ANTIGENIC VALUE OF LUNG EXTRACT.

By

Helene A. Thomas.
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Approved by: Noble P. Sherwood
Head of Department.

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INTRODUCTION.

Many years ago, tissue extracts were found to be effective in hastening the coagulation of blood but no worth while investigations were made until the time of Wooldridge, (1883). Since then, although additional work has been done on the mechanics of blood clotting and various factors influencing it, tissue extracts did not again come into prominence until the investigations of Mills in 1921.

Although these workers took into consideration the chemical nature of tissue extracts and their effects upon blood coagulation in vitro, even to the formulating of a theory of blood clotting, apparently nothing was done from the standpoint of immunity. There was little knowledge concerning the possible role of antisera to tissue extracts in blood coagulation and little as to their effect upon the digestive properties of pepsin hydrochloride. This investigation, accordingly, was undertaken in an endeavor to clarify these two questions.

As a preliminary survey of the work bearing upon these subjects, the following phases of it will be touched upon in the succeeding pages:

(1) Theories of blood coagulation.

(a) Wooldridge.

(b) Morawitz.

(c) Howell.

(dO Mills.

(2) Accelerators of blood coagulation.

(3) Retarders of blood coagulation and anti-coagulants.

Wooldridge worked with aqueous extracts of thymus, lymph glands, and red blood cells. He found that 1.5 gram of the active material precipitated by strong acetic acid would kill an ordinary sized dog and that one gram was sufficient to kill a rabbit. He concluded that the important material was a protein-phospholipin compound, that the phospholipin fraction was essential for coagulative action but that it could not alone produce intravascular clotting. He called this active substance tissue fibrinogen because the tissue material entered into the formation of the fibrin.

The next notable investigator in this field, Morawitz, apparently ignored the findings of Wooldridge and advanced the following theory of blood coagulation (McLeod): Fibrin, is formed by the union of a nucleo-protein, (thrombin), calcium, and a protein from the blood known as fibrinogen which has the properties of a globulin. Calcium and fibrinogen occur normally in the blood but thrombin is present only in an inactive form called prothrombin. However, prothrombin may be converted into thrombin by the addition of a thrombokinase which is an activator released from blood platelets upon their disintegration.

A short time later, Howell (McLeod, Howell, 1911-1912) elaborated on the theory of Morawitz. He agreed with him

that blood normally contained prothrombin, calcium and fibrinogen but he contended further that these substances were prevented from reacting and the normal fluidity of the blood maintained by an anti-thrombin which he found present in normal alian plasma, bird plasma, and peptone plasma. This anti-thrombin holds the prothrombin in combination and thus prevents its' conversion into, or its' activation to, thrombin. When blood is shed, disintegration of platelets furnish thromboplastin which combines with antithrombin and liberates more prothrombin; the latter is then activated by calcium and acts on fibrinogen.

The most recent theory of blood coagulation and the one upon which the experimental work of this paper is based is that of Mills. (Mills, 1921 A). He harked back to the findings of Wooldridge and states that tissue fibrinogen does not produce coagulation of the blood by removing an anti-coagulant or an anti-thrombin, but in itself unites directly thru calcium with blood fibrinogen to form fibrin. This theory will be better understood by considering each of the interacting substances separately.

(1). The first substance concerned in the formation of fibrin is fibrinogen. Fibrinogen is a simple protein, related to the globulins, differing chiefly in its' ready coagulability by fibrin ferment, heat, salt, and other coagulants. It may be precipitated out with sodium chloride or sodium sulphate. It does not coagulate spontaneously by itself.

(2). Soluble calcium salts are also essential for blood coagulation as proved by the fact (Mills, 1921 B) that the addition of tissue fibrinogen to oxalated plasma does not result in fibrin formation until the plasma has been recalcified. In Howell's theory, calcium activates prothrombin to thrombin. In Mills' theory, it acts merely as a connecting link between blood fibrinogen and tissue fibrinogen.

It is interesting to note the comparative decrease in effectiveness of other salts which are in the same group in the periodic system as calcium. Howell (1914 B) found that the addition of barium chloride to fibrinogen gave no clot in twenty-four hours, magnesium chloride gave a feebly floating clot in six to twenty hours, and strontium chloride a floating clot in the same period, as compared to a clot in ten minutes when calcium chloride was added and fifteen minutes upon the addition of calcium nitrate. This shows the part played specifically by calcium in blood coagulation.

(3). The third constituent to be considered in the formation of fibrin, is tissue fibrinogen. This has already been mentioned in connection with the work of Wooldridge and Mills (Mills 1921 B). He found that lung extract contained more tissue fibrinogen than any other organ extract similarly prepared and therefore was the most effective tissue used as a blood coagulent. He separated this active

substance by half saturation with ammonium sulphate; complete saturation with magnesium sulphate; or sodium chloride; precipitation with one per cent. mercuric chloride solution; sulphosalicylic acid or very dilute acid (N/500 to N/1000).

Upon analysis, Mills (1921 A) found it to consist of 41.6% phospholipin and 58.4% protein or one mole of protein to thirteen moles of phospholipin. He found the protein to be definite and specific. The phospholipin, he considered to be cephalin. This purified substance possesses the soluble character of the globulin class of proteins and is thermo-labile.

It must be remembered that the activity of this substance on blood coagulation depends on the union of the phospholipin and protein fraction, as each by itself possesses only a small degree of activity. The union of the two, however, will restore the greater part of the activity, although not all of it. (Mills, 1921 A).

After determining the effectiveness of tissue extracts in hastening blood coagulation in vitro, Mills (1921 C) determined it in vitro by injecting one cubic centimeter or more of a 1 - 10 solution of lung extract intravenously into a rabbit with the result that death occurred within a minute due to clots in the portal system in the inferior Vena Cava and the right heart.

Thus, it can be seen that so potent was the lung ex-

tract that the formation of an anti-serum was not possible by intravenous injection but judging from the success of Hektoen in developing specific precipitins to leukecytes (Hektoen, 1922) and to hemoglobin (Hektoen and Schulhuf, 1923), it seems probable that specific preciptins could also be developed to tissue fibrinogen could the tissue extract be injected in such a manner that absorption would be so slow as not to excite intravascular clotting. Accordingly, the species or organ specificity or non-specificty of possible tissue antisera becomes of interest in the light of blood coagulation since Loeb (as cited by Mills) found only a definite class specificity to exist for tissue coagulants, that is, extracts of the tissues of one species will hasten the clottingof the blood of other animals of the same class but not of different classes. Dorst and Mills (1923) held that there may be a specificity as regards speed of coagulation, but not as regards ability of the coagulants to enter into union with the blood fibrinogen to form fibrin.

Not only must an anti-serum against the lung extract as a whole be considered but also the possibility of developing anti-sera to each fraction taken alone. Since the protein is soluble, no question need be raised as to its' antigenic properties but there is room for doubt that the phospholipin extract possesses an antigenic capacity although Jobling and Carroll (1912) found a specific immune

lipase in erythrocytes and Chung Yik Wang (1919) an antigenic capacity in the lipoidal extract of dried blood. It is interesting to note in this connection, the wide variation in the coagulent activity of the two fractions as well as the variation in antigenic properties. The protein fraction (Mills, 1921 A) of tissue extracts acts as an anti-coagulant in blood clotting while the phospholipin portion seems to possess slight accelerating properties.

Although this investigation deals primarily with the effects of tissue extracts and their anti-sera upon blood coagulation, a comprehensive understanding of the situation necessitates a knowledge of other substances and conditions which may accelerate or retard clotting, namely:

Accelerators.

Blood Serum

The influence of blood serum on the coagulation activity of tissue extracts is of practical interest. Mills and Mathews (1921) found that normal rabbit serum is capable of causing as high as a thirty fold increase in the coagulative activity of lung extract. This effect is gradually replaced by a diminuation in the activity of the extract below the original if the mixture is left standing. Normal human sera, also syphilitic serum, possesses something of the same power but the increase is only four fold.

Cephalin.

Cephalin has a great accelerating effect on the coagu-

lation of recalcified oxalated plasma. (Gratia and Lavene)

Pancreatic Rennet.

Pancreatic Rennet shows great activity as a coagulant for normal and hemophilic blood. Moderate intravenous injections diminish coagulation time and excessive doses increase coagulation time after an initial shortening. Rennet produces no intravascular clotting. (Epstein and Rosenthal).

Bacterial products may also modify coagulation. Staphlococcus aureus is the most powerful of these coagulants. Typhoid, diphtheria, tubercle, and xerosis bacilli are without apparent effect. Pyocyaneous, prodigeosis, and the colon bacilli occupy an intermediate position. After the organisms are killed by boiling, the effect is greatly reduced. (Loeb).

The clotting time of blood is shortened about an hour after each meal. (Mills, 1923 E).

Anti-Coagulants.

In all disease connected with suppuration and leucocytosis, the amount of fibrinogen is increased, particularly in pneumonia. The fluidity of the blood in septicemia may depend upon the appearance of the coagulation inhibiting phase that follows the action of the products of cell destruction or because of the presence of an anti-thrombin in excell. (Dochez, 1912).

Blood may become non-coagulable after phosphorus poi-

soning due to a fibrin destroying ferment in the liver which causes an absence of fibrinogen in the blood. (Zeit, 1900).

Intravenous injection of thrombin into the circulation within limits of 20 to 56 milligrams per 1000 grams animal, does not cause intravascular clotting. In fact, the coagulation of blood is diminished for a certain short period of time. (Appears in 7 to 28 minutes, lasts 3 minutes to an hour or more). The injection of thrombin calls forth a production of anti-thrombin which may come into play normally to protect the body from intravascular clotting. (Daniel Davis, 1911-1912).

Howell (1918) has found two new substances concerned in blood coagulation.

(1). A phosphotid called heparin since it is obtained from dog's liver. It's characteristic reactions are:

(a) Retards or prevents coagulation in the body or without.

(b) Causes marked increase of anti-thrombin when added to blood serum.

Heparin inhibits clotting by preventing the activation of prothrombin to thrombin. The action of heparin is not destroyed at 100°.

(2). A mother substance for Anti-thrombin. This substance is present in plasma and to a considerable amount and is called pro-anti-thrombin.

Pro-anti-thrombin + Heparin = Anti-thrombin.

Theoretical.

Heparin and pro-anti-thrombin are normal constituents of the circulating blood for the purpose of safe guarding the fluidity of the blood, i.e., prevent intravascular clotting. Pro-anti-thrombin by conversion to anti-thrombin protects against thrombin.

In vitro, proteins such as Caseosan and Aolan delay coagulation, also non-proteins, Yatren, Placenta-optone and Pituglandol. On diluting to one-tenth, the opposite effect was obtained of hastening coagulation. This is recommended before operations. (Salamon and Vey, 1923).

Arsenobenzols exhibit anti-coagulating properties. (Pomerat, 1922).

Blood in contact but a few moments with pleura is rendered non-coagulable. (Herwerden, 1922).

The time element or age of a serum enters into it's coagulation ability, i. e., its' coagulation activity decreases as its' age increases. (Effront and Prescott).

Heating serum for several hours at even a moderate temperature of 38° markedly decreases its' coagulative ability. (Effront and Prescott).

EXPERIMENTAL WORK.

The scope of this investigation is outlined as follows:

(1) Is it possible to develop anti-serums for tissue fibrinogen, against tissue anti-coagulants?

(2) Would such anti-serums show species specificity, organ specificity, or neither, taking into consideration the chemical specificity of proteins?

(3) Would the rise of the immune bodies be due to the protein fraction, the phospholipin fraction, or a combination of both?

(4) What results might be obtained by combining the protein fraction of one species with the phospholipin fraction of another?

(5) What effect, if any, might the anti-serums produced have upon blood coagulation?

(6) What effect does the tissue extract and its' anti-serum have upon enzyme activity as regards the digestion of fibrin with pepsin hydrochloride?

The methods used in this work as regards preparation of material and coagulation tests in vitro are the methods of C. A. Mills of the Biochemical Laboratory, University of Cincinnati. The methods of injection and later absorption tests have been developed as the work progressed.

So far, lung extract has been the only organ extract used, as lung tissue has been found to contain much more

of the active coagulant material than any other tissue of the body. The lungs of cattle, sheep, hogs, dogs, rabbits, and guinea pigs were used, all of about equal activity except rabbit lung which were found to be much stronger - probably due to the presence of more or less rabbit serum which in itself is sufficient to increase the coagulative activity of lung extract thirty fold. (Mills and Mathews).

The lungs were obtained from local meat markets, The Swift Packing Company, and University experimental animals. As can be seen, it was usually impossible to obtain them before the blood had clotted in the tissue. In an endeavor to remove as much blood as possible, the veins were slit open and washed thoroughly with water. Soaking over night was also resorted to. They were then ground in a meat grinder and dried at room temperature by means of an electric fan.

The crude saturated tissue extract was made by powdering the dried lung and extracting with .85% sodium chloride for thirty minutes after which the liquid was drained off through several layers of cheese cloth. (4 c.c. saline per one gram dried lung tissue). It was then centrifuged or sedimented in the ice box over night and the clear solution pipetted off. In spite of all efforts made to remove the blood, the color of the extract showed them to have been futile.

The phospholipin fraction was freed as follows:

TO PARTIALLY EXTRACT PHOSPHOLIPIN:

Lung extract dried at room temperature.
(Powder in mortar)

+

Benzene at room temperature for two minutes. (200 c.c. Benzene per 100 grams dried lung.

Decant off benzene and evaporate extract to dryness at room temperature.

Residue contains 4.8% phospholipin.

MORE THOROUGH EXTRACTION:

Lung tissue (dried, powdered.)

+

Benzene (room temperature) for four days. Same proportions as above. Change two or three times a day.

Decant off benzene and evaporate to dryness at room temperature.

Residue contains 7% to 8% phospholipin.

(I was able to secure 4 grams phospholipin from 46 grams of

dried lung or a yield of 8.6%

Lung are about 76% water).

PROTEIN EXTRACTION: (1)

(After phospholipin extraction.)

(1) Remove benzene from lung tissue by evaporation at room temperature.

Extract with .85% NaCl.

+

Half saturate with ammonium sulphate.

(Gives gray white precipitate containing all the active material.)

Redissolve in 1% NaCl.

(Due to the presence of the ammonium sulphate, this extract proves toxic upon injection unless dialyzed.)

OR

(2) Precipitate the protein out by acidifying with N/2 H_2SO_4 to give a final normality of 500. (Collect)

Wash with N/500 H_2SO_4.

Sediment overnight.

Decant off supernatant fluid. Dry by placing in shallow pans in air

current.

Yield = 1.9% fresh lung.

EXTRACT OF PURIFIED ACTIVE MATERIAL FROM DRIED LUNG:

(1) This method corresponds exactly to the preceeding one with the exception that the active material is precipitated from the saline extract of whole dried lung.

Since .1 c.c. of the saturated lung extract, when injected intravenously into a rabbit, is sufficient to kill it in a minute (1), all injections were made intraperitoneally and in relatively small amounts. This treatment, however, brought no results so that larger and ever increasing amounts of the extract were resorted to. (This is made possible by the slowness of absorption.)

The injections were made as follows:

RABBIT A.

Saturated HOG lung extract (2 c.c.)

Died.

RABBIT B.

Saturated DOG lung extract.

2...2...2...2...2...2 c.c.

1...1...2...1...1 Day intervals.

No titer.

2...3...5...8 c.c.

6...8...2 Day intervals.

Died.

RABBIT C.

Saturated BEEF lung extract. (pH = 7.4.)

2...2...2...2...2...2 c.c.

1...1...2...1...1 Day

Intervals.

No titer.

2...4...4...4...6.5...10...12...13.5 c.c.

2...6...5...3.....2....3....3 Day in-

tervals.

Titer = 400.

Since it had proved impossible to entirely remove the blood from the lung tissue, there was the possibility that the anti-serum had been developed against the beef SERUM and not against the lung tissue fibrinogen. Accordingly, the following precipitin reactions were set up:

TABLE I.

	0	2	5	10	25	50	100	200	400	800	1600	3200	6400
A N T I - B E E F S E R U M .													
Beef Serum 1	2	3	3	3	3	3	3	2	2	1	1	1	0
Beef lung 2	2	2	2	2	2	2	1	1	0				
A N T I - B E E F L U N G .													
Beef Serum 1	1	1	1	1	1	1	1	2	2	2			
Beef Lung 3	3	3	3	3	3	3	3	1	1	1			

This proved beyond doubt that beef serum had played an important part. To determine whether all the anti-bodies in the anti-lung serum were due to the presence of blood in the lung extract injected, the antibodies to beef serum were absorbed out by using equal volumes of anti-beef lung serum and beef serum (Dilution of 1 to 200). This was left at room temperature for one hour, set in the ice box over night and centrifuged in the morning.

A number of precipitin tests were then set up using this absorbed anti-serum:

TABLE II.

ABSORBED ANTI-BEEF LUNG SERUM

0	2	10	25	50	100	200	400	800	1600	3200	6400	Controls
Beef Serum -	-	-	-	-	-	-	-	-	-	-	-	-
Beef lung 2	2	2	2	2	1	1	1	-	-			-

A month an a half later, the same rabbit was again injected with crude lung extract as follows:

2...5...5 c.c.

2...5 Day

intervals.

Further injections were made impossible as the rabbit exhibited Arthur's Phenomenon, an evidence of anaphylaxis shock.

The animal was then bled, the serum absorbed out with

beef serum(1 - 200) as previously described, and then titrated with beef lung extract. It titered 1600 as against 400 from the last injection.

It was noticed at the time of drawing blood from the ear of the rabbit that its' coagulation time was markedly increased. Whereas normally its' clotting time was three minutes, it now had been increased to a range varying from 10 minutes to several hours. The blood in these instances was collected when flowing freely from the vein through a piece of capillary tubing into a test tube. When the blood was allowed to flow over the surface of the ear, its' coagulation time was decreased to 1 to 3 minutes, thereby showing the effectiveness of tissue extracts in hastening coagulation.

This prolongation of clotting time of the rabbit's blood was repeated in the case of another rabbit which had been similarly injected. After standing twenty minutes, the blood was centrifuged. The supernatent fluid was colorless, clear plasma, showing that the coagulation time had been so lengthened that even after standing, no clot had, as yet, been formed.

Four rabbits were injected with crude beef lung extract during the course of this work. The method of injection which proved the most effective is as follows:

2...5...8...10...10 c.c.

2...2...3...3 Day

Intervals.

Titrate on the sixth day.

SPECIES SPECIFICITY: Extracts of the lungs of other species were made in a manner similar to beef lung extract and precipitin tests set up against anti-beef lung serum.

TABLE III.

Species Specificity.

	ANTI - BEEF LUNG.												
	0	2	5	10	25	50	100	200	400	800	1600	3200	Controls
Dog Lung	-	-	-	-	-	-	-	-	-	-	-	-	-
Hog Lung	-	-	-	-	-	-	-	-	-	-	-	-	-
Rabbit Lung	-	-	-	-	-	-	-	-	-	-	-	-	-
Guinea Pig Lung	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep Lung.	+	+	+	+	+	+	+	+	+	+	+	-	-

The above results seem to point toward a species specificity with the exception of the positive reaction to sheep lung extract. This might have been expected, however, since beef and sheep hemolysins are in the same class and since in this case the blood had not been absorbed previously with blood serum. The question now is: Is a positive precipitin reaction due only to this similarity of beef and sheep hemolysins or do sheep and beef lung extract possess the same inter-reactive characteristics also? That the latter supposition might be true is shown in the following absorptions

and precipitin reactions.

TABLE IV.

	0	2	5	10	25	50	100	200	400	800	1600	3200	Control.
(1) Anti-beef lung serum absorbed with sheep lung extract (1 to 200).													
Sheep Lung - Control.	-	-	-	-	-	-	-	-	-	-	-	-	-
Beef lung -	-	-	-	-	-	-	-	-	-	-	-	-	-
(2) Anti-beef lung serum absorbed with beef lung extract (1 to 200)													
Beef Lung Control -	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep Lung -	-	-	-	-	-	-	-	-	-	-	-	-	-
(3) Anti-beef lung serum absorbed with beef serum (1 to 200).													
Beef Serum - Control.	-	-	-	-	-	-	-	-	-	-	-	-	-
Beef Lung + Extract	+	+	+	+	+	+	+	+	+	+	-	-	-
Sheep Lung + Extract.	+	+	+	+	+	+	+	+	+	+	-	-	-
(4) Anti-beef lung serum absorbed with sheep serum (1 to 200)													
Sheep Serum-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beef Lung +	+	+	+	+	+	+	+	+	-	-	-	-	-
Sheep Lung +	+	+	+	+	+	+	+	-	-	-	-	-	-

(5) The anti-beef lung serum was tested repeatedly for beef and sheep hemolysins and in all cases the results were negative.

Thus, it appears that the active antigenic elements of beef and sheep lung extracts are inter-reactive.

To determine what effect, if any would result from reconstructing lung extract from the fractionated lung extracts of different species, rabbit D was injected with a mixture composed of beef lung protein (5 c.c.) and hog lung phospholipin (.1 gram) as follows:

3...5...3...5 c.c.

2...1...1 (hr.)

Days Interval.

3.....5.....3.....5 c.c.

2 day.1 day.1 hr. Intervals.

The beef lung protein was prepared as described on page 14 of this article, and the hog lung phospholipin as on page 13. Six days after the last injection, the rabbit was bled and the following precipitin reactions set up with the antiserum:

TABLE V.

TITRATION OF BEEF-HOG LUNG ANTI-SERUM

	ANTI - SERUM.										
	0	5	10	25	50	100	200	400	800	1600	3200	Controls
Hog Lung	+	+	+	+	+	+	+	+	-	-	-	-

TABLE V.

(Continued).

	0	5	10	25	50	100	200	400	800	1600	3200	Controls.
Beef Lung	+	+	+	+	+	+	+	+	+	+	+	-
Beef Lung Protein	+	+	+	+	+	+	+	+	+	+	-	-
Hog Lung Protein.	-	-	-	-	-	-	-	-	-	-	-	-
Anti-serum absorbed with beef serum (1 to 200)												
Beef Serum	-	-	-	-	-	-	-	-	-	-	-	-
Beef Lung	+	+	+	+	+	+	+	+	+	-	-	-
Beef Lung Protein.	+	+	+	+	+	+	+	+	-	-	-	-

The anti-serum was not titrated against hog lung phospholopin because of the impossibility of getting the phospholipin into solution without destroying any precipitin activity it might have possessed.

Crude beef liver, kidney, and brain extracts were prepared similar to crude lung extract for the purpose of investigating organ specificity. Since these extracts contained some beef blood and the anti-beef lung serum has been demonstrated to contain anti-bodies to beef serum, a positive precipitin reaction was inevitable except in the case of the brain extract which appeared to contain no blood.

However, after absorption of the anti-beef lung serum with beef serum (1 to 200) and consequent titration with the three respective organ extracts, all precipitin reactions were negative. This work has been repeated at various intervals, each time with freshly prepared extracts. The results throughout have been consistently negative. (It should be stated that accompanying all absorption tests as a means of further control, equal volumes of the anti-serum and saline are incubated, refrigerated, and set up against their respective antigens to make certain that the anti-serums have retained their strength).

TABLE VI.

ORGAN SPECIFICITY.

ANTI - BEEF LUNG SERUM.										
	10	25	50	100	200	400	800	1600	3200	Controls.
Beef Liver	+	+	+	+	+	-	-	-	-	-
Beef Kidney	+	+	+	+	-	-	-	-	-	-
Beef Brain	-	-	-	-	-	-	-	-	-	-
ANTI-BEEF LUNG SERUM ABSORBED WITH BEEF SERUM (1 to 200)										
Beef Serum	-	-	-	-	-	-	-	-	-	-
Beef Liver	-	-	-	-	-	-	-	-	-	-
Beef Kidney	-	-	-	-	-	-	-	-	-	-
Beef Brain	-	-	-	-	-	-	-	-	-	-

Thus, it would appear that lung extracts demonstrate organ specificity.

This work bears out Mill's work in vitro (Mills, 1923 E). He found that liver extract did not decrease the coagulation time of blood even when lung phospholipin fractions were added, seeming to indicate that the protein fraction of the lung extract is different from that of liver extracts (Mills, 1923 E).

Mills found kidney extract effective in hastening blood coagulation in vitro although not to so great an extent as lung extract.

I found brain extract as active as lung extract in this particular.

PURIFIED ACTIVE MATERIAL:

The purified active material was separated from the crude lung extract according to the method of Mills on page 15 in this article. This was injected into a rabbit intraperitoneally as follows:

2.....1.....2.....4.....6.....1.....3.....8.....5.....2 c.c.
2 day.1 hr..1hr...1 hr..2 day.1 hr..4 day.4 day.1 day Intervals.

After six days, the rabbit was bled from the ear and the anti-serum titrated against the purified active material and against crude beef lung extract. In the former case, the titer was 3200, in the latter, 1600.

Crude beef lung extract was broken into its' component

parts by the use of Mills' methods. (Pages 13 and 14 of this article.)

The waxy phospholipin fraction contained no demonstrable protein as evidenced by its giving negative Biuret, Millon. Xanthoproteic, Adamkiewicx, or Cole-Rosenheim tests.

An emulsion, satisfactory for injection purposes, was finally obtained by emulsifying the phospholipin in olive oil. It was injected intraperitoneally as follows:

.05---.1---.3---.5---.5---.5 gram

2----4----2----4----2 Days

Intervals.

After six days, the anti-serum was titrated using complement fixation reactions, in which an alcoholic extract of phospholipin served as the antigen. These results were negative.

However, when the anti-serum was titrated against the whole beef lung extract in a precipitin reaction, it showed a titer of 200.

Accordingly, the same rabbit was injected again two weeks later:

.5----.75 gram.

2
Day Interval.

Using the same titration methods as above, the complement fixation tests were again negative, but the precipitin reaction against the whole beef lung extract showed a raise in titer to 400.

The protein fraction, which Mills found to be an anti-coagulant (1), was injected intraperitoneally into a rabbit in the following amounts:

3.....(1.....3).....6.....5.....(2.....5) c.c.

2 day..1 hr...4 day.3 day.1 day..2 hr. Intervals.

After six days, the rabbit was bled from the ear thru a capillary tube into a test tube. When flowing freely, several samples were taken for determining coagulation time. The blood clotted in three and one-half minutes which was practically normal. The anti-serum was titrated against the protein fraction and against crude beef lung extract. In the case of the former, the titer was 800; the latter, 400.

Since the importance of lung extract is due chiefly to its' property of hastening the coagulation time of blood, an investigation was made of the effects of anti-seras, normal seras, bacterial suspensions, bile, and extracts of other organs upon clotting time. Whole blood, oxalated to .5%, and 1% calcium chloride were used. All tests were conducted in a 40° water bath in small test tubes, complete coagulation being at that moment when the tube could be inverted without losing any of its' contents.

Table VII is a summary of this work. The blood of four different people was used, each sample being tested several times with each of the foreign coagulants, anti-coagulants, and non-active substances added.

TABLE VII.

SHOWING EFFECT OF VARIOUS SUBSTANCES UPON COAGULATION TIME OF BLOOD.

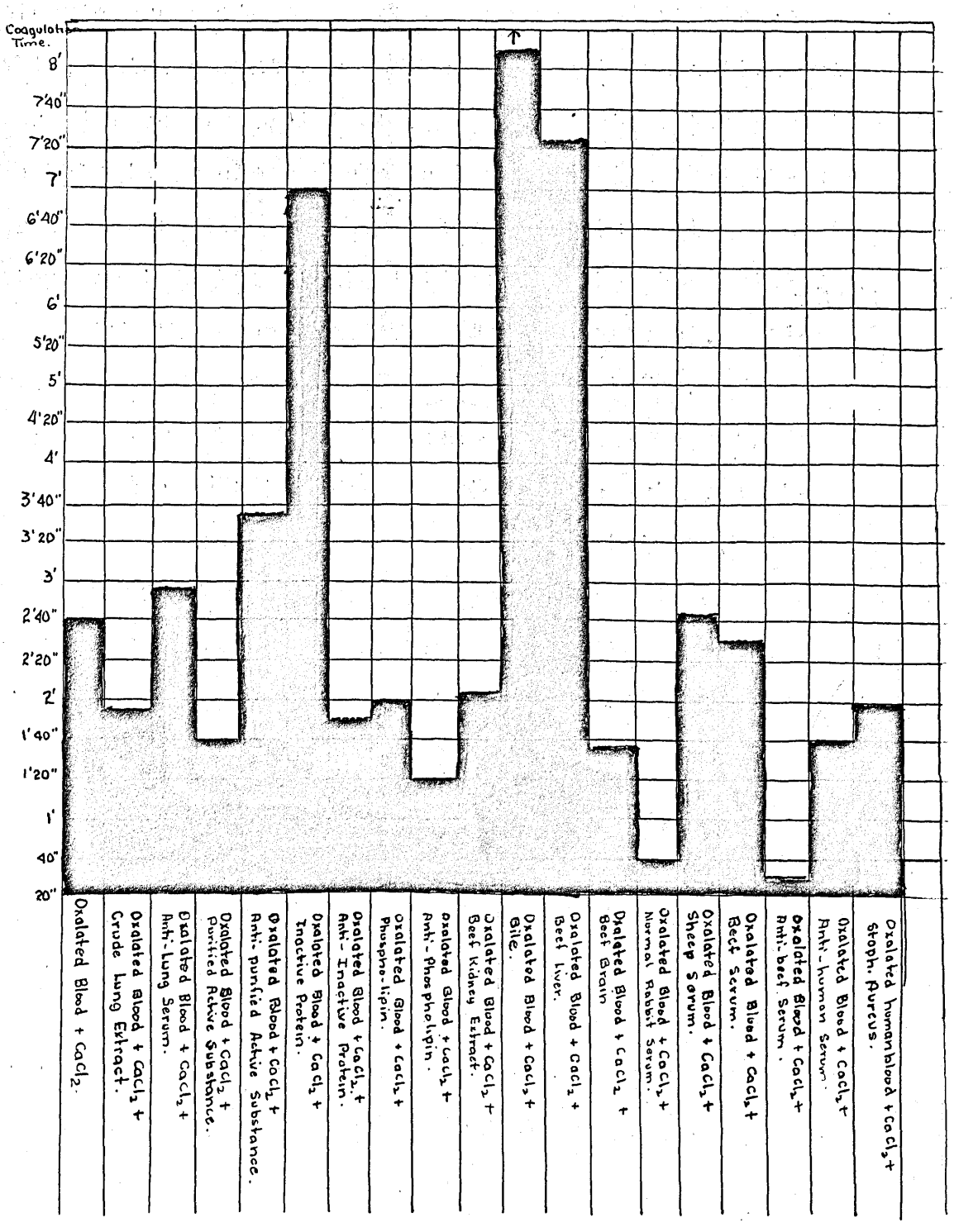

TABLE VIII.

EFFECT OF VARIOUS SPECIES OF LUNG EXTRACT UPON COAGULATION.

Blood (Citrated to 1%).	CaCl (1%)	Tissue Extract	Time
1 c.c.	1 c.c.	0	3'35"
1 c.c.	1 c.c.	.5 beef	1'35"
1 c.c.	1 c.c.	.5 dog	1'45"
1 c.c.	1 c.c.	.5 rabbit	40"
1 c.c.	1 c.c.	.125 gm. Pl	2'

The following tubes were set up, incubated at 37° for one hour, and centrifuged at a high rate of speed.

(A) Oxalated Human Plasma (.5 c.c.) - .85% NaCl (.25 c.c.).

(B) Oxalated Human Plasma (.5 c.c.) - Anti-human serum (.25 c.c.).

(C) Oxalated Human Plasma (.5 c.c.) - Anti-beef lung serum (.25 c.c.).

Five-tenths cubic centimeter calcium chloride was added to the supernatant fluid in each case and the coagulation time tested with the following results:

Tube A clotted in 4 minutes.

Tube B clotted in 6 minutes.

Tube C clotted in 10 minutes and 35 seconds.

THE EFFECT OF TISSUE EXTRACTS UPON PEPSIN-HYDROCHLORIDE DIGESTION.

Since fibrin is very insoluble in all substances except pepsin hydrochloride and since tissue fibrinogen enters into the formation of fibrin, the effect of tissue extracts upon the enzymatic activity of pepsin hydrochloride was investigated, also, the ability of tissue extract to act as a protective agent to the fibrin. Accordingly, tubes were set up as indicated in Table IX. With the exception of Tubes 9 and 10, which were each incubated one hour before addition of the sheep fibrin, the contents of all tubes were added at the same time and left to act at room temperature.

The fibrin from Tubes 4, 5, and 6 (Table IX) was removed and one half the fibrin from each was thoroughly washed in water. Pepsin hydrochloride (fresh) was then added to both the washed and unwashed portions with the following results:

TUBE	COMPLETE DIGESTION.	
	WASHED	UNWASHED
4	6 minutes	11 minutes
5	14 minutes	14 minutes
6	14 minutes	19 minutes

TABLE IX.

SHOWING THE EFFECT OF LUNG, LIVER, AND KIDNEY EXTRACTS ON THE ENZYME ACTIVITY OF PEPSIN HYDROCHLORIDE.

Tube #	Pepsin - HCl	Pepsin	HCl	Beef Lung	Anti-Beef Lung Serum	Phospho-lipin.	Fibrin	Beef Liver	Beef Kid-ney.	Time	Digestion.
1		+ 2 cc.					+			72 hr.	None
2			+ 2 cc.				+			72 hr.	Very slight
3	+ 2 cc.						+			24'	Complete
4	+			+			+			72 hr.	None
5	+				+		+			72 hr.	Almost Comple-te.
6				+			+			72 hr.	None
7	+ 2 cc.			+ 1 cc.	+ 1 cc		+			25'	Complete
8	+					+	+			2 hr16'	Complete
9	+ 2 cc. ← Incubated 1 hr. →			+ 2 cc.			+			72 hr.	None
10	+ 2 cc. ← Incubated 1hr. + 2cc. Saline.						+			22'	Complete
11	+						+	+		15 hr.	Complete
12	+						+		+	60 hr.	Complete

DISCUSSION.

Although it would be a most difficult matter to produce anti-bodies against lung tissue extract by intravenous injections, since the amounts injected must necessarily be so minute as not to cause intravascular clotting, it is somewhat simpler to produce them by intraperitoneal injections. In the latter case, apparently the absorption is so slow that it is necessary to inject relatively large amounts.

Although it is relatively simple to produce low titered serums, it becomes increasingly difficult to produce serums of a titer above 3000 as the rabbits do not survive the necessary increased amounts and injections must cease lest the rabbit die.

It was to be expected that anti-serums could be developed against the protein fraction and possibly against the phospholipin fraction as Jobling in his work, as well as others, have found the antigenic properties of lipins to be well established.

It was impossible to determine whether any precipitins were developed against the phospholipin as it is soluble only in organic solvents which in themselves tend to prohibit the reaction. Although no anti-body production was demonstrated by the complement fixation tests, this failure might be attributed to the unsatisfactoryness of the alcoholic extract of phospholipin as antigen since the positive

precipitin reaction with crude lung extract showed without doubt that there was some anti-body production. Although the phospholipin extract gave no test for protein, its' antigenic properties may yet be due to a minute trace of protein, which amount was sufficient for immunization but not for visible chemical tests. Again, part of the protein may be held in so close a chemical combination that it is extracted out with the phospholipin, again making immunization possible but chemical identification impossible.

With the exception of sheep lung extract, anti-beef lung serum showed marked species specificity. The cause of the one exception has not been determined as yet.

As far as this investigation was carried, anti-beef lung serum showed remarkable organ specificity. Care was taken that all organs used were from beef in order that specificity should not influence the reaction. These results might have been somewhat anticipated from Mills' work since he found such wide variation in the effects of the extracts of various organs upon coagulation time of blood. In the case of liver, particularly, he proved that this difference was due, not to the absence of phospholipin, but to the presence of a different type of protein in the liver than was in the lung. Since the precipitin reaction is protein specific, it can readily be seen that the possibilities of other organs reacting with anti-beef lung serum are greatly reduced.

COAGULATION.

It is very difficult to draw conclusions from coagulation tests as serum is not a substance of constant composition. According to its origin, it may contain besides thrombin, either serozyme or thrombokinase. Also, there may be a difference in calcium content.

It is well known that various tissue extracts hasten coagulation, in fact, most coagulation theories include thromboplastic substances in their mechanism. Table VII shows this again to be true with the exception of liver extract. Its' strong anti-coagulant nature may be explained by the presence of bile and a phosphotid called Heparin. The marked acceleration following the addition of brain extract to plasma is probably due to the cephalin present.

The slight hastening of coagulation time upon the addition of Staphlococcus Aureus verifies the work of L. Loeb.

Normal serums, according to MacLeod slightly prolong clotting time. Mills (1923 E) found an exception to this in the case of normal rabbit serum which hastens coagulation about thirty fold.

The latter effect must be kept somewhat in mind in considering the coagulation of plasma by anti-serums. The fact that beef lung extract increases coagulation and anti-beef lung serum prolongs it, seems to be due to a specific change which takes place in the animal body due to the injection of the lung extract. In other words, it might be plausible to suppose that since blood plasma containing fibrinogen

was present in the lung extract injected which in itself contains tissue fibrinogen, that to prevent clotting, the body is forced to develop antagonistic substances capable of neutralizing the effect of the added fibrinogen. Effront and Prescott explain it as follows: "Thus it is that the serums of animal A, especially prepared with plasma of species B, precipitate the plasma of species B. According to Camus, the anti-coagulating action of these serums on plasmas results from the fact that, by precipitating fibrinogen, they have removed the capacity for coagulation." It might be possible that the serums hinder coagulation m more in that they eliminate the thrombin than in that they transform fibrinogen.

The clotting ability of the phospholipin and the anti-coagulating ability of the protein are not specific since, due to the treatment with benzene necessary to separate the fractions, both may be rearranged and changed in character from their structure in the whole lung extract. This, of course, is applicable also to their anti-serums.

PEPSIN HYDROCHLORIDE DIGESTION:

From Table IX, it is apparent that fibrin can not be digested with pepsin hydrochloride in the presence of lung extract or its' anti-serum. Two theories may be advanced in explanation: (1) Lung extract reacts with the pepsin hydrochloride in such a manner that the digestive ability of the latter is inhibited. (2) Lung extract surrounds the

fibrin as protective agent, thus preventing the pepsin hydrochloride from digesting it.

This investigation points to the first theory as the most plausible. In Tube 6, beef lung extract was left in contact with fibrin for 72 hours to give time for any combination which might take place between the two. Apparently, there was no such union since upon removal of the fibrin, the lung extract could be easily washed out. When fresh pepsin hydrochloride was added, digestion went on at normal rate.

However, as in Tube 9, when pepsin hydrochloride and beef lung extract were incubated together for one hour at room temperature and then fibrin was added, the activity of the pepsin hydrochloride seemed to be entirely inhibited as no digestion took place. That the incubation did not in itself inactivate the pepsin hydrochloride was proved by control tube 10 in which pepsin hydrochloride and saline were treated as in Tube 9, but in which the digestion of fibrin went on at normal rate.

It will be noted that in those tubes in which no digestion had taken place, when the fibrin was removed and placed in fresh pepsin hydrochloride, (Table IX B) digestion began at once.

It is an interesting fact that anti-beef lung serum seems capable of so neutralizing the beef lung extract that fibrin digestion is not interferred with in the least. Dr.

Sherwood called to my attention the fact that this series of tubes does not include a control substituting normal rabbit sera for anti-beef lung sera. This should be included in any complete investigation of this phase of lung anti-serum activity.

An inhibitive action upon pepsin hydrochloride is possessed to a slight degree by liver extract and to a greater degree by kidney extract but in neither case is the inhibition so pronounced as with lung extract.

CONCLUSIONS:

(1) By intraperitoneal injection, anti-bodies may be developed against whole crude lung extracts and against its' protein and phospholipin fractions whether from the same species or a combination of the fractions of two species.

(2) Anti-beef lung serum showed species specificity except in the case of sheep lung extract.

(3) Anti-beef lung serum exhibited organ specificity.

(4) Anti-serums markedly affect the coagulation time of human blood. In all cases, in this investigation, the anti-serum produced the opposite effect of the antigen, i.e., those substances which hastened clotting time in vitro, produced anti-serums which prolonged it, and vice versa.

(5) Lung extract seems to inhibit the enzyme action of pepsin hydrochloride so that blood fibrin digestion is prevented.

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