the reservoir function of the spleen which can be called into action to cause a great increase in platelet count and cause it quickly.

THE SPLEEN AND DISINTEGRATION OF PLATELETS.

Platelets play a role in initiation of clotting by producing tissue fibrinogen and thrombokinase. However, it is evident that the platelets must be disintegrated in order to liberate these materials. In this sense the stability of the platelets takes on a great significance. As noted above: in

the distribution curve of animals against their platelet counts (graph II), the peak of the curve for splenectomized animals parallels very closely the major peak of the normal curve; in the distribution curve of animals against their clotting times (graph I), the peak of the curve for splenectomized animals is sharply displaced, showing a delay in clotting time for splenectomized animals beyond the value for normal animals. This distinct difference between the curve of platelet counts and the curve of clotting times for splenectomized animals, indicates a function of the spleen in controlling coagulability other than the effect of increasing the number of platelets in circulation. It has been reported that the red cells are more stable in the absence of the spleen, and I believe it is reasonable to conclude that the same would be true of platelets. Such a condition would cause a displacement of the curve for splenectomized animals in the same direction as observed above.

CONCLUSION.

There is at least one substance, possibly several, which may be obtained from partial decomposition of tissue, and which causes an increase in the coagulability of the blood on internal injection. Some evidence has been found to indicate that this material is a decomposition product of cephalin, possibly the lyso-compound or the unsaturated fatty acid. The active preparations effect their change through mediation of the spleen as they are ineffective when this organ is removed. They may act directly on the spleen, or through either the digestive mechanism or the suprarenals. The action of the spleen is manifested as an increase in the platelet count. The spleen may also aid in coagulation by augmenting the disintegration of Platelets.

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