NOTE TO USERS

This reproduction is the best copy available.

UMI®

I. THE ACTIVITY OF LUNG EXTRACT, AS COMPARED TO EXTRACTS OF OTHER TISSUES, IN INDUCING COAGULATION OF THE BLOOD

II. CHEMICAL NATURE OF TISSUE COAGULINS

III. THE ACTION OF TISSUE EXTRACTS IN THE COAGULATION OF BLOOD

BY

C. A. MILLS

(SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY, UNIVERSITY OF CINCINNATI, MAY 31, 1920)

REPRINTED FROM
THE JOURNAL OF BIOLOGICAL CHEMISTRY
VOL. XL, NO. 2, DECEMBER, 1919
VOL. XLVI, NO. 1, MARCH, 1921

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. XL, No. 2, 1919

THE ACTIVITY OF LUNG EXTRACT, AS COMPARED TO EXTRACTS OF OTHER TISSUES, IN INDUCING COAGULATION OF THE BLOOD.

BY C. A. MILLS.

(*From the Department of Biochemistry, University of Cincinnati, Cincinnati.*)

(Received for publication, October 14, 1919.)

Wherry and Ervin (1), in experimenting with the intravenous injection of extracts of tuberculous lung tissue, found that very small doses of an extract of normal as well as tuberculous lung tissue would produce death in a few seconds in rabbits and guinea pigs. It was at their request that I undertook to discover the cause of the sudden death in these cases.

The extracts were made by grinding the fresh lung tissue well with sand in a mortar, adding gradually while stirring 10 cc. of 0.9 per cent NaCl for each gm. of tissue taken. The mixture was then centrifuged for 20 to 30 minutes at about 3,000 revolutions per minute and the slightly cloudy, reddish solution used for the injections. It was found by trial that 0.3 cc. of this solution injected *rapidly* into the ear vein of a rabbit weighing 1,000 to 1,500 gm. caused respiratory symptoms of irregular breathing and uneasiness in 20 to 30 seconds, weakness and prostration shortly afterwards, and death with convulsions and respiratory spasms usually within 1 minute after the injection. If a larger dose, such as 0.5 cc., was injected rapidly into a rabbit of this size respiratory symptoms began in 20 seconds and death, accompanied by violent convulsions and spasms, followed within 10 seconds. For rabbits of 1,800 to 2,500 gm. the dose necessary to produce death was 0.4 cc. so that the reaction is in a measure quantitative.

On examination of the rabbits immediately after cessation of the spasms, the heart was usually found beating rhymically, although there were sometimes arrhythmias and fibrillation. All organs appeared normal, the lungs always being found collapsed

after opening the thorax. On opening the heart or vessels, larger or smaller clots were always found and sometimes the whole blood was found to be coagulated, especially if more than the minimum fatal dose was given. In cases where the whole blood was not clotted, the escaping fluid portion clotted more slowly than normal, the length of time before the occurrence of spontaneous clotting varying from a few minutes to a day.

These observations lead to the conclusion that in these cases death was due in a large measure at least to intravascular clotting, the symptoms probably resulting from the complete asphyxia of the nervous system. Although the death resembled that of anaphylaxis, yet in contradistinction to the latter the lungs were always found to collapse on opening the thorax, whereas in deaths from anaphylaxis the lungs do not collapse.

I therefore undertook the study of the particular power of lung tissue in causing thrombosis, as I was unable to find any reference to the peculiar toxicity of lung extracts in this respect. This study has resulted in showing the very remarkable thromboplastic power of the lungs, a power far surpassing that of any other tissue of the body.

Intravascular coagulation from the intravenous injection of tissue extracts has been known to be possible for many years. Quoting from Carpenter (2),

> "The contact of dead animal matter with the blood appears to promote the coagulation of its fibrin in a very remarkable degree; occasioning coagula to form, whilst it is yet actively moving in the vessels of the living body. Thus M. Dupuy found that the injection of cerebral substance into the veins of an animal occasioned its death almost as instantaneously as if prussic acid had been administered; the circulation being rapidly brought to a stand, by the formation of voluminous clots in the heart and large vessels. These experiments were repeated and confirmed by M. de Blainville. (Gazette Medicale, 1834, p. 521.) The same effect is produced with still more potency, when the substance injected is rather undergoing degradation, than actually dead; for it then seems to act somewhat after the manner of a ferment, producing a marked diminution in the vitality of the solids and fluids with which it may be brought in contact. Such is pre-eminently the case with *pus*, as was long ago observed by Hunter and as Mr. H. Lee has since determined more precisely. It was found by the latter, that healthy blood received into a cup containing some offensive pus coagulated in *two* minutes; whilst another sample of the same blood, received into a clean vessel of similar size and shape, required *fifteen* minutes for its complete coagulation."

Wooldridge (3), working from 1881 to 1889, was the first, however, to attempt to isolate the active thromboplastic material from the tissue extracts and to make a thorough study of the action of such extracts. He used extracts prepared from testes, thymus, and lymph glands, extracting the fresh tissues with water and precipitating the active material from the solution by making it strongly acid with acetic acid. The precipitate was then washed in water and dissolved in very dilute carbonate solution. Such solutions he found to produce thrombosis throughout the whole vascular system of rabbits after rapid intravenous injections, but in dogs, clots were usually found only in the portal system. In any case, if the animal survived the thrombosis, a second injection within 24 hours was without effect, and blood drawn after such injection had a greatly diminished coagulability, spontaneous coagulation often being delayed as long as 24 hours. The remarkable fact was discovered that the addition of more of the tissue extract to this blood outside the body produced coagulation in a few minutes. He observed that the degree of non-coagulability of the blood was in a measure proportional to the extent of the thrombosis.

In studying the extracts to determine the active substance, he decided that a protein-phospholipin compound was responsible for the results. This compound could be extracted from the acetic acid precipitate with dilute alkali. He states that the solution was not a true solution since the dissolved substance would not pass through a clay cell. If he extracted this precipitate with alcohol and ether the activity of the undissolved residue was lost, so that the phospholipin must be a necessary part of the compound. Also on digestion of the solution with pepsin and hydrochloric acid, the phospholipin, with a small amount of the protein, was precipitated, and the remaining solution had lost its activity. The precipitate was active. Examination of the phospholipin convinced him it was a lecithin-like substance, although purified lecithin from egg yolk was not active. Lecithin prepared from other tissues gave him the same results as the tissue extracts. Wooldridge was not familiar with cephalin at that time, so that he did not identify the active phospholipins as cephalin which was present as an impurity in his tissue lecithin, although he did show that not all lecithin prepa-

rations were active. More recent work in Howell's laboratory (4) indicated that the phospholipin inducing coagulation discovered by Wooldridge is cephalin, but the strange fact appeared from my experiments that although the nervous tissue is the tissue believed to be the richest in cephalin, yet extracts of the brain made in the same way as these lung extracts did not cause intravascular coagulation and death in rabbits in doses up to 3 cc. for an 1,800 gm. rabbit. It was therefore deemed advisable to compare as quantitatively as possible the thromboplastic activity of lung tissue extracts with that of extracts of other tissues of the body.

Fresh tissues of dogs killed in ether anesthesia and rabbits were used in making extracts similar to the lung extract described above, that is, for every gm. of the fresh tissue, after grinding thoroughly with sand, 10 cc. of 0.9 per cent NaCl solution were added, mixed well, and centrifuged to remove all solid particles. Extracts thus made were found to undergo a gradual loss of their power to induce coagulation when injected into the circulation or of hastening coagulation when added to blood outside the body. When standing at 5°C. very little change occurred in the first few days, but activity had almost disappeared at the end of 3 weeks. This loss of activity was not due to a settling out of the active matter formerly in suspension, since on shaking and injecting, or adding precipitate and solution mixed, the diminution of activity persisted. It might be, however, that a gradual agglomeration of particles had occurred leading to a smaller number of larger aggregates with a resulting diminution of surface and hence of activity. This rather than a chemical change might explain the progressive loss of power. This possibility will be further investigated.

The activity of the tissue extracts prepared as described were tested in two ways, (1) by injections into the blood stream of dogs and rabbits, and (2) by testing their power of hastening coagulation when added to peptone and oxalate plasma outside the body. In the latter case the test-tube method for determining the coagulation time was used, coagulation being considered complete when the tube could be inverted without spilling the contents. If care was taken in shaking the tubes and in keeping all other factors constant, this method was considered sufficiently accurate for this work.

Peptone plasma and oxalated plasma of dog's blood were used in most of the tests, although two sets of rabbit tissue extracts were tested on rabbit blood rendered partially non-coagulable by a process which will be described in another paper.

In order to compare the activity of the extracts of the different tissues, one method was to find the amount of the various extracts necessary to induce coagulation of 1 cc. of peptone plasma in the same time as a definite amount of lung extract. Another method used was to add the same amount of the different extracts to oxalate plasma and compare the amount of acceleration of coagulation of the plasma by serum. All tests were carried out in a water bath at 38–40°C.

Tables I and II indicate the results of the tests carried out with the extracts of the tissues of two different dogs.

The pancreas, skeletal muscle, thyroid, and omentum contained so little of the thromboplastic material that they would not accelerate the coagulation to 30 seconds in any amount, so the figures for them are approximations derived from the degree of lessening of coagulation time by increasing amounts of the extracts.

TABLE I.

Tissue extract used (dog).	Time of coagulation of 1 cc. of oxalate dog plasma, by 3 drops of extract and 6 drops of serum.*	
	Tissues of Dog I.	Tissues of Dog II.
	min.	*min.*
Lung	½	½
Kidney	4	4
Testes	7	6
Brain	8½	6
Heart	8	7
Spleen	9	4
Bone marrow	7	7
Adrenal	8	8½
Liver	11	8
Omentum	9	16
Skeletal muscle	14	12
Pancreas	14	14
Thyroid	14	15

* 1 cc. of oxalate plasma and 6 drops of serum (with no extract) showed no coagulation in 1½ hours, but were coagulated in 24 hours.

TABLE II.

Tissue extract used (dog).	Amount of extract necessary to coagulate 1 cc. of peptone dog plasma in 30 seconds.	
	Tissues of Dog I.	Tissues of Dog II.
	gtt.	*gtt.*
Lung	2	2
Kidney	5	5
Testes	8	15
Brain	7	6
Heart	20	12
Spleen	4	4
Bone marrow	20	20
Adrenal	6	20
Liver	25	20
Omentum	30 +	30 +
Skeletal muscle	30 +	30 +
Pancreas	40 +	30 +
Thyroid	30 +	20 +

The activity of extracts of the tissues of two rabbits on partially non-coagulable rabbit blood is shown in Table III.

TABLE III.

Tissue extract used (rabbit).	Amount of extract necessary to clot 1 cc. of rabbit blood.	
	90 seconds. Tissues of Rabbit I.	70 seconds. Tissues of Rabbit II.
	gtt.	*gtt.*
Lung	1	1
Kidney	5	5
Heart	5	5
Thymus		3
Spleen	8	4
Brain	8	13
Skin		10
Testes		20 +
Ovary	22	
Uterus	20	
Liver	30 +	30
Bone marrow		20 +
Pancreas		20 +
Adrenal	26 +	20 +
Omentum	30	
Skeletal muscle	30 +	30 +

Here again some of the tissues were so poor in the active substance that they would not clot the blood in the specified time in any amount, so that the figures given are only approximations.

In every case studied the lung extract was found to be much stronger than that from any other tissue, being from two to thirty times as strong as the other tissues in accelerating coagulation. Kidney, heart, brain, spleen, thymus, and skin come next in activity, somewhat in the order named. Then, certain other tissues, pancreas, skeletal muscle, liver, bone marrow, omentum, and adrenal, showed only slight thromboplastic activity as compared to the lung tissue.

Having thus established the predominant thromboplastic power of the lung extracts I next attempted to discover whether the toxicity from intravenous injections of these extracts paralleled their thromboplastic activity. To this end injections were made into rats and rabbits. In using rats the injections were made directly into the heart, but with rabbits injections were into an ear vein. Adult white rats, weighing about 350 gm., were used. They were found to be much less affected by partial coagulation of the blood in the vessels than rabbits, quick death being produced only by almost solid intravascular coagulation following injections of relatively large doses of the extracts. Most of the extracts of rabbits and rat tissues would not produce coagulation in rats in doses up to 3 cc. when made up in the usual way, so double strengths of these extracts were used, that is, only half the usual amount of saline solution was used for each gm. of fresh tissue. These extracts were labeled ($\times 2$). Table IV indicates the dosages of the various extracts necessary to produce rather extensive coagulation in the vessels of the rats.

In intravenous injections into rabbits only two of the tissue extracts proved fatal, death occurring in rabbits after only slight thrombosis. Lung extract was fatal in doses of 0.3 to 0.4 cc. and kidney extract ($\times 2$) was fatal in a dose of 2.0 cc. producing only a slight amount of thrombosis, and with death occurring after a delay of $2\frac{1}{2}$ minutes. None of the other tissue extracts was fatal in doses up to 3.0 cc. Wooldridge reports solid thrombosis of the whole system in rabbits with injections of thymus, testes, and lymph gland extracts, but he used many times the amount of material for injections that was used in these cases.

Thus he used ½ to 1 gm. of the washed acetic acid precipitate from the extracts to obtain such solid intravascular coagulations.

From these intravenous injections into rats and rabbits as described above, it is evident that the extracts are toxic in much the same order as they exhibit thromboplastic activity in extravascular coagulation, lung extract being much the most active of all tissue extracts in each case.

Most hemostatic preparations on the market today are prepared from brain material, but from the results of the above experiments it would seem that lung tissue would afford a much stronger hemostatic. Battelli (5) describes a method for preparing such material from fresh horse or sheep lungs.

TABLE IV.

Extract used.	Coagulative dosage for rats.
	cc.
Rat lung extract	0.5
" kidney "	1.0
" " " (×2)	0.9
" brain " (×2)	1.1
Rabbit lung extract	0.4
" kidney " (×2)	0.9
" brain "	3.0
" " " (×2)	1.0
" heart " (×2)	1.5
" liver " (×2)	2.0

The significance of such a strong thromboplastic activity in lung tissue, and not so very much less in kidney tissue, is an interesting question. Is it a protective mechanism against possible hemorrhages in these organs in which the rich capillary network is covered only by a one celled layer of epithelium? This high activity of the lung tissue may perhaps have an especial function in certain diseases, such as pneumonia, where there occurs such an extensive destruction of lung tissue. With the extremely rich blood supply in all parts of the lungs one would expect severe hemorrhages in these cases, but the hemorrhages are, in fact, not one of the main characteristics of this disease. May it not be the liberation of this active material by the tissue destruction which protects against extensive hemorrhages? The

one extract of the skin tested was also found to possess considerable thromboplastic activity, again denoting a possible protective mechanism.

The investigation of the nature of the active principle in lung extract is being carried further and will form the subject of another paper.

SUMMARY.

1. Sudden death from the intravenous injection of lung tissue extract is apparently due to intravascular coagulation, although there is a possibility of anaphylactic effect on the lungs, not sufficient, however, to keep them from collapsing on opening the thorax.

2. Tissue extracts were tested as to their thromboplastic activity on the blood both intra- and extravascularly. Lung extracts were found far more active than the extracts of any other tissue, kidney coming second, and then heart, brain, spleen, thymus, testes, skin, somewhat in the order named. The remaining tissues were weakly active as compared to lung, some of them showing very slight thromboplastic action.

3. Lung tissue offers a possible source for the preparation of a strong hemostatic.

4. The strong coagulative activity of lung and kidney tissues, and to a lesser degree of skin, is suggestive of a possible protective mechanism against hemorrhage.

BIBLIOGRAPHY.

1. Wherry, W. B., and Ervin, D. M., *J. Infect. Dis.*, 1918, xxiii, 240.
2. Carpenter, W. B., Carpenter's principles of human physiology, London, 1869, 239.
3. Wooldridge, L. C., On the chemistry of the blood, and other scientific papers, London, 1893, Croonian lecture on the coagulation of the blood.
4. Howell, W. H., *Am. J. Physiol.*, 1912–13, xxxi, 1.
5. Battelli, F., *Compt. rend. Soc. biol.*, 1910, lxviii, 789; abstracted in *Chem. Abstr.*, 1910, iv, 2513.

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. XLVI, No. 1, March, 1921

CHEMICAL NATURE OF TISSUE COAGULINS.*

BY C. A. MILLS.

(From the Biochemical Laboratory, University of Cincinnati, Cincinnati.)

(Received for publication, August 18, 1920.)

The importance of tissue extracts in hastening the coagulation of the blood has roused interest for a great many years. Carpenter in his "Principles of human physiology," 1869, states that in 1834 it was known that brain tissue, or an emulsion of it, often caused intravascular clotting of the blood when mixed with it by accident or intention (for exact quotation see (1)). Nothing was done, however, to determine the chemical nature of this active substance or the manner of its action on the blood until 1883 when Wooldridge (2) began his work in this field. The author wishes to state here that Wooldridge's views on the coagulation of blood and the action of tissue extracts have been of the greatest value and inspiration during the course of the present work. Very little has really been added to the knowledge of intravascular coagulation since his death.

Working with watery extracts of the testes, thymus, lymph glands, and red blood cells, Wooldridge found that the active material was precipitated by strong acidification with acetic acid, and this precipitate, washed in water, was soluble in dilute sodium carbonate. He stated that 1.5 gm. of this precipitate would kill a dog of ordinary size by intravascular clotting and that 0.5 to 1.0 gm. was sufficient to kill a rabbit. Extraction of the precipitate with alcohol and ether to remove the phospholipins was found to destroy its power to produce intravascular clotting, although it did not render it entirely insoluble in dilute alkali. Digestion with pepsin-HCl produced a precipitate which contained all the phospholipin content of the material together with a little of the protein. Neither the solution nor the precipitate from the digestive mixture would cause clotting in the vessels, so he concluded that the important substance was a protein-phospholipin compound and that the phospholipin fraction was essential for its coagulative action although it alone would not produce intravascular coagulation. It would, however, accelerate clotting *in vitro* of peptone plasma. Wooldridge called this phospholipin lecithin, although he showed that pure lecithin from the yolks of eggs was inactive. He was not familiar with the cephalin class of phospholipins at that time and so could not know

* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, University of Cincinnati, May 31, 1920.

that it was probably cephalin he was working with. This has been shown to be the case by more recent workers, especially by Howell (3). But later workers have missed an important point in Wooldridge's work and have assigned to cephalin the entire coagulative action of tissue extracts. Yet Wooldridge satisfied himself that the active agent was a protein-lecithin combination and that the lecithin present was essential to the activity of the compound. The present work will be seen to bear out Wooldridge's views in regard to the relative importance of the protein and phospholipin in the compound, since a crude laboratory preparation of cephalin does not possess 1/1,000th the activity toward oxalate plasma that is found in a certain protein-phospholipin compound isolated from tissue extracts.

As for Wooldridge's views on the manner of action of this material in causing intravascular clotting, they will be reviewed in the following paper dealing with the author's work in that line (4).

Following up the work of Wooldridge, Wright (5) brought forth the view that the substance was a nucleoalbumin, although nucleic acid alone possessed no activity in coagulation.

Brieger and Uhlenhuth (6) claimed to have discovered the toxicity of organ extracts, making no mention of Wooldridge's work. They produced death in animals by the subcutaneous injection of the toxin, and so missed the coagulative action of the material. It is likely that they were working with the same sensitive compound, however, since they found that it could be extracted with dilute alkalies, was destroyed by acids, was thermolabile, losing its toxicity at 80°C. (30 minutes), and was precipitated almost quantitatively by $(NH_4)_2SO_4$ and chlorides of the heavy metals. Since it was not affected by dialysis, and from the other facts mentioned above, they classed it with the toxalbumins. Dold (7) studied this same toxic material, noting that its toxicity was decreased by treatment with serum. No coagulative action was noted. Dold and Ogata (8) noticed the intravascular coagulation and the delayed coagulation of the remaining fluid blood. They found hirudin to prevent the toxic action of the extracts, as also did a previous injection of peptone. Extraction with ether and alcohol destroyed the activity so they decided it was of a ferment nature. Dold and Kodama (9) rediscovered the fact that treatment with acid to precipitate the proteins removed the toxin, but that resolution of the acid precipitate in dilute alkali restored the toxicity. They also found the material to be completely precipitated by saturation of the extract with $MgSO_4$. Heating to 60–70°C. destroyed the toxicity of rabbit lung extracts, but extracts of guinea pig lungs were not completely detoxicated by heating to 100°C. for some time. Izar (10) removed the toxin from extracts by shaking with chalk, animal charcoal, or brain emulsions. He also found that the cations of certain salts detoxicated the material. Blood serum did the same. Dold and Ogata (11) found that it was not necessary to break up the cells to obtain a toxic extract. By work on different parts of the eye, they concluded that the amount of toxic substance obtained was proportional to the lymph present rather than the cells. Popielski (12) described vasodilatin, which he obtained from peptone solutions and from tissue extracts.

This gave the same fall in blood pressure as did tissue extracts, but was without their coagulative action, or possessed a negative effect on coagulation. He claimed that it was not histamine although their actions were similar. Aronson (13) also isolated a toxic organic base from tissue extracts, which acted like histamine, giving bladder peristalsis and acute death. Biuret test was negative on this substance. Dale and his coworkers (14) isolated a number of toxic amines from putrid tissues, chief among these amines being histamine. They also found this amine to be present in the walls of the intestine and in extracts of other tissues, but believed it was not present except as formed from histidine by bacteria. Ackermann (15) and Mellanby and Twort (16) showed that such a formation of histamine by decarboxylation of histidine was readily brought about by bacterial action, especially by a certain bacillus of the colon group. Abel and Kubota (17) decided that histamine was present in all tissue extracts, especially extracts of the posterior portion of the pituitary gland, hydrolysis alone, without bacterial action, being sufficient to free the histamine from the protein material. Jackson and Mills (18), however, demonstrated that pituitary extracts need not contain any histamine in order to possess their typical action, although some pituitary preparations do apparently contain very small amounts of the base.

Morse (19) in a recent article refers to work in which he took part a few years ago and in which it was concluded that the toxic agent of tissue extracts was related to the phospholipin content of the tissues, being found bound with nucleoproteins, so that it precipitates with them. Different samples of nucleic acid were found to be without action. The inference is that the toxicity of the extracts is due to their phospholipin content, although Wooldridge showed clearly about 35 years ago that the protein fraction was as essential as the phospholipin.

There seem, then, according to the work in the past, to be two different toxins present in tissue extracts, one causing blood to coagulate, and the other causing a great fall in blood pressure and giving other histamine-like reactions. The latter toxin was found by several investigators to delay the coagulation of blood.

It will be shown in the present communication that Wooldridge was essentially correct in his conclusion that the active agent of tissue extracts was a protein-phospholipin compound, and that the phospholipin alone, although possessing a slight effect on blood coagulation, can account for only a very small fraction of the total activity of the extract. It will also be shown that splitting of the compound destroys its activity, and that reunion of the two fragments restores its action. Neither the protein nor phospholipin alone shows more than a slight activity, not sufficient to cause death when injected in any reasonable amount; but the

combination of the two forms an exceedingly powerful coagulant for blood.

As to the possible presence of histamine in tissue extracts, it will only be shown here that there is none in the purified coagulant. This does not exclude its presence among the other constituents of the extracts.

EXPERIMENTAL.

Since lung tissue was found to contain much more of the active material than any other tissue of the body, it was used principally in the course of this work. The lungs of dogs, rabbits, and cattle were used, all being about equally active. All tests for the active material were made on oxalated beef plasma since it was found possible to obtain fresh plasma in any amount and at any time from the abattoir. This gives in some ways an ideal testing fluid when fresh, for by means of it a noticeable shortening of the coagulation time can be detected with lung extract diluted several thousand times. The coagulation time of the plasma by calcium is found to lengthen gradually on standing in the ice box, due to a settling out of a granular precipitate—the A-fibrinogen of Wooldridge, which is so important in coagulation. This fact does not in the least interfere with its value as a test fluid for the presence of coagulation-accelerating agents. With proper care in measuring the fluids used, in keeping a constant temperature, and in handling the tubes, duplicate tests will usually vary less than 5 seconds in the coagulation time. With very rapid clotting the variation is much less.

Table I will serve to show how the coagulation time varies with the strength of the tissue extract used.

The lung extract used in these tests was a saturated extract made by powdering beef lung, previously dried in an air current at room temperature, and grinding well with 0.9 per cent NaCl solution in the ratio of about 4 cc. of salt solution to 1 gm. of dried lung tissue. Only a few minutes were necessary to insure saturation of the extract with the active substance, so that it must be readily soluble in dilute salt solution. Using a smaller proportion of salt solution does not result in a stronger or more active solution, as comparative tests on the coagulative activity showed. A larger amount of salt solution per gm. of dried lung

may be used and a saturated extract still be obtained, if a longer time is given for extraction.

From such a dilution table, the amount of active material in other extracts may be estimated merely by determining the amount of hastening of coagulation produced by them and then finding the dilution of lung extract that will produce coagulation in the same time.

TABLE I.

Dilution of Lung Extract.

1 cc. oxalate plasma, 0.04 cc. lung extract, 0.2 cc. 1 per cent $CaCl_2$.

Dilution.	Log of dilution.	Coagulation time.	Log of coagulation time.
		seconds	
1/1	0.00000	10	1.00000
1/2	0.30103	12	1.07918
1/4	0.60206	14	1.14613
1/8	0.90309	17	1.23045
1/16	1.20412	20	1.30103
1/32	1.50515	25	1.39794
1/64	1.80618	30	1.47712
1/128	2.10721	40	1.60206
1/256	2.40824	55	1.74036
1/512	2.70927	65	1.81291
1/1,024	3.01030	80	1.90309
1/2,048	3.31133	100	2.00000
1/4,096	3.61236	120	2.07918
1/8,192	3.91339	140	2.14613
1/16,384	4.21442	170	2.23045
1/32,768	4.51545	200	2.30103
1/65,536	4.81648	240	2.38021
1/131,072	5.11751	250	2.39794
None.		250	2.39794

A chart, showing that the logarithm of the dilution of the extract plotted against the logarithm of the coagulation time gives almost a straight line, is to be found in the following paper (4). A formula for the line is also suggested there, together with the method of calculating the strength of any solution in terms of lung extract, and also for calculating the actual concentration of the active material as mg. per cc.

1. Phospholipin Extraction of Dried Tissues.—In order to study the rôle of cephalin in the action of tissue extracts on blood clot-

ting, phospholipin extraction by ether and alcohol was carried out. Dried lung tissue was extracted four or five times successively with boiling 85 per cent alcohol and ordinary anesthesia ether under a reflux condenser. Quantitative figures for this extraction will be given in a later paper. This crude phospholipin material, after evaporation of the ether and alcohol on a water bath, was tested for activity on blood coagulation. About 0.5 gm. of it was rubbed up thoroughly with 10 cc. of 0.9 per cent NaCl solution to a fine milky emulsion which remained emulsified for weeks.

Intravenous injections of this emulsion into rabbits in amounts up to 5 cc. at a single injection were ineffective, whereas 0.1 cc. of the saturated lung extract would produce death in less than a minute. Blood clotting *in vitro* was quickened slightly, but quite definitely, by the phospholipin emulsion, the quickening being about the same as that induced by saturated lung extract diluted 10,000 times with 0.9 per cent NaCl (see Table II).

TABLE II.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Coagulation time.
	min.
None	$4\frac{1}{2}$
0.2 cc. phospholipin emulsion	$3\frac{1}{2}$
0.04 " lung extract (× 1/10,000)	3

Phospholipin extracts were also made at room temperature by the use of absolute ether, benzene, chloroform, carbon bisulfide, and carbon tetrachloride on separate portions of the dried lungs. The solvent in each case was evaporated at room temperature in an air current. The extracted phospholipin was tested as described above.

The results were similar to those obtained in the preceding experiment. It was noticed that this activity of the phospholipin decreased on standing exposed to the air.

"Laboratory cephalin," obtained from Armour and Company, was also emulsified similarly and tested. The same, or slightly less, activity was found.

The lung tissue freed from phospholipins by boiling alcohol and ether was finely ground with sand and extracted with 0.9 per cent

NaCl solution to see if any active material was left. Such extraction yielded practically nothing, since the proteins had been rendered insoluble by the boiling alcohol. No trace of activity was found in the saline extract. Similar extraction of the lung tissue previously extracted with benzene, chloroform, etc., at room temperature, yielded an extract rich in proteins, but with only about 1/10,000th the activity possessed by a similar extract of untreated beef lungs.

It was possible that the phospholipins were oxidized or otherwise changed in the process of their removal from the lung tissue and evaporation of the solvent. Still the dried lung tissue can be kept exposed to the air and light at room temperature for months with only a slow loss of its action on blood coagulation.

2. Shaking the Watery Lung Extract with Ether.—Since it was thought that the active compound might be extracted from the watery extract, prolonged and vigorous shaking with ether was tried. Following such shaking three layers always separated. The top layer consists of ether with some dissolved fatty material which is without detectable action on blood coagulation. The bottom layer is the watery extract, but with its activity considerably reduced. Repeated extractions with ether will remove very nearly all the active material from the watery extract. The middle layer consists of solid protein material which is very active. It is not coagulated or changed by the ether, apparently, but separates out because of having collected in the surface film surrounding the ether globules and as these coalesce and rise to the surface, the material in the surface films is carried along.

This separation out in a surface film on shaking with another liquid insoluble in water is not peculiar to this protein, but will also occur with the casein of milk and egg albumin in solution.

3. Precipitation of the Active Material by Salting out.—The protein fraction of the extract was now examined.

Half saturation with $(NH_4)_2SO_4$ gave a grayish white precipitate which contained all the active material. The precipitate, redissolved in 1 per cent sodium chloride solution and made back to the original volume, contained all its original activity in hastening coagulation. Complete saturation of the extract after removal of the precipitate separating at half saturation gave about the same amount of protein precipitate, but this was inactive.

Complete saturation with $MgSO_4$ or NaCl gave the same results as half saturation with $(NH_4)_2SO_4$.

As regards "salting out," the active material behaves like a globulin. It does not redissolve well in pure water, but requires the addition of a small amount of NaCl.

4. Precipitation with $HgCl_2$ and Sulfosalicylic Acid.—To a 0.9 per cent NaCl solution extract of finely ground lung tissue was added a 1 per cent $HgCl_2$ solution, drop by drop, until no further precipitation resulted, being careful to avoid any large excess of the $HgCl_2$. The precipitate was collected on a filter, suspended in salt solution again, and the mercury removed by H_2S. The HgS was allowed to settle out and the protein solution decanted. No attempt was made to remove the excess of H_2S, as it seemed to be without effect on the activity of the material. The solution thus obtained possessed the full activity of the original extract. The same volume was maintained so that comparative tests could be made.

The filtrate, after precipitation of the extract with $HgCl_2$, was without a trace of activity on blood coagulation.

Precipitation of another portion of the same extract with 3 per cent sulfosalicylic acid, added carefully drop by drop to avoid excess, removed all the active material in the precipitate, the clear filtrate being left with no trace of activity. No effort was made to recover the active substance from its union with the sulfosalicylic acid. This could probably have been done by making slightly alkaline with ammonia and dialyzing to remove the ammonium salt of the sulfosalicylic acid.

Either of these two methods would be very likely to remove the phospholipins from a solution with the proteins so that such precipitaton has no bearing on the question whether the activity depends on the proteins present, or on the phospholipins.

5. Effect of Heat on the Activity.—By keeping the extract in a water bath and slowly raising the temperature it was found that the greater part of the proteins coagulated at 56–57°C. only a small amount being left to coagulate at 70–75°C. After removing the proteins coagulating at 56–57°C., the remaining solution has only a faint trace of activity on coagulation.

Heating the extract below 57°C. slowly destroys its activity without actually producing a precipitate. Table III shows this.

The heating above 45°C. produces an opalescence in the extract which probably indicates a partial coagulation of the proteins, without an actual precipitation until 57°C. is reached. This slight change in the proteins, however, at once decreases the coagulative activity of the extract.

Heating dried lung tissue also decreases the amount of active material that may afterward be extracted from it. Heating at 100°C. for $2\frac{1}{2}$ to 3 hours leaves only about one-sixteenth of the original amount of active substance to be extracted by 0.9 per cent NaCl solution. Heating for 24 hours at 110°C. completely destroys all activity of the material. All the proteins are coagulated, apparently, for extraction with salt solution yields practically nothing.

TABLE III.

1 cc. oxalate plasma, 4 drops 1 per cent $CaCl_2$.

Lung extract (0.9 per cent NaCl).	Coagulation time.
None	7 min.
1 drop	45 sec.
1 " (kept at 46°C. for 30 min.)	60 "
1 " (" " 50°C. " 12 ")	60 "
1 " (" " 54.5°C. " 8 ")	2 min.
1 " (" " 56°C. " 10 ")	$2\frac{3}{4}$ "
1 " (" " 56–57°C. " 12 ")	$4\frac{1}{2}$ "

Autoclaving the fresh lung at 15 pounds pressure for 15 minutes leaves only a trace of active material to be extracted.

6. Berkefeld Filtration.—Filtering the extract through a Berkefeld filter leaves only a faint trace of activity in the filtrate. The substance must be in large molecules or aggregates, although it will pass through ordinary filter paper until the pores of the paper become clogged with the material, after which filtration even under suction is very slow. The activity of the filtrate decreases as the filter becomes clogged with the material.

7. Effect of Hydrolysis or Digestion on the Activity of the Extract.—Roger (20) showed that sterile autolysis of tissue extracts served to destroy their toxicity. Wooldridge also showed that digestion with pepsin-HCl destroyed the activity by digesting off the protein fraction. However, in autolysis cephalin might also be hydrolyzed, and in digestion by pepsin-HCl, the acid might

affect the cephalin fraction so neither of these methods will serve to prove whether cephalin is necessary for the activity of the compound.

The experiments detailed below will show the effect of different digestive or hydrolytic agents on lung extract.

Experiment 1. Autolysis.—To 25 cc. of lung extract (0.9 per cent NaCl solution) were added 5 cc. of toluene and 8 drops of 10 per cent HCl. The mixture was well shaken, placed in an incubator at 38°C., and left for 27 hours. Then 10 cc. of the fluid were removed, and the toluene was removed as completely as possible by evaporation *in vacuo* and by air currents.

The HCl was neutralized by adding 10 per cent Na_2CO_3 drop by drop, and the liquid tested for activity on coagulation. The autolyzed extracts had lost their activity.

TABLE IV.

1 cc. oxalate plasma, 5 drops 1 per cent $CaCl_2$.

Amount of solution used.	Kept at 38°C.	Coagulation time.
drops		
2	1 min.	12 sec.
2	1 hr., 15 "	25 "
2	2 hrs., 15 "	30 "
2	4 "	1 min.
2	6 "	6 "
4	11 "	8 "
10 (neutralized by Na_2CO_3).	28 "	No clot.
None.		15 min.

Injected intravenously into a rabbit, 0.3 cc. of the original extract killed a 2,500 gm. rabbit in 20 seconds by intravascular clotting. Injection of 3 cc. of the neutralized extract after autolysis was entirely without effect on a rabbit.

Experiment 2. Action of HCl.—To 10 cc. of lung extract were added 5 cc. of 0.1 N HCl. The mixture was kept at 38°C. and the test made on the activity as shown in Table IV.

The acid caused a gradual decrease in the activity of the extract until it not only did not accelerate blood coagulation, but seemed actually to retard it. A precipitate formed in the tube, but both it and the liquid above were unable to accelerate blood clotting.

Since acid alone destroyed the activity of the material, it was necessary to find an enzyme that would act in neutral solution. It was desired to find an enzyme that would possess proteolytic

action, but be lipase-free, or else to find a protease-free lipase. This was finally accomplished by using hogs' pancreas.

Experiment 3. Lipase-Free Trypsin Solution.—This trypsin solution was made according to the directions of Cole (21). It was carefully tested and found to be lipase-free but strong in tryptic action.

Pancreatic Lipase Solution.—The lipase was prepared according to the directions of Cole,[1] and tested for lipase and protease activity. Very little protease action was shown, but the lipolytic activity was high. Thus, 2 cc. of the solution added to 5 cc. of neutralized olive oil and kept at 43°C., with occasional shaking, for 1 hour, developed an acidity equivalent to 8.3 cc. of 0.1 N NaOH in excess of that developed by the control tube containing boiled lipase and olive oil.

Watery Pancreatic Extract.—30 gm. of fresh fat-free hogs' pancreas were hashed and finely ground with sand. To this were added 90 cc. of distilled water, and the mixture was allowed to stand 20 hours in the ice box with occasional shaking. It was then strained through eight layers of cheese-cloth and tested exactly as were the two previous extracts. The results showed that 8.25 cc. of 0.1 N NaOH should be taken as a measure of the lipolytic activity of this extract. This activity is almost exactly the same as that of the alcoholic lipase tested just previously. This extract was tested on fibrin, now, together with the other two extracts. The results on the fibrin digestion experiments showed that the lipase-free trypsin solution and the watery extract of the pancreas possessed equal proteolytic activity, while the alcoholic lipase solution, very nearly trypsin-free, and the watery extract had equal lipolytic powers. That is, the watery extract possessed the equivalent of the combined digestive powers of the other two solutions.

These three pancreatic extracts were next tested as to their power to destroy the activity of lung extract in blood clotting.

The following tubes were prepared and placed in a water bath at 43°C. The blood clotting tests were also carried out in the same water bath.

A. 10 cc. lung extract + 2 cc. 0.9 per cent NaCl solution.
B. 10 " " " + 2 " lipase-free trypsin solution.
C. 10 " " " + 2 " watery extract of pancreas.
D. 10 " " " + 2 " alcoholic lipase solution.

Table V shows the rate of inactivation of the lung extract in the different tubes.

Here, then, we have complete and very rapid destruction of the activity of lung extract by a trypsin solution which was shown to be absolutely lipase-free, so that protein digestion alone is shown to be sufficient for inactivation of the material. There was no apparent change in the character of the mixture in this

[1] Cole (21), p. 158.

tube, that is no precipitation occurred, so that the phospholipins apparently were left in solution as finely divided as ever. If the

TABLE V.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Kept at 43°C.	Coagulation time.
		seconds
None		105
0.04 cc. original lung extract		18
0.04 " watery extract of pancreas		100
0.04 " cephalin emulsion		100
0.04 " of A	5 min.	18
0.04 " " "	19 "	18
0.04 " " "	39 "	19
0.04 " " "	50 "	19
0.04 " " "	60 "	20
0.04 " " "	75 "	20
0.04 " " B	1 "	20
0.04 " " "	13 "	30
0.04 " " "	24 "	60
0.04 " " "	30 "	100
0.04 " " "	45 "	100
0.04 " " "	67 "	100
0.04 " " C	10 sec.	18
0.04 " " "	14 min.	60
0.04 " " "	27 "	95
0.04 " " "	45 "	100
0.04 " " "	60 "	100
0.04 " " D	½ "	18
0.04 " " "	13 "	24
0.04 " " "	24 "	30
0.04 " " "	30 "	33
0.04 " " "	45 "	37
0.04 " " "	60 "	50
0.04 " " "	2 hrs.	100
None		105

activity of the extract was dependent on the phospholipin content alone, it should not have been affected here, and yet we see complete inactivation in 30 minutes. Likewise, with the lipase

solution, which was practically trypsin-free, the inactivation of the substance was complete although brought about at a much slower rate. Therefore, it appeared that digesting off the phospholipin fraction, leaving the protein in solution, also destroyed the activity. Both protein and phospholipin fractions appeared essential to the activity, neither alone being effective. It is possible that the activity of cephalin preparations on blood clotting may be due to a slight trace of the protein fraction still present in the material.

8. Precipitation by Acids. Isoelectric Point.—If CO_2 is passed through the solution of the active material for $\frac{1}{2}$ to 1 hour there is almost complete precipitation of the active material, leaving the coloring matter in the solution.

Sulfuric acid produces similar precipitation at N/250 to N/1,000, with most complete and rapid separation at N/500. The precipitated material retains all its activity, and most of the coloring matter remains in the solution. Optimum acidity for precipitation varies with the concentration of material, weaker extracts precipitating best at N/1,000 to N/1,500. The final H^+ concentration of the solution after the precipitate has settled is always 10^{-5} to 10^{-6} N.

9. Effect of Treatment of Lung Extract with Acid or Alkali.—If the activity of the tissue extract depended on the protein content it would probably be affected by changing the neutral proteins to acid or alkali *m*-proteins. For testing out this possibility a series of tubes was prepared, with the calculated acidity varying as follows: 10 N, 4 N, 2 N, N, N/2, N/10, N/50, N/100, N/500, N/1,000, N/5,000, N/10,000. H_2SO_4 was used.

The precipitates found in Tubes 8, 9, 10, and 11, when dissolved in 0.9 per cent salt solution, were found to be very active. The contents of Tube 7, when neutralized with NaOH, were found to be slightly less active than Tube 12 similarly treated, while Tube 6 was found to be almost devoid of activity. All tubes above Tube 6 were entirely lacking in coagulative power on blood. It is seen then, that acid added to give a calculated acidity of N/50 or greater destroys the power of the substance to hasten blood coagulation, even while leaving a perfectly clear solution. Since the proteins precipitate from these solutions on neutralization it is evident that acid *m*-proteins have been formed.

Whether this change alone is responsible for the inactivation cannot be stated.

The precipitation that occurred in Tubes 8 to 11, with the maximum in Tube 9 at a calculated acidity of N/500, probably represents the same sort of precipitation as occurred with CO_2, since all the activity of the precipitated material was retained.

The action of different concentrations of NaOH was also tested in the same manner. Lung extracts are always opalescent, no matter how long they are centrifuged, provided they are very active on blood coagulation. This normal opacity is not affected by alkalies until NaOH has been added sufficient to produce N/100 NaOH, when a slight clearing of the solution is seen. At N/50 NaOH the clearing is more marked and at N/10 the solution is almost transparent, having lost almost all its opalescence. There is little or no loss of activity of the material with the increasing concentration of alkali, until this clearing of the solution starts, and from then on the activity decreases as the clearing increases. At N/10 NaOH only a trace of activity of the material remains. Neutralization of the contents of Tubes 1, 2, and 3 with H_2SO_4 leads to the formation of a protein precipitate, so that here again it may be assumed that *m*-protein formation has taken place, alkali proteins being formed at the same time that a loss of activity of the material results.

This loss of activity with a presumably slight change in only the proteins of the solution would certainly seem to indicate that some protein in the extract is highly important in the production of the activity on the coagulation of blood.

10. Cataphoresis Experiment with Lung Extract.—Cataphoresis experiments with fresh lung extract in 0.9 per cent NaCl showed that the proteins moved toward the anode. Therefore these proteins, including the active substance, are electronegative in neutral saline solution.

11. Final Method of Purification Adopted.—The precipitation of the material at its isoelectric point was chosen as the method of its purification for several reasons. First, such precipitation removed the material completely from the solution without any apparent change in it as far as its activity was concerned. Second, although the substance possesses the characteristics common to all globulins as far as concerns salting out of solution with

neutral salt such as NaCl, $MgSO_4$, and $(NH_4)_2SO_4$, it differs from other globulins in its precipitation at very faint acidity. Thus, if blood plasma is half saturated with $(NH_4)_2SO_4$, a very heavy precipitation of globulins occurs, whereas if to another sample of the same plasma N/2 H_2SO_4 is added to give a calculated acidity of N/500, the only result is the formation of a very small amount of precipitate which, when collected and redissolved in 0.9 per cent NaCl solution, hastens the clotting time of normal oxalate plasma. In blood plasma there are, then, at least three different globulins: fibrinogen, precipitated by half saturation with NaCl; the globulin active in quickening blood coagulation, which precipitates in weakly acid solution; and other globulins forming the greater part of the total globulins of the plasma, which are not affected by such weak acidity, but are salted out by saturation with NaCl or $MgSO_4$, or by half saturation with $(NH_4)_2SO_4$.

Since the substance active in hastening clotting could be so clearly isolated from such a composite protein solution as the blood by this method, it was considered sufficient for the purification of the material from lung extract. In precipitating lung extract in this manner with weak acid, the surprising observation was made that this one active substance was practically the only globulin present in the extract. That is, if the extract was first half saturated with $(NH_4)_2SO_4$, and the resulting globulin precipitate redissolved in 0.9 per cent NaCl and made very slightly acid in the usual manner, the whole of the globulins present precipitated. Also by first removing the active substance from lung extract by acid precipitation, and then half saturating the remaining solution with $(NH_4)_2SO_4$, it was shown that the acid precipitate contained the total globulin content of the extract.

The method adopted in the purification was as follows: Extracts of fresh or dried beef lungs were made by finely grinding the material and adding 0.9 per cent NaCl solution. After settling thoroughly in the ice box, the supernatant extract was siphoned off and tested to find the optimum concentration of acid for precipitation of the active material. The first few extracts of the lung material usually require a concentration of N/500 acid for precipitation. If the extract is very strong, the first precipitation will not remove all the active material so that a second

addition of acid is necessary. H_2SO_4 was used as the acid in all this work. The lung tissue was extracted repeatedly with 0.9 per cent NaCl solution until the active material was practically exhausted, as shown by testing the extract for its action on the clotting of blood.

In this way 566 gm. of beef lungs, dried at room temperature, were finely powdered and extracted with 0.9 per cent NaCl solution, using 8 liters for each extraction. The seventh extract contained only a trace of activity so no further extraction was made. These extracts were acidified as described, and the precipitate collected and washed twice with distilled water made about N/5,000 acid by adding N/2 H_2SO_4. This removes all traces of salt from the precipitate and thus leaves it insoluble in pure water. Washing several times with distilled water now removes most of the acid combined to the precipitate and all else except the materials insoluble in pure water. The precipitate was recovered each time by allowing it to sediment in the ice box and then removing the supernatant liquid through a tube attached to a suction pump. Filtration was not used because of the nature of the precipitated material, the pores of the filter rapidly becoming clogged so that filtration was extremely slow even under suction. The material was finally dried by placing the precipitate, after drawing off all the water possible, in several shallow evaporating dishes and allowing a warm air current to play over it until the water had been completely removed, as far as possible. The material thus dried was collected and weighed. A yield of about 45 gm. was obtained from the 566 gm. of dried lung. This represents a yield of about 8 per cent from the dried lung, or about 1.9 per cent of the weight of the fresh lung. (Fresh beef lungs in drying at room temperature lose about 76.4 per cent of their weight.)

The material finally obtained has a rather dark gray color when powdered. When dissolved in 0.9 per cent NaCl solution it exhibits the usual activity on oxalated plasma and intravascular clotting. The sample was kept in a stoppered bottle in the ice box for analysis and tests.

12. Analysis of the Purified Material.—The material prepared as described above was used for the analysis. It was first tested qualitatively to determine its constituents. The following facts were established.

The biuret, xanthoproteic, and Millon's tests were very strong. Tryptophane tests weak. Molisch test for carbohydrates quite distinct. Reduced sulfur tests fairly strong and phosphorus very strong. Ehrlich's test for imidazole compounds negative. Traces of iron found.

Since tests for phosphorus in the substance were so strong, it was desired to see in what form this phosphorus occurred. Phospholipin extraction with boiling 80 and 95 per cent alcohol, and absolute ether gave yields of 39.9, 41.3, and 41.6 per cent phospholipin, depending on the duration of the extraction. It was very difficult to be certain that the last traces of phospholipin had been removed from the compound.

Quantitative analysis of the whole compound and of the protein and phospholipin fractions gave the following results:

Nitrogen determinations on the whole compound carried out by the Arnold-Gunning modification of the Kjeldahl process showed (1) 10.63 per cent N, and (2) 10.76 per cent N_2, average 10.7 per cent N.

Like determinations carried out on the phospholipin-free fraction gave (1) 16.10 per cent N, and (2) 16.01 per cent N, average 16.06 per cent N.

Phosphorus percentage in the whole compound, determined by the Pemberton-Neumann method, was (1) 1.52 per cent P, and (2) 1.558 per cent P, average 1.539 per cent P.

Phosphorus percentage in the phospholipin-free fraction, determined by the same method, was (1) 1.056 per cent P, and (2) 1.061 per cent P, average 1.058 per cent P.

Phosphorus percentage in the phospholipin fraction was found to be (1) 2.12 per cent P, and 2.13 per cent P, average 2.125 per cent P.

A complete analysis of the active substance will be carried out and reported in full at a later date.

The finding of so much phosphorus remaining in the protein fraction raised the question as to the form in which it was present. Tests for phosphoprotein (boiling with NaOH of different concentrations for different periods) and for nucleoproteins (examination for purine bases after boiling with 14 per cent HNO_3) showed that the phosphorus remaining in the protein fraction is not there as ordinary phosphoprotein or as nucleoprotein. Either it must be present in a very stable form of phosphoprotein, or else as phospholipin which cannot be removed by extraction with boiling alcohol and ether. This latter possibility is made improbable, however, by the fact that the nitrogen percentage of the compound (16.06 per cent) is such as to indicate a rather pure protein,

while if enough phospholipin were present to account for the 1 per cent of phosphorus the nitrogen percentage would be considerably lower than this.

13. Is the Phospholipin Fraction Necessary for the Activity of the Compound?—The finding of as much as 41.6 per cent of phospholipin in the substance at once raised the question as to the importance of it there. The only way to determine the relative importance of the protein and phospholipin fractions would be to separate them without change in either and study their action separately. This is rather difficult, since many methods of removing the phospholipin alter the protein in some way. Extraction with boiling solvents is excluded because of the coagulation of the protein resulting. Alcohol as a fat solvent is excluded for the same reason. Ordinary ether likewise alters the protein, leaving it very much less soluble, probably as a result of the action of the impurities, aldehydes, etc., present. A number of other solvents for the phospholipin were tried, dried beef lung tissue being extracted for several days at room temperature with successive portions of the solvents until the filtered solvents showed only a faint color due to phospholipin. Extraction with 0.9 per cent NaCl solution of the lung tissue so treated gave varying results. Absolute ether, chloroform, carbon disulfide, carbon tetrachloride, and benzene were used as the fat solvents. Each of them left the proteins little altered as far as their solubility in salt solution is concerned, although ether showed the greatest effect in this respect, the saline extract appearing poorer in proteins than normal. Saline extracts of the lung tissue extracted by the other solvents, CS_2, CCl_4, etc., looked just as strong as normal lung extract, but, when tested for their action on blood clotting, were found to possess only a slight action, comparable to the normal lung extract diluted more than 1,000 times. To show that the protein portion of the active substance was present in the inactive extracts they were treated with acid to give N/500 calculated acidity, at which point the precipitation occurred just as in normal extracts, most of the proteins present remaining in solution.

The isoelectric points of the protein alone and of the protein-phospholipin compound are the same. This would lead to the supposition that the protein alone is responsible for the precipita-

tion in slightly acid solution. The protein fraction is present then, unchanged, and possessing only a very slight degree of activity as compared to that of the whole compound.

If both protein and phospholipin have remained unchanged during the extraction they should reunite when mixed together. This was tried by thoroughly rubbing up a small amount of the extracted phospholipin with the inactive protein in a mortar. An immediate and marked increase in activity resulted. Frequent shakings of this mixture by hand caused its activity to continue to increase for several minutes, equilibrium finally being reached with its action on coagulation about 250 times as strong as that of either of the fragments alone. This was still

TABLE VI.

Table Showing Reactivation.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Kept at 40°C.	Coagulation time.
None		6 min.
0.04 cc. inactive protein solution		3 min., 30 sec.
0.04 " phospholipin emulsion		3 " 30 "
0.04 " protein + phospholipin	1 min.	70 "
0.04 " " + "	7 "	45 "
0.04 " " + "	13 "	40 "
0.04 " " + "	50 "	40 "
0.04 " " + "	15 hrs. in ice box.	40 "
0.04 " normal lung extract		12 "

somewhat below the strength of the normal lung extract, so that apparently the normal amount of phospholipin was not yet attached to the protein.

The tests given in Table VI show this reactivation.

The inactive protein solution was obtained by thoroughly extracting dried powdered beef lung tissue at room temperature with benzene, freeing the extracted tissue from all traces of benzene by an air current, and then grinding the tissue well with 0.9 per cent NaCl solution in quantity small enough to give about a saturated solution of the soluble proteins. The phospholipin emulsion was made by taking the phospholipin obtained by evaporating the benzene extracts at room temperature in an air

current, and grinding it well with 0.9 per cent NaCl solution in the proportion of about 0.1 gm. of phospholipin to 20 cc. of salt solution. This gave an emulsion of milky appearance after the excess phospholipin had risen to the surface and been removed.

In adding the phospholipin to the protein solution, about 0.1 gm. of the phospholipin was thoroughly rubbed up with 5 cc. of the inactive protein solution in a mortar, and then transferred to a test-tube and well shaken by hand. The test on the normal lung extract (saturated) is given to show that the complete activity had not been obtained in the reactivation. By reference to Table I it will be seen that the increase in activity of the combined fractions over either separately was fully a 250-fold increase.

Benzene and carbon tetrachloride were found to be the fat solvents that left the two fractions least altered, since reactivation occurred most readily after their use in the extraction of the phospholipins. It might be added that great care was taken throughout in making the different saline extracts in order to keep as nearly as possible the same concentration of the substances concerned as occurred in the normal extract.

It might be argued here that the protein merely acts by finely emulsifying the cephalin and thus increasing its activity, considering the total effect of the solution on coagulation as being due to the cephalin. Experiments were tried, therefore, in which the phospholipin extracted from lungs as described above, was shaken in a machine for 30 minutes with solutions of soluble starch, soap, and 0.9 per cent NaCl.

A rather thick milky emulsion of the phospholipin in 0.9 per cent NaCl solution was made, and these mixtures made:

(*a*) 1 cc. of phospholipin emulsion + 1 cc. of 5 per cent solution of soluble starch.

(*b*) 1 cc. of phospholipin emulsion + 1 cc. of 1 per cent soap solution.

(*c*) 1 cc. of phospholipin emulsion + 1 cc. of 0.9 per cent NaCl solution. When shaken as described above, a fine and stable emulsion was obtained with the soluble starch, less so with the soap solution, and still less with only the 0.9 per cent NaCl, but the activity on blood clotting remained the same for the three solutions.

14. Increasing the Activity of Lung Extract.—The conclusion that a protein-phospholipin compound is the active principle led naturally to the question whether the compound as it occurs in tissues and tissue extracts is saturated with phospholipin, or whether the addition of more of this fraction would still further enhance the activity of the extract on the clotting of blood. To test this out, saturated lung extract, made as described in the first section of this paper, was thoroughly rubbed up in a mortar with a small amount of phospholipin freshly extracted from dried beef lungs by benzene at room temperature, the benzene being evaporated in an air current at room temperature. Thus to 5 cc. of such saturated lung extract was added about 0.2 gm.

TABLE VII.

Activation of Lung Extract.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Dilution of extract, 0.9 per cent NaCl.	Coagulation time.
0.4 cc. lung extract		10 sec.
0.4 " " "	2 times.	12 "
0.4 " " "	4 "	14 "
0.4 " same extract + phospholipin		8 "
0.4 " " " + "	4 "	10 "
0.4 " " " + "	8 "	12 "
0.4 " " " + "	16 "	14 "
None		4 min.

of fresh phospholipin. The mixture was well shaken by hand in a test-tube (air being present) for several minutes, and was then tested for its activity as indicated in Table VII.

This same fourfold increase in activity was obtained by adding phospholipin to more dilute lung extracts. Kidney extracts were also rendered more active in the same manner. An analysis to determine the phospholipin percentage in the active substance after such activation as shown in Table VII has not yet been made.

The effort was next made to increase the strength of brain extracts in the same way, but much less of an increase in activity resulted, the activity after phospholipin addition being less than

double the original activity of the extract. This was to be expected since the brain was so very rich in phospholipin already. There is probably an excess of this fraction in brain extracts, so that the addition of more phospholipin has little further effect. Possibly the addition of the inactive protein constituent of the lung would increase the activity of brain extracts, but this has not yet been tried. It would be expected that the active substance as it occurs in brain extract would have the same phospholipin percentage as the lung compound after activation with further phospholipin. It is intended to make such comparative analyses soon.

15. Is the Protein Fraction of a Specific Nature, or May Different Proteins be Used?—Whether the protein in the active compound is of a specific nature is difficult to prove, since methods for isolating and identifying animal proteins of closely similar character are very unsatisfactory. Experiments were carried out in which phospholipin freshly extracted from dried beef lungs with benzene was thoroughly shaken with a 2 per cent solution of dried egg white in 0.9 per cent NaCl solution, and with a 2 per cent casein solution. An emulsion of the phospholipin in 0.9 per cent NaCl was first made and to this milky emulsion the other solutions were added as follows:

(*a*) 2 cc. phospholipin emulsion + 2 cc. 2 per cent egg white solution.
(*b*) 2 " " " + 2 " 2 " " casein solution.
(*c*) 2 " " " + 2 " 0.9 " " NaCl.

After shaking well by hand for about 15 minutes, these different mixtures were tested for their action on blood coagulation as shown in Table VIII.

After standing for a few minutes following the shaking, it was noticed that there had occurred a heavy precipitation in the tube containing the egg white, probably resulting from the union of the colloidal phospholipin with the colloidal protein.

No such precipitation occurred in the casein solution, so it cannot be stated whether union occurred between the casein and phospholipin.

Another interesting experiment pointed to the specificity of the protein fraction. Liver extracts contain a large amount of globulins which possess the same solubility characteristics as the

active compound in the lungs. In fact they could not be differentiated except by the difference in activity, the liver extracts possessing only a slight trace of activity in hastening the clotting of blood. It was thought this lack of activity might be due to a lack of phospholipin, and so this was added just as it was added to lung extract. But only a faint increase in activity resulted, no more than might be accounted for by the free phospholipin added. Kidney extracts, on the other hand, possess considerable activity, and this can be increased markedly by the addition of further phospholipin.

It would seem, therefore, that the protein fraction is of a definite and specific nature, even very similar globulins, such as those from the liver, being ineffective. This gives a clear-cut proof of the importance of the protein fraction of the compound.

TABLE VIII.

1 cc. oxalate plasma, 0.2 cc. 1 per cent CaCl.

Other addition.	Coagulation time.
None.	5 min., 30 sec.
0.2 cc. of (*c*).	3 " 30 "
0.2 " " (*a*).	3 " 30 "
0.2 " " (*b*).	3 " 40 "

16. The Nature of the Phospholipin Concerned.—Howell has showed that of all the phospholipins present in various tissues, only cephalin possesses the power of hastening blood coagulation. This conclusion has been widely accepted and with justification. It is only his statement that cephalin is the active principle of tissue extracts which requires further proof. This is discussed elsewhere in this paper.

In order to see whether it is cephalin that is concerned in the formation of the active material described in these pages, the phospholipin was extracted from the protein fraction, dissolved in ether, and four volumes of absolute alcohol were added. Very nearly the whole of the dissolved material was precipitated and may therefore be considered to be cephalin. It is probably all cephalin, since the last traces of this substance are never entirely precipitated from ether by alcohol addition.

The character of this cephalin and of the total phospholipins of the lungs is very different from brain phospholipins which contain much lecithin. The lung phospholipin is a yellowish brown, sticky, waxy material, very difficult to dry. Some of it was dissolved in ether and absolute alcohol added as above to see what part of the mixture was cephalin. Practically complete precipitation again resulted just as with the phospholipin from the pure compound. It is rather surprising that only cephalin should occur in this tissue to any great extent. It is very possible that all, or nearly all this cephalin, will be found to be present in the new compound described in this paper. The work so far has given that impression. Quantitative work to determine to what the phospholipins are attached in the lung tissue is being carried out at present.

17. Comparative Tests on the Different Coagulant Preparations in Use at Present.—Samples of coagulant preparations were kindly furnished by a number of firms. Tests were made on these different preparations in the usual way, and the results are given in Table IX.

Thromboplastin (Squibb), Hemostatic Serum (Mulford), and Hemogulen (Lilly) are all brain extracts in different degrees of saturation. Hemogulen probably represents a complete saturation of salt solution with the active principle of the brain. These three solutions are similar to lung extract in that boiling destroys their activity and that the active material may be isolated in the same way. Coagulen-Ciba, Coagulose (Parke, Davis Company), and Thromboplastin Hypo. (Squibb) may be boiled without effect on their action so that they contain none of the protein responsible for the action of the tissue extracts. Their activity is comparable to that of cephalin and it is very likely that they are composed of this in large measure. Coagulen and Coagulose are prepared from the blood, supposedly from the platelets, but we might also say from the A-fibrinogen of Wooldridge. However, in the preparation, the protein fraction of the compound is lost and with it goes the greater part of the activity on coagulation. These two preparations, then, would probably be safe for intravenous use, just as is cephalin, unless an abnormal tendency toward clotting was present in the blood. Intravenous injection of any coagulant, however, should be undertaken with great

care, on account of the serious results that often follow even slight thrombosis. What has just been said of Coagulose and Coagulen holds good for Thromboplastin-Hypo. (Squibb), although it is prepared from the brain. It will be shown that the A-fibrinogen

TABLE IX.

Comparative Tests on Coagulant Preparations.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Coagulation time.
None	6½ min.
1 drop Coagulen-Ciba solution	5 "
5 drops " "	4½ "
10 " " "	5 "
1 drop Coagulose, Parke, Davis Co.	4 " 30 sec.
5 drops " " " "	2 " 45 "
10 " " " " "	2 "
1 drop Hemostatic Serum, Parke, Davis Co.	8 "
5 drops " " " " "	8 "
10 " " " " " "	8 "
1 drop Kephalin solution, Armour Co.	6 "
5 drops " " " "	4 " 30 sec.
10 " " " " "	3 " 30 "
1 drop Thromboplastin-Hypo., Squibb	5 " 40 "
5 drops " "	4 "
10 " " "	3 "
1 drop Thromboplastin, Squibb	20 sec.
5 drops " "	12 "
1 drop Hemostatic Serum, Mulford	40 "
5 drops " " "	20 "
10 " " " "	18 "
1 drop Hemogulen, Lilly	14 "
5 drops " "	10 "
1 drop lung extract	10 "
1 " " " (activated)	8 "
1 " " " (diluted four times with 0.9 per cent NaCl)	10 "

of the blood is identical with the active material of the tissues. This was also stated by Wooldridge.

Solutions as active as the brain extracts tested, or lung extract, can be used only for *local* application to arrest hemorrhage, unless they be greatly diluted, in which case the danger of thrombosis

from intravenous injection is very slight. Application of such materials for intravenous use, however, will be discussed by the author in another paper.

A few points in regard to the local application of this active tissue material in hemorrhages are: First, it is the only substance, apparently, in the tissues of the body and in the body fluids which will markedly accelerate the clotting of blood. It is then the principal factor in the natural mechanism for the arrest of hemorrhages. Second, it is by far the strongest blood coagulant known. Third, it greatly increases the amount of fibrin to be formed from a given plasma, thus assuring a firmer clot to withstand the pressure of the blood from behind. These three points, when properly appreciated, should make the use of tissue extracts, or of the purified material, almost universal in all types of local hemorrhage, whether in surgical or accidental cases. Whether this will apply to hemophilia cases as well has not yet been investigated by the writer.

The thing of importance to remember in their use is that the strongest preparation possible should be applied to the bleeding area, since it is certain to be greatly diluted by the blood issuing from the small vessels. Back in the open ends of the vessels is where the clots must form to be effective and here is where the concentration of the material applied will be least. Pressure to slow the flow of the escaping blood would result in much less dilution of the active material and give quicker clotting. For the purpose of obtaining this maximal concentration in the blood to be clotted, lung extract activated by the addition of further phospholipin would appear to be the ideal preparation to use since it may be diluted fully sixteen times and still be of equal strength with the strongest brain extract undiluted. Reference to Table I will show the slight effect which dilution has on the action of the material. A comparison of the different commercial preparations, as shown in Table IX, with different dilutions of lung extract will show their relative value in directly accelerating blood coagulation.

DISCUSSION.

The experimental facts presented are in direct conflict with very few of the observations made in the past as to the nature of the active coagulant in the tissues. It can readily be shown how

many of the conclusions of others have been erroneous, although their observations were usually correct. Take, for instance, the widely held opinion as to the importance of cephalin in coagulation. On the basis of Howell's very valuable work it has been generally conceded that this phospholipin is the active material of the tissues, with the result that cephalin preparations have appeared on the market as blood coagulants. Cephalin has indeed some action but by no means so powerful as when united with the protein. Even preparations made supposedly from the blood platelets (A-fibrinogen of Wooldridge) were always carefully purified of coagulable proteins so that they could be boiled without injury, with the idea that the all important cephalin was the only constituent that needed to be retained. The reason for all this importance being assigned to cephalin lies in the fact that in its extraction from the tissue the real active protein-phospholipin compound was split into its two fragments each of which possesses slight activity under the best of conditions, but as the extraction was really conducted all the proteins were coagulated by heat and alcohol and that fraction of the active material was lost completely. Of the phospholipins thus extracted cephalin alone showed coagulative activity and so to it was assigned the all important rôle mentioned. It is difficult to see how this conclusion could be arrived at on such grounds, since a simple comparison of the cephalin with tissue extracts would have shown that it alone could not account for a thousandth part of the activity of certain tissue extracts. Apparently no such comparative tests were made in a quantitative way.

Regardless of the work of Wooldridge in showing both the protein and phospholipin to be necessary for a maximum activity, it has been concluded that methods of removing the proteins from active solutions, such as by boiling, by precipitation with alcohol, or by salting out, removed the activity only because the cephalin was adsorbed onto the protein aggregates and thus removed from an active state. Why this idea should have persisted is difficult to see, since any method of removing the proteins from solution or of splitting them by hydrolysis will invariably destroy the activity of the material. It is true that the cephalin is removed along with the proteins but why assign to cephalin all the importance, rather than to the proteins? Evi-

dently it was because cephalin had been shown to possess some slight action while no pure protein had ever been found to possess such, although this point had really hardly been touched, except by Wooldridge. The proof presented in this paper as to the equal importance of the phospholipin and protein in producing the activity should be conclusive. The slightest change in the nature of the protein as by the production of acid or alkali proteins destroys the activity of the material, even though the state of solution of the substance is markedly improved. This loss of activity is impossible to explain if cephalin alone is considered responsible for the action of the compound. Also the hydrolysis of the protein by a trypsin solution almost or entirely lipase-free, very rapidly destroys the activity of the compound and furnishes further proof of the importance of the proteins. But the final and conclusive proof comes when it is shown that extraction of the phospholipins, without injury to the proteins, destroys most of the activity, while reunion of the separated fragments restores the original action.

The importance of the amount of phospholipin thus combined with the protein is clearly shown also by the fact that addition of further amounts of it to the active compound in lung extract results in a fourfold increase in the specific activity. It must be remembered, however, that it is not the cephalin alone that is responsible for this increase, but the union of the phospholipin with the specific protein molecules. Emulsification of the phospholipin with other agents, or union with other proteins does not thus increase its action. Even emulsification of it with brain extract gives no results, probably because the specific protein in such extracts is already saturated with such material.

From a scientific point of view, the proof of the nature of this material should be of interest. It has been thought that the phospholipins of the tissue cells were very probably united with the proteins in some sort of a union forming a very complex molecule, but the actual proof for this was lacking. If such a state of actual union could be proved, it might supply a basis for explaining many pathological changes in functions of the cell, especially for the effects of the anesthetics, at least those which are fat-soluble. The facts here presented should furnish this needed proof, since for the specific action of this substance on coagula-

tion, the close union of the phospholipin and protein has been shown to be absolutely essential. That the compound occurs in the tissues in this form is proved from the fact that the tissue material itself, upon disorganization of the cell structure with a minimum of chance for chemical change, as by merely grinding in isotonic salt solution, exhibits this same marked action on coagulation. There are probably other such compounds between phospholipins and proteins present in the cells, but this is difficult to prove on account of there being no specific test for them such as we have for this material. It is hoped soon to see just how much of the total phospholipin of the tissues is present in this one compound.

It is likely that there may be a practical application of some value of the discovery described here by which a coagulant is produced at least sixteen times as strong as any at present on the market, and 1,000 times as strong as several that have been widely used in the past. When it is remembered that the greatest possible concentration of the active material in the escaping blood is needed to insure quick coagulation, the possible value of thus increasing the power of these coagulants is clear.

The synthesis of such a substance as this active material has proved to be, cannot occur until specific proteins and phospholipins can be built up. At present the science is far from this perfection.

SUMMARY.

The results of this work may be summarized briefly as follows:

1. The phospholipins to be extracted from tissues possess only a very small part of the total activity of the tissue material on the clotting of blood.

2. The active principle of tissue extracts is in part protein in nature, so that any treatment that removes the proteins from solution or alters them in any way destroys or greatly reduces the activity of the material.

3. The protein nature of the compound must be considered in its purification, if its activity is to be retained.

4. The isoelectric point of the compound lies between $N \times 10^{-5}$ and $N \times 10^{-6}$ acidity. At this point the material precipitates from its solutions without loss of its activity on coagulation.

This fact is made use of in the isolation and purification of the active principle.

5. The purified substance possesses the solubility characteristics of the globulin class of proteins and is heat-coagulable.

6. It consists of about 41.6 per cent of phospholipin and 58.4 per cent of protein, the protein fraction containing about 1.06 per cent of phosphorus. This phosphorus is apparently present as very stable phosphoprotein, since no purine bases were found after thorough hydrolysis of the protein by acid. That the phosphorus is very firmly combined in the phosphoprotein is evident from the fact that several hours boiling with 5 per cent alkali fails to split off more than a small part of it.

7. The activity on blood coagulation depends on the union of the phospholipin and protein fractions. Separation of the two parts as completely as can be by extraction at room temperature with fat solvents, leaves each with only a very small part of the original activity. Reunion of these two fractions restores the greater part of this activity.

8. Addition of further amounts of phospholipins to the active material as it exists in the lungs increases its activity about fourfold. It is therefore evident that the percentage of phospholipin united to the protein fraction is a very important factor in the production of the activity.

9. This further activation of tissue extracts should be of considerable practical importance, since it is possible thereby to get a solution fully sixteen times as strong in activity as the best of those at present on the market, and about 1,000 times as strong as many that have been widely used.

10. The presence in the tissues of an actual union between the protein and phospholipin elements is here clearly shown. It is also shown that any alteration in this relation causes a great change in the specific activity of the compound. This fact may afford a future basis for working out the reasons for many cell reactions, especially reactions to any fat solvent materials.

The work reported in this and an accompanying paper on the action of tissue coagulins, as well as an earlier paper on the distribution of the active material in the various tissues, has been carried out under the direction of Dr. A. P. Mathews, for whose

valuable aid and numerous suggestions the author here desires to express his deep appreciation. Much credit is also due Dr. Shiro Tashiro for many valuable suggestions during the course of the work.

BIBLIOGRAPHY.

1. Mills, C. A., *J. Biol. Chem.*, 1919, xl, 425.
2. Wooldridge, L. C., On the chemistry of the blood and other scientific papers, London, 1893, The Croonian lecture on the coagulation of the blood.
3. Howell, W. H., *Am. J. Physiol.*, 1912–13, xxxi, 1.
4. Mills, C. A., *J. Biol. Chem.*, 1921, xlvi, 167.
5. Wright, A. E., *Lancet*, 1892, i, 457, 515.
6. Brieger and Uhlenhuth, *Deutsch. med. Woch.*, 1898, xxiv, 163.
7. Dold, H., *Z. Immunitätsforsch., Orig.*, 1911, x, 53.
8. Dold, H., and Ogata, S., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 667.
9. Dold, H., and Kodama, H., *Z. Immunitätsforsch., Orig.*, 1913, xviii, 682.
10. Izar, G., *Z. Immunitätsforsch., Orig.*, 1912–1913, xvi, 557.
11. Dold and Ogata, *Z. Immunitätsforsch., Orig.*, 1912, xiv, 138.
12. Popielski, L., *Bull. int. Acad. Sc., Cracovie*, 1912, 1157; abstracted in *Chem. Abstr.*, 1914, viii, 1463.
13. Aronson, H., *Berl. klin. Woch.*, 1913, l, 253.
14. Dale, H. H., *J. Physiol.*, 1910, xl, 503. Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910–11, xli, 318; 1918–19, lii, 355. Barger, G., and Dale, H. H., *J. Physiol.*, 1910–11, xli, 499. Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918–19, lii, 110.
15. Ackermann, D., *Z. physiol. Chem.*, 1910, lxv, 504.
16. Mellanby, E., and Twort, F. W., *J. Physiol.*, 1912–13, xlv, 53.
17. Abel, J. J., and Kubota, S., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 243.
18. Jackson, D. E., and Mills, C. A., *J. Lab. and Clin. Med.*, 1919–20, v, 1.
19. Morse, P. F., *J. Lab. and Clin. Med.*, 1919–20, v, 319.
20. Roger, H., *Arch. méd. exp. et anat. path.*, 1918, xxviii, 325.
21. Cole, S. W., Practical physiological chemistry, Cambridge, 3rd edition, 1913, 212.

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. XLVI, No. 1, March, 1921

THE ACTION OF TISSUE EXTRACTS IN THE COAGULATION OF BLOOD.*

BY C. A. MILLS.

(From the Biochemical Laboratory, University of Cincinnati, Cincinnati.)

(Received for publication, August 18, 1920.)

It has often been demonstrated that blood drawn from a vessel without contact with any injured tissue surface and kept in such a manner as to prevent destruction of the blood corpuscles, exhibits a considerable delay in its coagulation. Bird blood especially will remain fluid almost indefinitely when drawn and kept under such conditions. If, however, the escaping blood flows over an injured tissue surface, or the corpuscles are broken up as by whipping, it is found that the blood exhibits a much more prompt coagulation. The addition of saline extracts of tissues, especially such tissues as the lungs and brain, accelerates the clotting still further, it being possible thus to shorten the coagulation time to a small fraction of a minute.

There is evidently then in tissues and cells in general a substance which possesses the property of quickening the clotting of the blood. This substance has been variously named. Wooldridge (1), who was the first to study the nature and action of it, held that it was a protein-phospholipin compound and that in the process of coagulation it reacted with the fibrinogen to form fibrin, an actual union occurring between the two substances with a loss of phospholipin. Since the tissue material entered into the formation of the fibrin, he termed it tissue fibrinogen. He also showed that there was a substance in the blood itself which was practically identical with the tissue fibrinogen, and this he termed A-fibrinogen. The reaction of this with the ordinary fibrinogen of the blood which he termed B-fibrinogen, resulted in the forma-

* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, University of Cincinnati, May 31, 1920.

tion of fibrin. Precipitation of this A-fibrinogen by letting the plasma stand in an ice box for some time destroyed the spontaneous coagulability of the plasma. Addition of a new supply of the substance or of the tissue fibrinogen restored the power of clotting to such plasma. Wooldridge was also the first to show that the intravenous injection of the tissue fibrinogen in sufficient amounts would upset the equilibrium existing in the blood between the A-fibrinogen and B-fibrinogen and result in the formation of fibrin in the vessels, causing death from thrombosis. The fluid blood remaining after such intravascular coagulation showed a delayed coagulation time, or absolute non-coagulability. The increased coagulability of the blood he termed the positive phase of coagulation, while the tendency toward non-coagulability was called the negative phase. The negative phase was also produced by sublethal injections of tissue fibrinogen without the formation of solid clots to any great extent, and he considered this to be a protective reaction on the part of the blood itself or of the vascular endothelium. Wright (2), following up the work of Wooldridge, considered the negative phase to be due to the partial digestion or hydrolysis of the tissue fibrinogen in the blood with the liberation of albumoses and peptones, these bodies being more directly responsible for the negative phase reaction on the part of the vascular system. His basis for this conclusion was that the urine always gave a reaction for albumoses after injection of tissue extracts and also that in pneumonia there occurred a delayed coagulation of the blood at the same time that albumoses and peptones were being excreted in the urine, the origin of these bodies being from the resorption of the imperfectly digested exudate in the lungs. Since the direct injection of such albumoses or peptones into the blood was known to produce varying degrees of non-coagulability in the blood of dogs, Wright considered the process here to be of the same order.

It would not be practical to review in detail all the more recent work on the action of tissue extracts, so only a few of the more important articles will be mentioned here. Morawitz (3) and Fuld and Spiro (4) held that the tissue substance acted in the rôle of a kinase to initiate and hasten the formation of thrombin from the calcium and prothrombin of the plasma. Fuld and Spiro termed the substance cytozyme while Morawitz called it thrombokinase. Many workers have since accepted this theory of the action of the tissue material and based upon it their explanations of ex-

perimental observations. Nolf (5) and Howell (6) hold views somewhat different from the above. Nolf believes that fibrinogen is held in solution by being combined with a substance, hepatothrombin, produced by the liver, and that the breaking of this union by removal of the hepatothrombin permits the fibrinogen to precipitate as insoluble fibrin. The hepatothrombin may be removed by a substance derived from the leucocytes and hence called leucothrombin. Howell considers that tissue extracts act by neutralizing the antithrombin present, the antithrombin acting to prevent the interaction of prothrombin and calcium in the formation of thrombin. On neutralizing the antithrombin, then, the thrombin formation proceeds and clotting results. He called the substance thromboplastin or thromboplastic substance. Loeb (7) terms the active tissue substance coagulin because he considers it to act in the same manner as thrombin. Thus he finds that it will clot a solution of fibrinogen in the presence of calcium, presumably without the presence of any prothrombin. Mellanby (8), however, has brought forth proof that the prothrombin is very intimately associated with the fibrinogen and probably is not removed in the purification of the fibrinogen.

According to the theories of Morawitz, Fuld and Spiro, Nolf, and Howell, the tissue substance is not concerned in the formation of fibrin except indirectly in the formation of thrombin. That is, it is not a fibrinogen in the sense that it enters directly into the formation of the fibrin. Wooldridge, on the other hand, considered it to be a true tissue fibrinogen, furnishing a part of the material that entered into the fibrin molecule. The present work will be found to support Wooldridge's work in this, as in many other respects, although differing from it in certain important points. Most attention in this work, however, was paid to the effects of tissue extracts on the blood *in vivo*, especially to determining the nature of the negative phase.

EXPERIMENTAL.

1. Relation of Concentration of Active Material to Time of Clotting.

In a previously published article (9) the author showed that different tissues of the body vary in their content of material active in hastening the clotting of blood. Lung extract was found to be considerably stronger in this respect than extracts of any other tissues made in a similar manner, so it was chosen for use in most of this work. The method found most efficient for testing the activity of these extracts, and that which most nearly approximates the normal conditions of blood coagulation was the use of fresh oxalated blood. Upon recalcification with the optimum amount of $CaCl_2$, clotting occurred in fresh oxalate plasma in approximately the same time as in normal blood drawn from a vein and let stand. Standing in the ice box causes a gradual

lengthening of the coagulation time of the oxalate plasma, the time ranging from 3 to 15 minutes during 2 to 4 weeks standing, the reason for this apparently being a gradual precipitation of the A-fibrinogen of Wooldridge which is essential for spontaneous coagulation. Addition of very small amounts of this substance or of tissue fibrinogen shortens the coagulation time, the shortening bearing a definite relation to the amount of tissue substance added.

The plasma used throughout this work as normal plasma was obtained from the abattoir by letting the blood flow directly from the slaughtered animals into a vessel containing sufficient potassium oxalate to make the whole amount collected about 0.5 per cent. This made the plasma alone, after centrifuging, about 0.8 per cent oxalate, and this gave too heavy a precipitate of calcium oxalate on recalcification to make a reliable test fluid for accurate coagulation time determinations. To such cell-free plasma $CaCl_2$ was added in amount just below that necessary for clotting, and the precipitate of calcium oxalate removed by sedimentation in the ice box and then by centrifuging out the last traces. This resulted in a fairly clear plasma which required only a small amount of $CaCl_2$ to induce clotting in a normal manner. It was found necessary to put so much oxalate in at the time of the drawing of the blood because it was mostly done by inexperienced hands, and with smaller amounts of oxalate there frequently occurred clotting due to insufficient mixing of the oxalate with the blood. The plasma finally used for the test required usually about 0.2 cc. of 1 per cent $CaCl_2$ per cc. of plasma for optimum recalcification. Such a plasma forms a very delicate medium for detecting the presence of any amount of active coagulant material.

Table I in the preceding paper (10) giving dilution of lung extract shows clearly that the shortening of the coagulation time bears a definite relation to the concentration of the tissue extract.

The lung extract used in these dilution tests was a saturated solution made by extracting dried ground beef lung tissue with 0.9 per cent NaCl solution (4 cc. of salt solution per gm. of dried lung tissue). Fresh beef lungs were thoroughly washed in tap water, hashed in a meat chopper, spread on a glass plate in a thin layer, and dried in a warm air current for 24 hours. This

drying caused a loss in weight of about 76 per cent, but did not affect the blood-coagulating properties of the active material as was determined by comparative tests. The dried material was now powdered and extracted with 0.9 per cent NaCl solution in the proportion stated above. The use of less salt solution per gm. of tissue did not increase the activity of the solution so saturation was probably complete. Only a few minutes standing was necessary to obtain this saturation, after which all solid tissue particles were removed by centrifuging the solution for 20 minutes at about 3,000 revolutions per minute.

The relation of coagulation time to the dilution of the extract may be represented as shown in Chart 1. The plot of the logarithm of the dilution against the logarithm of the clotting time is nearly a straight line.

Considering the line in Chart 1 to be a straight line, the following formula was very kindly suggested by Dr. Mathews:

$$\text{Log } x = 3.4 \log y - 3.4$$

where x represents the dilution of the extract, and y the coagulation time in seconds. By using this formula it would be possible to express the amount of active material in any extract in terms of lung extract, merely by determining its degree of acceleration of the normal coagulation time, and, letting the observed coagulation time equal y, calculate x. However, it is readily seen that this would only hold good for tests in which the normal clotting time of the plasma used was 250 seconds. If it differed from this figure, which it would do in all likelihood, an approximate value could be arrived at by simple proportion.

Thus, if an extract of unknown strength is tested on oxalate plasma having a normal clotting time of 5 minutes, *i.e.* 300 seconds, and it is found that 0.04 cc. of the extract (or about 1 drop) accelerates the clotting time to 80 seconds, the strength of the extract as compared to saturated lung extract could be found as follows:

$$300 : 250 :: 80 : y$$
$$y = 67 \text{ seconds}$$

Substitute this value of y in the formula

$$\text{Log } x = 3.4 \log y - 3.4$$
$$\text{Log } x = (3.4 \times 1.82607) - 3.4$$
$$\text{Log } x = 2.80864$$
$$x = 644$$

That is, the extract tested is equivalent in strength to saturated lung extract diluted with 643 volumes of 0.9 per cent NaCl solution.

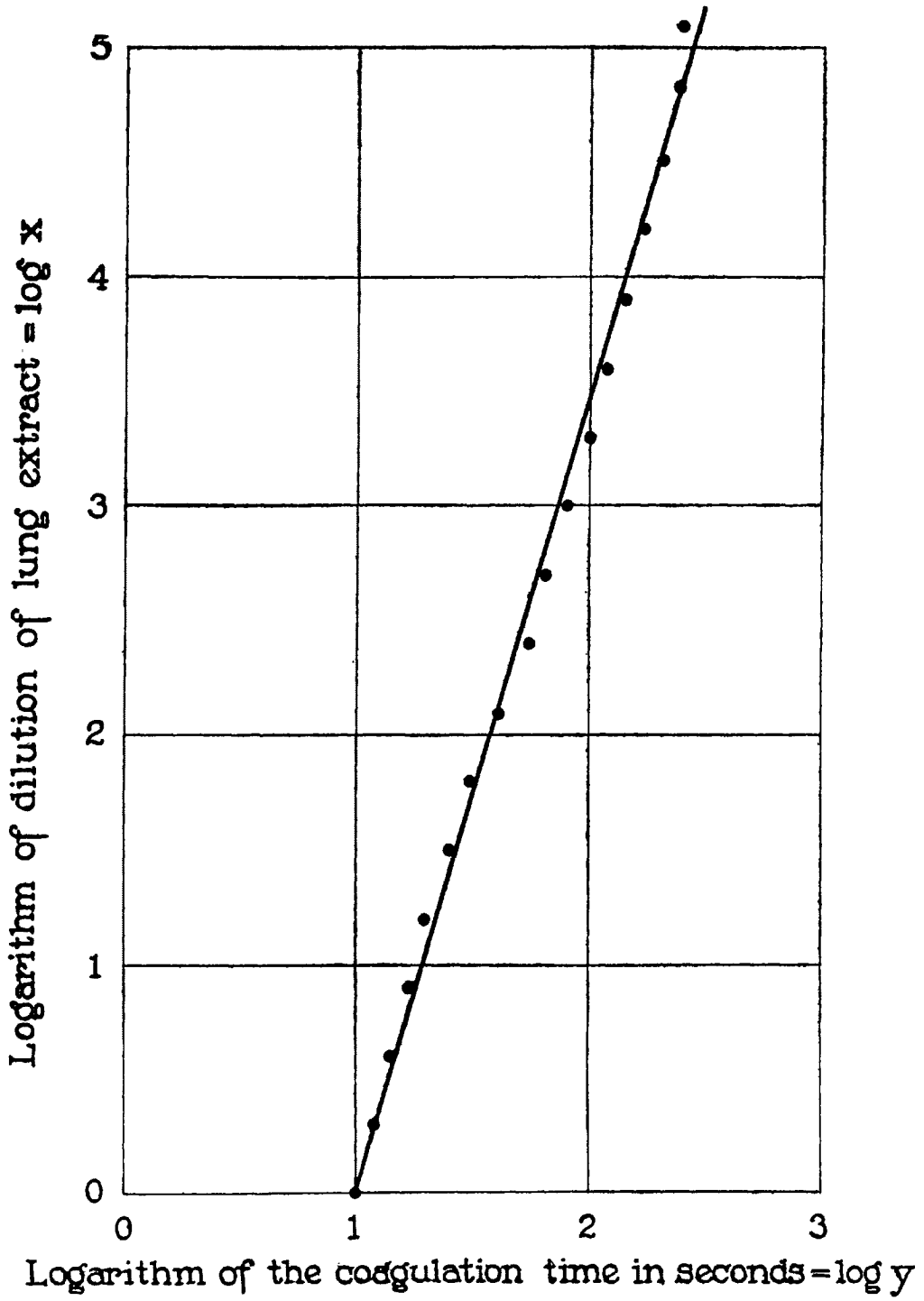

CHART 1.

A method of arriving at a quantitative expression of the amount of active substance in any solution would be to define arbitrarily a unit of the substance as that amount present in a certain amount of a given dilution of lung extract, as, for instance, in 0.04 cc. of lung extract diluted 100,000 times, and then express the activity of other solutions in terms of this unit. However, it is hoped that a definite statement can be made very soon as to exactly how much active substance by weight is present in any strength of extract, and thus save establishing any arbitrary unit of measuring the activity.[1]

[1] Since the preparation of this manuscript, experiments have been carried out to determine the concentration of active tissue substance in solutions of known effect on hastening coagulation of blood. For this determination, about 300 gm. of fresh beef lungs were hashed, ground well with sand, and extracted twice with 0.9 per cent NaCl solution. These two extracts, after removal of all solid particles by sedimentation in the ice box over night, were acidified with 0.5 N H_2SO_4 to make a calculated acidity of 0.002 N. The resulting precipitate of the active substance was washed free of other materials, as described in a later section of this paper. The active material, thus purified, was not dried, but was redissolved in 0.9 per cent NaCl solution, 0.5 N NaOH being added to give a calculated concentration of 0.002 N to aid the resolution of the material. This solution of the active principle diluted to the same volume as the combined original extracts possessed the same amount of activity in hastening coagulation as did the combined extracts before precipitation of the active substance.

Duplicate determinations of the N content of the solution were now made in order to compute the concentration of the active material present. In the preceding paper it is shown that the active substance contains 10.7 per cent N. Using this figure as a basis, the average of the two determinations gave 4.16 mg. of active substance per cc. of solution.

This solution was now tested for its accelerating influence on clotting, and the coagulation time was found to increase almost exactly the same, with dilution, as it did in diluting lung extract, as given in Table I of the preceding paper. Apparently it was about one-fourth as strong, undiluted, as was the saturated lung extract used in the tests recorded in Table I (10).

The saturated lung extract used contained about $4 \times 4.16 = 16.64$ mg. of active substance per cc. of solution. Diluted 65,536 times, at which point the last trace of acceleration of clotting was in evidence, there was then $16.64 \div 65{,}536 = 0.000256$ mg. of active substance per cc. of the diluted solution. 0.04 cc. of this was added to 1 cc. of oxalate plasma in the test, so that this final concentration in the plasma was $0.000256 \div 25 = 0.00001024$ mg. per cc., or approximately 1 part of active substance to 100,000,000 parts of plasma.

2. Is the Active Tissue Substance a True Tissue Fibrinogen?

In all the tests with active tissue extracts the clots formed were very much firmer and more resistant to breaking up on shaking than were clots formed from the same plasma without tissue extract addition. This suggested that this active material not only hastened the clotting of the plasma, but also increased the fibrin yield. Schmidt (11), in 1872, described what he termed a "fibrinoplastic substance" which he obtained from blood plasma by dialysis or treatment with weak acid, and which probably corresponded, in part at least, to the active material from the tissues. He claimed to obtain as high as 1 per cent yield from beef plasma, which many times exceeds the amount the writer has been able to demonstrate in such plasma. However, Schmidt demonstrated increases in fibrin yields from given plasmas of from 10 to 30 per cent, under the influence of the addition of different amounts of his "fibrinoplastic substance." Rettger (12), in 1909, showed that from a given fibrinogen solution varying amounts of fibrin could be produced by adding varying amounts of thrombin. However, he says that probably not all the fibrinogen had been changed over to fibrin with the addition of the smaller amounts of thrombin, thus showing that the thrombin acts in a quantitative manner, rather than that it actually increases the amount of fibrin to be obtained from a given amount of blood fibrinogen.

A number of experiments have been carried out to determine the effect of tissue extract addition on increasing the fibrin yield from a given plasma, and results showing increases up to 156 per cent in the fibrin yield have been produced by varying the amount of active tissue substance added. A number of other substances, such as starch paste, boiled dilute egg albumin, and milk, have also been added to plasma in order to see if such inactive solutions or suspensions would increase the weight of the fibrin to be obtained. Thus, a 0.4 per cent starch suspension (boiled) mixed with citrated horse plasma in equal volumes causes only 3.66 per cent increase in the fibrin yield. Out of 1.6 gm. of starch so added, only 0.0868 gm. was retained in the fibrin after thorough washing with 0.9 per cent NaCl solution and distilled water. With the use of lung extract, containing 1.8

per cent of proteins, in the same way, an increase of 152 per cent in the fibrin yield was obtained, 1.641 gm. of the lung material out of 5.448 gm. being bound in the fibrin. Fresh egg white, which is without any marked effect on the clotting time of horse plasma, was diluted with five volumes of distilled water, filtered, and boiled. A milky opalescent suspension resulted, acting like lung extract as far as passing through a filter is concerned; that is, a few cc. pass through readily and then the pores of the filter become blocked and filtration practically ceases. Such a suspension of boiled egg white, containing 2.13 per cent of solids, was added to horse plasma and the fibrin yield observed. An increase of 39.5 per cent in the fibrin occurred, 0.466 gm. of the egg albumin being bound out of 6.366 gm. added.

These experiments, together with many more, will be reported more in detail in the near future. It is greatly to be desired that definite proof be found whether the active coagulant of tissue extracts produces the increase in fibrin by chemically uniting with the latter, or is merely held mechanically due to its coarse state of dispersion in the extracts. Further experiments on this subject are at present being conducted with the aid of Mr. G. M. Guest.

The few results cited above seem to indicate strongly that the active coagulant of lung extract is present in the fibrin in some other form than as mechanically held material. That is, the substance appears to be a true tissue fibrinogen, the name given to it many years ago by Wooldridge. If it does enter chemically into the fibrin formation, such a fact would be difficult to explain under the theories of coagulation that are commonly accepted at present.

3. Tissue Fibrinogen and Blood Fibrinogen Unite only in the Presence of Calcium.

The addition of tissue fibrinogen to oxalate plasma does not result in the formation of fibrin until the plasma has been recalcified, nor does the active material appear to be bound in any way, since its activity persists for days and weeks and it may be precipitated out in active form by making the plasma very weakly acid (10).

Experiments were also tried on the blood of rabbits *in vivo* to see if the blood could be rendered non-coagulable by decalcification and death from lung extract injection thus be avoided.

Attempts were therefore made to decalcify the blood of rabbits by intravenous injections of potassium oxalate and sodium citrate. The former proved too toxic to permit of even close approach to complete decalcification. After the injection of as much oxalate as the rabbit could withstand, thrombosis resulted when lung extract was injected. The blood of a rabbit completely prostrated by oxalate, clots in the normal time after withdrawal from the vessels, so that this salt is not at all suitable for intravenous use as desired here. Sodium citrate, on the other hand, is much less toxic, as high as 9 cc. of a 5 per cent solution having been injected into an 1,800 gm. rabbit intravenously over a period of 15 minutes, with only spasmodic tremors and twitchings resulting. The following protocol of an experiment will serve to show the effects of lung extract injection after such citrate treatment.

Rabbit, male, 1,800 gm. 9 cc. of 5 per cent sodium citrate injected intravenously over a period of 15 minutes, keeping the rate of injection just below that necessary to produce muscular twitching or evolutions.

This was followed at once by an injection of 1 cc. of lung extract (saturated). Lethal dose of this extract in a normal rabbit was less than 0.1 cc.

Spasms occurred 20 seconds after the extract injection, and the rabbit was dead in $1\frac{1}{2}$ minutes.

Immediate examination for clots showed only slight fibrin strings suspended from the chordæ tendineæ of the right heart. Remainder of blood was fluid, and a sample of it kept in the ice box showed only a slight clot in 24 hours. To another sample was added 1 drop of serum. Clotting occurred in 3 hours. To a third sample were added 2 drops of 1 per cent $CaCl_2$ which resulted in coagulation in 15 minutes.

40 cc. of the blood were oxalated to 0.5 per cent and centrifuged to obtain a clear plasma. The following tests were performed on this plasma:

1 cc. plasma + 2 drops 1 per cent $CaCl_2$ → clot in 65 sec.

1 cc. normal beef oxalated plasma + 2 drops of 1 per cent $CaCl_2$ → clot in 6 min.

1 cc. normal beef oxalated plasma + 5 drops of rabbit plasma + 2 drops of 1 per cent $CaCl_2$ → clot in 65 sec.

The rabbit plasma contained plenty of fibrinogen to form a normal clot, but was lacking in calcium. Therefore the decalcification by the citrate injection was almost complete. The rabbit plasma contained sufficient tissue fibrinogen to accelerate

markedly the clotting of normal beef plasma. Whether the slight clots which were found were sufficient to cause death cannot be stated. There is also the possibility that something else in the tissue extract, other than the tissue fibrinogen, might have caused the death of the animal.

4. Results of Intravenous Injections of Tissue Extracts.

Since the work of Wooldridge and of Wright on intravascular coagulation following injections of tissue fibrinogen little has really been added in this field, except repeated demonstrations that thrombin injections will not cause coagulation. Gutmann (13) found that rabbits killed by single injections of rabbit lung extract exhibited the negative phase of coagulation in the portion of the blood remaining fluid, and that this non-coagulability was due to a decrease in fibrinogen, the amount being decreased nine to eleven times. He supposed that this loss was to be accounted for by absorption of the fibrinogen by the clots formed in the vessels. Mellanby (14) showed that certain snake venoms were strongly coagulative for blood and that slow repeated injections of very small amounts rendered the blood of animals non-coagulable (negative phase) by gradual removal of the fibrinogen as fibrin, but without solid clot formation. It will be shown here that tissue extracts possess the same power of defibrinating the blood.

All the lung extracts used in the following injections were of about equal strength, ten parts by weight of 0.9 per cent NaCl solution being used for each gm. of lung tissue taken. This gives a much weaker extract than that used in the preceding section to study the effect of dilution. These extracts possess about one-thirty-second of the activity of the saturated extract.

A. Single Injections of Lethal Doses.—Injections of large doses of lung extract, that is 1 cc. or more, into rabbits intravenously cause respiratory symptoms in 20 seconds, followed about 10 to 15 seconds later by violent spasms and convulsive struggles. Death usually occurs within 1 minute of the time of injection. Defecation and urination usually occur during the struggles. On opening the thorax and abdomen immediately, it is noticed that the intestines are in very active peristaltic motion and that

the heart is usually beating weakly. Clots are always found in the portal vein, usually in the inferior vena cava and right heart, and sometimes in the veins above the heart. Only in a very few cases was the blood in the left ventricle found clotted. Wooldridge commented upon the frequency with which the clotting occurred in the portal vein and stated that it occurred here even more readily when the animal was in full digestion. Wright held that the CO_2 content of the blood was the main factor in deciding its tendency to coagulate, thus accounting for the prevalence of clotting in the venous system but its absence from the arterial system.

The blood remaining fluid in the vessels, after death from clotting as described above, showed a partial or complete negative phase of coagulation. Such blood contains no substances which will inhibit the coagulation of normal blood, but instead it still contains some of the active tissue fibrinogen so that its action is to accelerate the clotting of normal blood. It is not deficient in calcium, but contains an amount sufficient to recalcify normal oxalate plasma and cause it to clot. The following tests demonstrate these facts:

1 cc. oxalate plasma + 2 drops 1 per cent $CaCl_2$ solution → clot in 4 min.

1 cc. oxalate plasma + 1 cc. non-coagulable plasma → clot in 2 min.

Here the 1 cc. of the non-coagulable plasma not only furnishes the calcium required for the clotting of the oxalate plasma, but also quickens the clotting time from 4 minutes to 2 minutes. Fibrinogen tests on the plasma, as by half saturation with NaCl, usually show some small amounts to be present, although in some cases there is not the slightest precipitate. Where there is some fibrinogen present, clotting occurs slowly if the blood is not disturbed, the time varying from a few hours to several weeks. These clots are not firm, and are easily broken up by shaking. The negative phase, or non-coagulability, then seems to be due to a lack of fibrinogen, either partial or complete. The fact that the clotting in the vessels occurs while the blood is actively circulating probably accounts for a portion of the blood not being caught in the clot. It is well known that agitation of plasma or blood during clotting will cause the fibrin to precipitate out more in strings than as a fine network, so that most of the fluid is left free.

B. Negative Phase Production in Rabbits.—Intravenous injection of sublethal amounts of tissue extracts, repeated at intervals of 1 to 2 minutes and in increasing amounts, causes the development of a certain immunity to the material after a few injections, so that about ten times the original lethal dose may be given without symptoms. The following protocol will illustrate this.

Rabbit, 2,500 gm. No anesthesia used. Injections were made into the marginal ear vein.

Lung extract injected. cc.	
0.2	9.20 a.m.
0.3	9.21½ "
0.5	9.22½ "
0.8	9.23 "
1.2	9.24 "
1.6	9.25 "
2.0	9.26½ "
2.6	9.28 "
2.6	9.29½ "
2.6	9.32 "
2.6	9.37 "

(0.3 cc. of this extract is the lethal dose for a 2,500 gm. rabbit.)

The only symptoms were a slight, but steadily increasing weakness. The animal was killed by a blow on the head 3 minutes after the last injection, and examined for the presence of clots. No trace of clotting was found anywhere. The blood drawn from the inferior vena cava was kept in the ice box for several days without evidence of coagulation occurring. It was very rich in the tissue fibrinogen, as shown by its power to hasten the clotting of normal blood, contained sufficient calcium to clot normal oxalate blood, but was found very poor in fibrinogen. It would not clot with fibrin ferment, CO_2 gas, dilution with water, or the addition of any amount of lung extract.

Thus, although the plasma is itself entirely non-coagulable, it contains sufficient calcium per cc. to recalcify the oxalate plasma and cause it to clot. It must then contain the equivalent of 4 drops of 1 per cent $CaCl_2$ per cc. Not only does it effectively recalcify the oxalate plasma, but it also quickens the coagulation time to half the normal period. This indicates the presence of some of the tissue fibrinogen still in the plasma. Similar experiments with peptone plasma from a dog show a similar recalcification of the oxalate plasma, but also indicate clearly the presence

of some powerful inhibitory substance or condition which retards or prevents the coagulation even with sufficient calcium present.

1 cc. oxalate plasma + 1 drop lung extract + 1 drop 1 per cent $CaCl_2$ → clot in 12 sec.

1 cc. oxalate plasma + 0.25 cc. peptone plasma + 4 drops lung extract → clot in 15 sec.

1 cc. oxalate plasma + 0.5 cc. peptone plasma + 4 drops lung extract → clot in 60 sec.

1 cc. oxalate plasma + 1.0 cc. peptone plasma + 4 drops lung extract → no clot.

The peptone plasma used here had stood several weeks in the ice box without clotting. It is evident from the above tests that 1 cc. of this plasma contains more than enough of the inhibitory material to counterbalance the accelerating effect of the 4 drops of the lung extract. The first test shows that the lung extract is very strong also, 1 drop of it causing 1 cc. of oxalate plasma to clot in 12 seconds when recalcified.

A further experiment was tried, using blood freshly drawn from a dog and mixing with it the peptone plasma and non-coagulable plasma used above.

1 cc. fresh dog blood drawn and let stand → clot in 4 min.

1 cc. fresh dog blood drawn into 1 cc. non-coagulable plasma → clot in 1 min.

1 cc. fresh dog blood drawn into 1 cc. lung extract → clot in 40 sec.

1 cc. fresh dog blood drawn into 1 cc. 0.9 per cent NaCl solution → clot in $4\frac{1}{2}$ min.

1 cc. fresh dog blood drawn into 1 cc. peptone plasma → very slight clot in 18 hrs.

Here it is evident that, while peptone plasma does strongly inhibit the clotting of normal blood, the non-coagulable plasma obtained by tissue extract injection is not only free from such action, but contains some of the tissue material in active form so that its action is to hasten normal clotting markedly. Since the decrease in fibrinogen in this plasma was demonstrated by half saturation with NaCl and comparison with normal plasma similarly treated, the non-coagulability was probably due to such fibrinogen deficiency.

This condition was induced in four rabbits, with complete non-coagulability of the blood resulting. In many other cases

the immunization was not so well accomplished, the animal being killed by too sudden an increase in the dosage. In such a case there were usually found slight clots either in the portal vein or on the chordæ tendineæ of the right heart. The fluid blood presented the characteristics described above, however. In no experiment was there any substance present in the non-coagulable blood which would inhibit the clotting of normal blood.

C. Negative Phase Production in Dogs.—This same immunization was carried out in seven different dogs with the animals under anesthesia. Blood pressure records were made and blood samples drawn at frequent intervals to study the blood changes. The following protocol will serve as a typical example of these experiments.

Dog, male, 9 kilos. Anesthetized with ether. Tracheal cannula. Blood pressure from right carotid artery. Injections from a burette into left femoral vein. Cannula in right femoral artery for blood samples. Table I shows the injections made and the results obtained.

In Table I, by lung extract ($\times \frac{1}{4}$) is meant lung extract diluted with three volumes of 0.9 per cent NaCl solution. Lung extract ($\times$ 1) is the undiluted lung extract. The coagulation time was taken as the time when the tube containing the blood could be carefully inverted without loss of its contents. By "activity" of the blood samples is meant their accelerating effect on the coagulation of normal oxalate beef plasma. All the samples drawn for these tests and for the alkali reserve were oxalated to about 0.5 per cent to prevent clotting, and the corpuscles centrifuged out to obtain clear plasma for the tests. Degree of activity of the plasma samples was tested as follows:

1 cc. oxalate beef plasma + 2 drops 1 per cent $CaCl_2$ → clot in $4\frac{1}{2}$ min.
1 " " " " + 5 " Plasma 1 + 2 drops 1 per cent $CaCl_2$ → clot in 2 min., 40 sec.

It is shown in the table of dilution of lung extract (Table I (10)) that the hastening of the coagulation time bears a very definite relation to the concentration of the active tissue fibrinogen present, so that, by testing the amount of shortening of the coagulation time induced by a given amount of the fluid to be tested, one may rather accurately estimate the amount of the active substance present. Now, by reference to the results listed in Table I of the

TABLE I.

Negative Phase in a Dog.

Time.	Lung extract injected.	Blood samples.			
		No.	Coagulation time.	Activity.	Alkali reserve.
	cc.				*cc.* CO_2
2.34 p.m.		1	4 min.	2 min., 40 sec.	38.6
2.50½ "	0.5 (×¼)				
2.51¼ "	0.5 (×¼)				
2.52 "	1.0 (×¼)				
2.53¾ "	1.0 (×¼)				
2.53½ "	1.2 (×¼)				
2.54 "	1.5 (×¼)				
2.54½ "	2.0 (×¼)				
2.55¼ "	3.0 (×¼)				
2.56 "		2	4 min., 10 sec.	2 min., 20 sec.	38.6
2.56½ "	4.0 (×¼)				
2.57 "	5.0 (×¼)				
2.58 "	6.0 (×¼)				
2.59 "	7.0 (×¼)				
3.00¼ "	8.0 (×¼)				
3.01½ "	9.0 (×¼)				
3.02½ "	10.5 (×¼)				
3.03½ "	12.0 (×¼)				
3.05 "	15.0 (×¼)				
3.09 "	1.0 (×1)				
3.11 "	2.0 (×1)				
3.12½ "	2.5 (×1)				
3.13¼ "		3	34 min.	1 min., 50 sec.	25.3
3.14½ "	3.5 (×1)				
3.16½ "	4.5 (×1)				
3.18½ "	6 (×1)				
3.21½ "	8 (×1)				
3.25 "	10 (×1)				
3.27 "		4	60 min.	1 min., 25 sec.	24.25
3.28½ "	12 (×1)				
3.31 "	15 (×1)				
3.33½ "	25 (×1)				
3.40 "	35 (×1)				
3.42 "	50 (×1)				
3.44½ "		5	Slight clot, 5 min.	45 sec.	23.8
4.06½ "		6	Slight clot, 15 min.	50 "	
4.12½ "	50 (1 per cent $NaHCO_3$)				

TABLE I—*Concluded.*

Time.	Lung extract injected.	Blood samples.			
		No.	Coagulation time.	Activity.	Alkali reserve.
	cc.				*cc. CO_2*
4.14½ p.m.		7	Slight clot, 14 min.	45 sec.	23.32
4.20 "	50 (1 per cent $NaHCO_3$)				
4.22 "		8	Slight clot, 9 min.	47 sec.	25.16
4.35½ "	50 (1 per cent $NaHCO_3$)				
4.38½ "		9	Slight clot, 10 min.	45 sec.	24.68
4.51 "		10	Slight clot, 13 min.	50 "	21.47
5.00 "	Death. Bled from heart.	11	Slight clot, 13 min.	50 "	29.32

present paper under "Activity," it is seen that the blood normally contained a quite definite amount of the material, since the clotting time of oxalate plasma was shortened from 4½ minutes to 2 minutes, 40 seconds, by 5 drops of the dog plasma. During the injections of the lung extract, this accelerating effect of the samples of dog plasma on normal clotting of beef oxalated plasma became more and more marked, so that it must be concluded that the concentration of the active substance in the blood of the dog was steadily increasing. Sample 5, taken 2½ minutes after the last lung extract injection, was able to shorten the clotting time of normal oxalate blood to 45 seconds, while Sample 6, taken 24½ minutes after the last injection, only shortens the clotting time to 50 seconds. Thus during that 22 minute period there occurred a noticeable decrease in the concentration of the tissue fibrinogen in the dog's blood. This decrease is probably to be accounted for by an excretion of the material into the urine, since the urine collected at the end of the experiment contained almost as much of the substance as the blood. 5 drops of the urine were able to

accelerate the clotting time of 1 cc. of normal oxalate plasma to 60 seconds, whereas the same quantity of blood plasma drawn at death gave a clotting time of 50 seconds to normal plasma. This excretion of the material in active form by the kidney is discussed later in this paper.

The coagulation time of the blood of the dog is seen to be unaffected by the injections until injections of 2 to 3 cc. of undiluted lung extract are used. (About 3 cc. of the extract are the fatal dose for a dog this size when only a single injection is given.) More frequent drawing of blood samples before this point always shows a quickening of the clotting time to $1\frac{1}{2}$ to 2 minutes, this then being followed by the negative phase. Samples 5 to 11 formed only slight clots which were readily broken up by the slightest agitation. Several days standing in the ice box failed to show any further clotting. Tests for fibrinogen in the corresponding oxalated samples showed the presence of a small quantity of fibrinogen, but nothing at all to be compared to the normal amount. The negative phase seems, then, to be due to a loss of fibrinogen from the blood. It is not due to any inhibiting substance present, or to lack of calcium, since this plasma will recalcify normal oxalate plasma and cause it to clot in a shorter time than will the addition of $CaCl_2$ alone. Tests similar to those given for the non-coagulable rabbit blood were carried out on this plasma and the results were so similar that they need not be given here. The fate of this lost fibrinogen is not known, since it is not present as visible clots anywhere in the vascular system. Sometimes slight fibrin strings were to be found attached to the chordæ tendineæ of the right ventricle, but no clots elsewhere. Furthermore the dog gave no symptoms of intravascular clotting during the injections. A possible explanation for the fate of the lost fibrinogen will be set forth in the discussion at the end of this paper.

The alkali reserve was determined with the Van Slyke CO_2 apparatus, following the method of Van Slyke and Cullen (15). There is a very sudden fall in the alkali reserve just at the time of the development of the negative phase in the blood. This occurred in each such experiment performed on dogs. It is not due to the anesthesia, for dogs kept under anesthesia, as controls, for 2 hours show a very much smaller fall. The signifi-

cance of such a sudden fall is unknown. It is probably due to negative phase production, since it is so constantly associated with it, but it is not the cause of the loss of coagulability, since bicarbonate injections later in the experiment show no effect on the coagulation time.

D. Effect of Acid and Alkali Injections. Asphyxia.—In order to see whether or not the fall in alkali reserve was a possible causative factor in the negative phase production, weak acid was injected into a dog until the alkali reserve reached an extremely low level. Instead of a lessened coagulability of the blood, there was found a greatly shortened clotting time. The protocol of the experiment follows.

Dog, male, 5 kilos. Anesthetized with ether. Blood pressure from carotid artery. Injections from burette into left femoral vein. Blood samples from right femoral artery.

The procedure followed and the results obtained are shown in Table II.

This experiment is inserted here merely to show that the great fall in alkali reserve during the production of the negative phase by tissue extract injections probably has in itself nothing to do with the non-coagulability. In this experiment it is to be noted that the CO_2-combining power of the plasma falls rapidly as the coagulation time of the blood markedly shortens, and again that it rises as the coagulation time lengthens.

Although this experiment has little bearing on the mode of action of tissue extracts on the blood *in vivo*, it merits attention as regards the action of acids and alkalies on the blood and its coagulability.

In text-books on physiology it is often found stated that death from asphyxia leaves the blood non-coagulable. In such a death the hydrogen ion concentration of the blood probably increases after the CO_2-combining power of the plasma has been exhausted, and so a preliminary experiment on asphyxia was carried out on this dog. Complete occlusion of the air supply for $2\frac{1}{2}$ minutes was all the dog could stand and still be able to recover. The blood became almost black in color, but the coagulation time was not affected. An increase of over 40 per cent in the CO_2-combining power of the plasma was found. Such an increase has been found by others in the past and has been taken to mean that the

corpuscles have contributed material to the plasma for binding the CO_2. It has been shown that the alkali reserve of the whole blood increases much less than does the plasma alone. It is doubtful whether in this experiment, the CO_2-combining power

TABLE II.

Time.	Treatment and injections.	Blood samples.		
		No.	Coagulation time.	Alkali reserve.
				cc. CO_2
3.10 p.m.	15 min. after etherization.	1	5 min., 30 sec.	37
3.16 "	Trachea clamped to cut off air.			
3.18½ "	Air supply restored.	2	5 min., 45 sec.	52.8
3.26 "	10 cc. 0.1 N HCl injected.			
3.27½ "		3	6 min., 30 sec.	31.4
3.30½ "	25 cc. 0.1 N HCl injected. (No more ether given.)			
3.33¼ "		4	5 min., 45 sec.	23.2
3.36¼ "	50 cc. 0.1 N HCl injected.			
3.41½ "		5	1 min., 30 sec.	18.4
3.43½ "			2 "	
3.50½ "	50 cc. 0.1 N HCl injected.			
3.56½ "		6	1 min.,	9.9
3.59½ "			1 "	
4.06½ "			1 "	
4.13 "		7	3 " 45 sec.	9
4.16 "	50 cc. 0.1 N $NaHCO_3$.			
4.20 "		8	12 min.	18.8
4.27 "	50 cc. 0.1 N $NaHCO_3$. (Ether given again.)			
4.31 "		9	23 min.	27.2
4.32½ "	35 cc. 0.1 N $NaHCO_3$.			
4.38 "		10	42 min.	31
4.49 "				
4.50 "	12 cc. 1 per cent $CaCl_2$. (Death during injection, intravascular clotting.)			
5.02 "	Blood taken from left heart.	11	2 min.	24.9

of the blood was exhausted, and, if it was not, the rise in H^+ concentration would have been negligible.

Injection of 0.1 N HCl was without marked effect until after 85 cc. had been given, when the blood in the vessels became so

viscid that it would scarcely issue from a medium sized cannula in the femoral artery. Breathing was very labored and no ether was necessary until during the alkali injections. Blood pressure fell to a very low level although the heart action was apparently well maintained. All these effects probably came as a result of the greatly increased viscosity of the blood. Injection of another 50 cc. of the 0.1 N HCl only increased the above effects. The blood would issue from the cannula so slowly that it would clot before it could be mixed with oxalate. It was with difficulty that enough could be oxalated, unclotted, for the alkali reserve determination. From the very great decrease in the CO_2-combining power of the plasma here it is probable that the H^+ concentration had risen considerably.

The first 50 cc. injection of 0.1 N bicarbonate restored the blood to its normal fluidity, caused a cessation of the labored respiration, and a marked elevation of the blood pressure. Further bicarbonate injections restored the dog to apparently normal condition so that it was necessary to restore the ether administration. The most noticeable peculiarity to be observed in the results is the marked slowing of the coagulation time as the proper bicarbonate concentration is restored to the blood. However, the injection of $CaCl_2$ that followed, with the resulting solid intravascular clotting in the heart and all large veins examined, showed that the delayed coagulation during the bicarbonate injections was probably due to a partial and increasing decalcification of the blood by precipitation of the calcium as $CaCO_3$. The $CaCl_2$ injection seemed to restore the coagulability to the blood through recalcification, and the intravascular clotting probably was caused by the presence throughout the blood of $CaCO_3$ particles which acted as centers for the initiation of the clotting process.

The action of the acid in so markedly increasing the viscosity of the blood suggested that the affair was probably a matter of increasing the hydration capacity of the blood proteins, and, since the coagulation time of the fibrinogen was affected, this protein must have been one of those affected. To see if a similar action could be found *in vitro* some fibrinogen was precipitated from oxalate plasma by half saturation with NaCl, and redissolved in 0.9 per cent NaCl solution. This fibrinogen solution

was placed in a series of tubes and acid added to give a calculated concentration ranging from 0.0002 to 0.5 N. No change in the contents of the tubes occurred until the acid concentration reached 0.01 N when a distinct opalescence was to be seen. At 0.02 N acidity this opalescence was more marked and at 0.1 N there was almost complete precipitation of the fibrinogen. Now the first effect of the acid injection on the dog occurred after 85 cc. of 0.1 N HCl had been injected. This 5 kilo dog had probably 400 cc. of blood, figuring one-tenth to one-thirteenth of the body weight as blood, and of this blood about 60 per cent, or 240 cc., would be fluid plasma. 85 cc. of 0.1 N HCl added to 240 cc. of plasma would make approximately a N/40 HCl solution, if no acid was lost. However, the blood proteins would bind considerable of the acid, and it would be lost rapidly into the tissue lymph and secretions. At any rate, it is seen that the acid concentration in the blood probably fell within the limits of concentration which cause an opalescence to appear in a fibrinogen solution *in vitro*.

E. Effect of Tissue Extracts on Blood Pressure.—One further point of interest in regard to the intravenous injection of tissue extracts is the effect on blood pressure. With the injection of amounts sufficient to cause extensive intravascular clotting, the blood pressure rises abruptly at the time of the clotting. With sublethal doses, however, when no clotting occurs, the blood pressure effects are almost identical with those of histamine (ergamine, β-imidazolylethylamine). There occurs the same abrupt fall in pressure followed by the same more gradual rise back to normal. Like histamine, also, lung extract will cause such an effect time after time, at intervals of 1 to 3 minutes; that is, the sensitivity of the vascular system to its effects is not diminished except very slowly. Besides this similarity to histamine in its vasodilator effect, there is also its stimulating action on other smooth musculature. Urination and defecation nearly always occur following the injection of a moderately large or lethal dose into rabbits while with much smaller doses the increased peristaltic movements of the intestines can be readily observed even without opening the abdomen. Also the gurgling of the contents becomes very marked as a result of such peristalsis.

As has been mentioned before, it is considered by some that tissue extracts contain a mother substance capable of freeing

histamine when injected into the blood, so that it is supposed to be the effects of the histamine that give the above described results. In another place the writer has mentioned that the purified tissue fibrinogen gives a negative test for the presence of imidazole ring compounds when tested by Ehrlich's diazo reaction, although the crude tissue extract gives a distinctly positive test when thus tested. Whether the positive test in the crude extract is due to histidine or to histamine has not been determined.

Since the purified material gave a negative test for histamine, it is interesting to note that intravenous injections of it into rabbits produced death in exactly the same fashion as did the crude lung extracts. It has not yet been tried on dogs, nor has its effect directly on smooth musculature been observed. This would make it seem as though the presence of histamine is not necessary for the characteristic effects of the substance, but rather that the effects probably depend on changes induced in the blood during the transformation of the fibrinogen to fibrin.

5. Excretion of Tissue Fibrinogen in Urine.—Tissue fibrinogen injected intravenously into dogs or rabbits so as to produce a non-coagulability of the blood always results in the excretion of some of the material unchanged in the urine. It so happened that all the urine samples collected from the animals were just about neutral to litmus. What would happen if the urine were acid cannot be stated, although it is likely the material excreted might lose some of its activity. The urine collected at the end of the dog experiment described on page 181, showed the presence of almost as much tissue fibrinogen as did the blood plasma. By reference to the protocol, it will be noted that the dog was not killed until 1 hour and 20 minutes after the last injection of lung extract, and that during this time the concentration of the material in the blood decreased, as shown by the activity tests. This active tissue fibrinogen in the urine retained all the properties of the material injected, the most characteristic of which was its activity on the clotting of blood. It could readily be salted out of the urine by half saturation with $(NH_4)_2SO_4$, just as it could from the lung extract.

It has been shown by the author in the preceding paper (10) that a presumably slight change in the protein-phospholipin compound,

which is here termed tissue fibrinogen, results in a partial or complete loss of its activity on coagulation. Since the size of the molecules of the substance must be very great (as judged by present molecular weight figures, the substance would probably consist of 1 protein molecule to which are united about 12 phospholipin molecules) it is extremely difficult to imagine any process by which it must have passed intact from the blood stream into the urine, unless the two living membranes through which it passed possess pores through which it might pass, just as the white corpuscles pass through the capillary walls.

6. Specificity of Tissue Fibrinogen.—Loeb (16) has shown that a substance derived from the tissues, or blood cells, is necessary for the completion of the coagulation process in the blood of various lower forms of animal life. He termed this substance "tissue coagulin." It is very likely identical with what is here termed tissue fibrinogen. Loeb (17) also states that he found a definite class specificity to exist for these tissue coagulins; that is, the extracts of the tissues of one species of animal will hasten the clotting of the blood of other animals of the same class, but will be without effect on the blood of animals of a different class. Thus the tissue extracts made from any mammalian tissue would hasten the clotting of the blood of any mammal but would be ineffective on bird, reptilian, amphibian, or fish blood.

The writer has not gone deeply into this question, but has found that extracts of the lungs of cattle, dogs, rabbits, rats, and guinea pigs all show marked activity in the coagulation of the blood of any of the animals mentioned, either in *vitro*, or when injected intravenously. The intravenous injections were made mostly into rabbits, although rats and dogs were used to some extent. The only animal outside the mammalian class so far tested was the frog. It was found that extracts of frog lungs would very readily clot frog blood *in vitro*, or cause solid intravascular clotting, but such extracts were entirely without effect on the coagulation of mammalian plasma. Mammalian lung extract, however strong, would not cause intravascular clotting of frog blood, although it would hasten the clotting of such blood *in vitro* to some extent.

SUMMARY.

1. Tissue extracts accelerate the clotting of blood in a very definite manner, the coagulation time of the blood increasing from a minimum of about 10 seconds up to the normal time as the tissue extract added is diluted. The logarithm of the coagulation time in seconds plotted against the logarithm of the dilution of the extract gives almost a straight line. It is possible to get a noticeable quickening of the clotting process with the active tissue substance added to blood plasma in the proportion of 1 part of active substance to 100,000,000 parts of plasma.

2. The active tissue substance will not react with the blood fibrinogen to form fibrin, either *in vitro* or *in vivo*, except in the presence of soluble calcium salts.

3. Injected intravenously, rapidly, and in sufficient amounts, tissue extracts, or the purified active substance, cause intravascular clotting and death in a very definite manner. Injected slowly and in smaller amounts the blood is rendered non-coagulable, partially or completely, the non-coagulability apparently depending on a gradual removal of the greater part of the fibrinogen from the blood stream. A marked decrease in the alkali reserve of the plasma develops along with the development of the negative phase of coagulation, but is apparently not the cause of it.

4. The injection of active tissue extracts into the blood stream so as to produce non-coagulability without clot formation, is followed by an excretion of the active tissue substance apparently unchanged in the urine (dogs and rabbit).

5. As observed by Loeb, there is present a class specificity in regard to the action of the tissue extracts on blood clotting. The specificity, however, is not absolute, since mammalian lung extract will accelerate the clotting of frog blood.

6. There is a latent period in the coagulation process which is remarkably constant under similar conditions. Upon this depended the success of showing accurately the result of dilution of the tissue extracts upon their coagulative action. But it may be demonstrated also for intravascular clotting of the blood. Thus, after the injection of a fatal dose of lung extract into the ear vein of a rabbit, the time that elapsed before the onset of the

convulsive struggles did not vary more than 10 seconds in different rabbits, being 15 to 25 seconds after the injection. This gave time for at least a partial distribution of the active substance through the blood with the resulting great dilution of it. Just as plasma *in vitro* cannot be made to clot much quicker than 10 seconds, regardless of the concentration of the tissue material, so it was also found to be with the blood in the vessels. Always if coagulation occurred in any degree in the vessels following tissue extract injection, it occurred within 30 seconds after the injection. If the amount injected was not sufficient to give results in that time, then no solid clots formed, but a negative phase of coagulation set in. Further injections into the same animal of like or gradually increasing amounts, only served to increase the negative phase, until finally the blood was totally non-coagulable. The diminution, or lack of fibrinogen in such blood appears to be the cause of its non-coagulability.

BIBLIOGRAPHY.

1. Wooldridge, L. C., On the chemistry of the blood and other scientific papers, London, 1893, The Croonian lecture on the coagulation of the blood.
2. Wright, A. E., *Lancet*, 1892, i, 457, 515.
3. Morawitz, P., *Beitr. chem. Physiol. u. Path.*, 1904, v, 133.
4. Fuld, E., and Spiro, K., *Beitr. chem. Physiol. u. Path.*, 1904, v, 171.
5. Nolf, P., *Arch. internat. physiol.*, 1906–07, iv, 165.
6. Howell, W. H., *Am. J. Physiol.*, 1911–12, 29, 187.
7. Loeb, L., *Biochem. Zentr.*, 1907, vi, 829.
8. Mellanby, J., *J. Physiol.*, 1908–09, xxxviii, 28.
9. Mills, C. A., *J. Biol. Chem.*, 1919, xl, 425.
10. Mills, C. A., *J. Biol. Chem.*, 1921, xlvi, 135.
11. Schmidt, A., *Arch. ges. Physiol.*, 1872, vi, 413; 1875, xi, 515.
12. Rettger, L. J., *Am. J. Physiol.*, 1909, xxiv, 406.
13. Gutmann, *Compt. rend. Soc. biol.*, 1914, lxxvi, 349.
14. Mellanby, J., *J. Physiol.*, 1908–09, xxxviii, 441.
15. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
16. Loeb, L., *Virchows Arch. path. Anat.*, 1903, clxxiii, 35; *Beitr. chem. Physiol. u. Path.*, 1904, v, 191, 534; 1905, vi, 260.
17. Loeb, L., *Med. News*, 1905, lxxxvi, 577.