THE COAGULATION OF BLOOD. Part II. THE ACTIONS OF SNAKE VENOMS, PEPTONE AND LEECH EXTRACT.

By JOHN MELLANBY, M.D., *George Henry Lewes Student.*

(*From the Physiological Laboratory, Cambridge.*)

CONTENTS.

Introduction.

In a previous paper[1] the phenomena observed when blood coagulates have been described. In this paper the actions of certain substances which influence the coagulation of blood *in vitro* and *in vivo* are considered.

The coagulation of blood is ultimately due to the action of fibrin ferment on fibrinogen. Fibrin ferment does not circulate in the blood but is produced when any tissue is injured so as to liberate thrombokinase in the blood stream. This kinase in conjunction with the calcium salts present in the blood generates fibrin ferment from the prothrombin associated with the fibrinogen and the production of fibrin ferment in molecular continuity with the fibrinogen ensures that coagulation will take place in the shortest possible time. Anti-fibrin-ferment is present in considerable quantities in normal blood but its properties indicate that its function is to prevent the occurrence of an extending coagulum due to the passage of fibrin ferment from its original seat of formation rather than to prevent coagulation at the injured tissue, *i.e.* where the kinase is liberated.

The preparation from stable birds' plasma of the substances involved in the coagulation of blood affords a means of analysing the phenomena which may be observed when such substances as snake venoms, leech extract, and peptone exercise an influence on the coagulation of blood. Fibrinogen may be readily prepared from birds' plasma by dilution with a large volume of distilled water made slightly acidic; and a dilute solution of testis extract affords a convenient source of kinase. Fibrin ferment is obtained after removing the fibrin from a solution of fibrinogen coagulated by kinase and calcium chloride; and a solution of prothrombin remains after coagulating a fibrinogen solution by fibrin ferment. Anti-fibrin-ferment has not been prepared but plasma free from this substance can be obtained by alcohol precipitation and redissolving the protein in water.

Since the substances whose properties are analysed in the following pages influence the coagulation of blood in a positive or negative way a general discussion of the methods in which the coagulation of blood can be affected may conduce to the clearness of the subject. Plasma may be coagulated by fibrin ferment or by kinase. Since plasma contains a large quantity of anti-fibrin-ferment but no anti-kinase and since a large quantity of ferment is adsorbed by the fibrin when formed, it

follows that kinase is a much more efficient coagulant of plasma than ferment. This conclusion is strikingly borne out by the effect of intravenously injecting kinase or fibrin ferment into an animal. The rapid injection of small quantities of kinase produces intravascular coagulation; the rapid injection of relatively large quantities of ferment only leads to an increase in the coagulability of the blood.

A substance which inhibits the coagulation of blood may produce its effects in various ways. In this complex process there are involved five different factors—(1) calcium salts, (2) kinase, (3) prothrombin, (4) fibrinogen, (5) fibrin ferment—and the absence of any one of them will prevent the onset of coagulation. (1) The production of stable fluid blood by the addition to it of potassium oxalate or other precipitant of calcium salts illustrates the necessity of the calcium factor. (2) Normally the cellular elements of blood are sufficiently unstable to ensure the liberation of kinase when blood is shed. But in birds' blood and generally in blood containing nucleated red blood corpuscles, the stability of the cellular elements is such that stable fluid plasma can be readily obtained. In these animals, therefore, the kinase must come from the wounded tissue rather than from the blood itself. But the addition of an adequate quantity of anti-kinase to any blood suffices to prevent the onset of coagulation. (3) The constant association of prothrombin with fibrinogen renders it doubtful whether an anti-prothrombin would prevent the coagulation of blood. (4) The coagulation of blood may be prevented by causing the destruction of its fibrinogen either actively or by combining an anti-fibrinogen with it. (5) The production of coagulation might be prevented by the action of an anti-fibrin-ferment. But since blood normally contains a large quantity of anti-ferment, which does not prevent the rapid onset of coagulation, it appears improbable that any substance which delays coagulation does so by means of a body possessing these properties. And finally the onset of coagulation might be delayed by the presence of a substance which prevented the production of fibrin ferment from prothrombin, or inhibited the action of fibrin ferment on fibrinogen, or retained in solution the fibrin when formed. Small quantities of acids or alkalies affect in various degrees these processes. Acids in very small quantities prevent the production of fibrin ferment from prothrombin; alkalies in minimal quantities retain the first formed fibrin in solution.

There is also the question of how the nature of a substance which coagulates a fibrinogen solution may be determined. The constant association of prothrombin with fibrinogen permits the coagulation of

a solution containing this substance to be effected by a mixture of kinase and calcium salts or by fibrin ferment. In pure fibrinogen solutions the nature of the coagulant can be readily determined since the kinase and calcium salts generate an excess of ferment which is left behind when the fibrin is removed. In plasma this effect is obscured owing to the presence of an excess of anti-ferment. With this fluid the nature of the coagulant may be determined by adding to it a quantity of potassium oxalate in excess of the calcium present. The oxalate prevents the action of the kinase but has no influence on the action of fibrin ferment. But the one test which determines the nature of a coagulating substance is whether it generates fibrin ferment from a solution of prothrombin. If it does so then it possesses the properties of a kinase; if it cannot do this but still coagulates blood then it possesses the properties of fibrin ferment.

VIPER VENOM (*NOTECHIS SCUTATUS* AND *ECHIS CARINATA*).

The properties of the dried venoms of two vipers are described in this section—an Australian viper (*Notechis scutatus*) and an Indian viper (*Echis carinata*). These two venoms possess similar qualitative properties and the experiments dealing with them are conveniently given at the same time. The properties of cobra venom are subsequently described since this venom exercises an entirely different action to the above venoms on the processes which occur in the coagulation of blood.

In 1893 C. J. Martin[2] published a series of experiments describing the effects produced by the intravascular injection of viper venom into animals. These effects were precisely analogous to those described by Wooldridge after the intravascular injection of tissue fibrinogen into animals. The similarity of the results led Martin to assume that viper venom when injected into the blood stream of animals caused the liberation of nucleo-protein and this nucleo-protein gave rise to the phenomena observed.

But in 1903 Lamb showed that viper venom coagulated blood in the presence of citrate or oxalate. Martin[3] confirmed and extended these crucial experiments of Lamb and came to the conclusion that viper venom contained a fibrin ferment.

From the earlier experiments of Martin on the production of intravascular coagulation and fluid blood—the so-called positive and negative phases of coagulation—after the intravascular injection of viper venom,

it was clear that viper venom possessed properties analogous to those ascribed to kinase on the present theory of the coagulation of blood. But from his later experiments on the coagulation of oxalate plasma by viper venom it appeared equally evident that this venom contained fibrin ferment. The interesting question then arose as to whether viper venom contained fibrin ferment or kinase, or a special substance which possessed the properties of both ferment and kinase. To solve the problem various experiments were made on birds' plasma, fibrinogen, and prothrombin.

The following abbreviations are used in the descriptions of the experiments:

The venom of *Notechis scutatus* (Australian viper)	= N. V.
The venom of *Echis carinata* (Indian viper)	= E. V.
The venom of the Cobra	= C. V.
Plasma	= Pl.
Fibrinogen in 1 % NaCl	= Fg—S.
Prothrombin	= Pro.
Fibrin ferment	= Ff.
Kinase	= K.
Serum	= S.

The coagulation of plasma.

Stable birds' plasma was prepared in the way previously described. To 1 c.c. of this plasma venom and water were added to bring the volume up to 2 c.c. and the time of coagulation was noted. All the experiments were done at 30° C.

The venom of Notechis scutatus. 3 mgrms. of *Notechis scutatus* venom were dissolved in 10 c.c. of distilled water. This solution was then diluted a thousand fold. 1 c.c. of this final solution (= N′ V′) contained ·0,000,003 grams of venom. A solution ten times as strong is denoted by the letters N.V.

Pl.	N′V′	H_2O	Coag. time
1 c.c.	·1 c.c.	·9 c.c.	4 minutes.
	·2	·8	3 ,,
	·4	·6	2 ,,
	·5	·5	1½ ,,
	1·0	·0	1¼ ,,
	·2 (N. V.)	·8	1 ,,

There are two points to be noted in these results. The first is the extraordinary activity of the venom of the Australian viper as a coagulant for birds' plasma. The amount of venom which coagulated 2 c.c. of diluted plasma in four minutes was ·00,000,003 grams. The second is the fact that a twenty fold increase in the quantity of venom added diminished the coagulation time from four minutes to one minute only. A corresponding increase in the quantity of fibrin ferment added to plasma would change the coagulation time from several minutes to a few seconds. But the change in the coagulation time is comparable to the results observed on adding varying amounts of kinase to plasma. The result therefore indicated that the venom of the Australian viper contained a kinase rather than a fibrin ferment. The venom of the Indian viper gave results similar to the above, the only difference being that the dried venom of this snake possessed about one twentieth of the activity of the Australian viper. E.V. denotes a solution of the venom of *Echis carinata* of the same strength as N.V., *i.e.* 3 mgrms. of dried venom in 10,000 c.c. of water.

The venom of Echis carinata.

Pl.	E.V.	H_2O	Coag. time
1 c.c.	·2 c.c.	·8 c.c.	8 minutes.
	·4	·6	6 ,,
	·6	·4	4 ,,
	·8	·2	3½ ,,
	·1 (E.V.+10)	·9	2 ,,
	·2 ,,	·8	1¼ ,,

The results are qualitatively comparable to those observed with the venom of the Australian viper.

The coagulation of fibrinogen.

But the coagulation of plasma by venom gave only general indications as to the nature of the substances contained in these venoms which effected the coagulation of blood. In order to analyse more accurately the properties of these coagulating substances various experiments on fibrinogen dissolved in sodium chloride were done. In all these experiments the fibrinogen was dissolved in 1% NaCl. To 1 c.c. of this solution varying quantities of venom solution and water were added to bring the final volume to 2 c.c. By this means the concentration of sodium chloride was reduced to ·5%.

Australian viper venom.

(Fg—S)	N'V'	H_2O	Coag. time
1 c.c.	·1 c.c.	·9 c.c.	50 minutes.
	·2	·8	30 ,,
	·4	·6	20 ,,
	·6	·4	18 ,,
	·1 (N. V.)	·9	11 ,,
	·2	·8	8 ,,
	·4	·6	6 ,,
	·6	·4	3½ ,,
	·8	·2	3 ,,
	1·0	·0	3 ,,

Indian viper venom.

(Fg—S)	E. V.	H_2O	Coag. time
1 c.c.	·2 c.c.	·8 c.c.	15 minutes.
	·4	·6	12 ,,
	1·0	·0	6 ,,
	·2 (E. V. + 10)	·8	4 ,,
	1·0	·0	2 ,,

From these results also it is evident how much weaker Indian viper venom is as a coagulant for fibrinogen solutions than Australian viper venom, although the effects produced by the two venoms are qualitatively parallel. But the results of these experiments appeared to prove conclusively that viper venom contained a fibrin ferment and that this was the nature of the active principle which caused the coagulation of blood. For it has been previously pointed out that fibrinogen solutions may be coagulated by fibrin ferment or by kinase working in conjunction with a calcium salt. Now a minimal quantity of calcium salt which can work in conjunction with tissue kinase as a coagulant for a fibrinogen solution is about one part in ten thousand. In the case of the venom of the Australian viper the total venom present when coagulation of the fibrinogen occurred in eight minutes was about one part in a million. Therefore if all the dried venom were assumed to consist of calcium salts the total quantity present would be too small to effect the coagulation of the fibrinogen even though this solution contained a maximal quantity of tissue kinase. The obvious conclusion to be drawn from these experiments therefore was that the venoms contained a fibrin ferment. But against this conclusion was the fact that even when the amount of venom added to the fibrinogen solution was increased a hundred fold coagulation took at least a minute to complete itself—

indicating that some preliminary procedure had to be undergone before coagulation could take place. And if the times of coagulation on varying the amount of venom were compared with similar experiments with fibrinogen and tissue kinase, the parallelism of the results indicated that the venom contained a kinase.

To investigate the problem more fully the fluids obtained after removal of the fibrin from solutions of fibrinogen which had been coagulated by venom were tested for fibrin ferment. If the fibrinogen coagulant contained in the venom consisted of fibrin ferment then the fluid expressed after coagulation should have contained prothrombin only: but if the initial coagulant possessed the properties of a mixture of kinase and calcium chloride then an active ferment solution should have been obtained. The following experiments give the results of these investigations.

A solution of fibrinogen was coagulated by the venom of the Australian viper.

(Fg—S)	N. V.	H_2O	Coag. time
1 c.c.	·2 c.c.	·8 c.c.	9 minutes.

After fifteen minutes the fibrin was removed and the activity of the remaining fluid determined after varying times.

(Fg—S)	Expressed fluid	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	13 minutes	15 minutes.
			6 ,,	30 ,,
			4 ,,	45 ,,
			3 ,,	60 ,,
			2½ ,,	75 ,,

A similar experiment was done with the venom of the Indian viper.

(Fg—S)	E. V.	H_2O	Coag. time
1 c.c.	·4 c.c.	·6 c.c.	15 minutes.

After thirty minutes the fluid was expressed and its activity determined.

(Fg—S)	Expressed fluid	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	65 minutes	30 minutes.
			40 ,,	60 ,,
			10 ,,	120 ,,
			8 ,,	180 ,,

The experiments conclusively show that these viper venoms contain substances which can generate fibrin ferment from prothrombin, *i.e.* that they contain substances physiologically equivalent to a mixture of tissue kinase and calcium salts. It may be noted that the rate of generation of ferment activity in the expressed fluids in the two cases is approximately of the same order as the times of coagulation of the original fibrinogen fluids. This result indicates that the coagulating activities

of the venoms depend upon the same substances which generate fibrin ferment from the prothrombin—that they contain only substances equivalent to kinase and calcium and do not contain fibrin ferment. The property of generating fibrin ferment from a prothrombin solution is also shown in the following experiments.

The generation of fibrin ferment from prothrombin by the venom of the Australian viper.

A solution of fibrinogen was coagulated by fibrin ferment. The expressed fluid contained no fibrin ferment but a quantity of prothrombin—a fact which was shown by its inability to coagulate fibrinogen solutions before it was acted upon by kinase and calcium salts.

Action of N.V. on prothrombin.

Pro.	N.V.	H_2O	
1 c.c.	·2 c.c.	·8 c.c.	= Pro. N.

The activity of this solution was tested after various times on fibrinogen solutions.

(Fg—S)	Pro. N.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	30 minutes	10 minutes.
			20 ,,	30 ,,
			15 ,,	50 ,,
			12 ,,	70 ,,
			10 ,,	90 ,,

The results show a slow but progressive increase in the ferment activity of the original prothrombin solution. A similar experiment was made with the venom on the Indian viper.

Pro.	E. V.	H_2O	
1 c.c.	·4 c.c.	·6 c.c.	= Pro. E.

After various times the activity of this fluid was tested.

(Fg—S)	Pro. E.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	70 minutes	60 minutes.
			34 ,,	90 ,,
			17 ,,	120 ,,
			9 ,,	150 ,,

In the case of this venom also a progressive increase of ferment activity may be noted in the prothrombin solution to which it had been added.

The results of all the above experiments indicate that the venoms of the Australian viper and Indian viper contain substances which are

physiologically equivalent to a mixture of tissue kinase and calcium salts. Such substances possess the properties of fibrin ferment insomuch that they can coagulate fibrinogen solutions alone. But such an effect does not depend upon the immediate action of the venom on the fibrinogen but is a consequence of the generation of fibrin ferment from the prothrombin associated with the fibrinogen. The proof that viper venom does not immediately act on fibrinogen cannot be adduced since fibrinogen free from associated prothrombin has not been prepared. But the observations that these venoms do generate fibrin ferment from prothrombin and that the times of coagulation of fibrinogen solutions are approximately of the same order as the times required to generate fibrin ferment from prothrombin afford strong evidence that the coagulation of fibrinogen by viper venom is preceded by the production of fibrin ferment from the associated prothrombin, and that the observed coagulation is due to the secondary action of this fibrin ferment on the fibrinogen. No experiment has yet been brought forward to indicate that the venom of the Australian viper differs from that of the Indian viper except that the former possesses about ten times the activity of the latter, weight for weight.

The deduction that these viper venoms contain substances which are physiologically equivalent to a mixture of kinase and calcium raised several interesting problems. In the first place the question naturally arose whether the active coagulating principle in the venoms contained calcium within its molecule and if so whether the proportion of calcium to kinase was such that the combination always possessed a maximum efficiency as a generator of fibrin ferment from prothrombin. One of the most interesting observations in the production of fibrin ferment from prothrombin by tissue kinase and calcium chloride was the reciprocal relation which existed between the kinase and calcium. If a minimal amount of kinase were present then at least one part of calcium chloride in two thousand of solution was required to quickly generate ferment activity; but if a large quantity of kinase were present then the calcium chloride could be reduced to one part in ten or twenty thousand; and by varying either the kinase or calcium coagulation in a definite time could be produced. In the second place the problem arose whether, if viper venom contained calcium in combination within its active coagulating molecule this calcium could be affected in any way by potassium oxalate—a precipitant of calcium salts which prevents the action of kinase in oxalated blood.

The influence of calcium chloride on the action of the venoms. The

reciprocal relation which may be observed between kinase and calcium chloride when these substances coagulate fibrinogen solutions and to which allusion has been made above is illustrated in the following experiments.

The coagulation of fibrinogen solutions by kinase and calcium chloride, the calcium being varied in amount.

(Fg—S)	$CaCl_2$	K	H_2O	Coag. time
1 c.c.	·2 $\left(\frac{N}{10}\right)$	·2 c.c.	·6 c.c.	2 minutes.
	·1 ,,		·7	4 ,,
	·5 $\left(\frac{N}{100}\right)$		·3	5 ,,
	·4 ,,		·4	8 ,,
	·3 ,,		·5	11 ,,
	·2 ,,		·6	75 ,,
	·0 ,,		·8	No coag. in 3 hrs.

The very large increase in the coagulation time in the above experiment occurred when the calcium chloride was diminished from $\frac{3N}{2000}$ to $\frac{N}{1000}$, *i.e.* from 1 part in 8800 to 1 part in 13,000. By making K smaller the increase in coagulation time would have taken place with a higher proportion of calcium chloride. The following experiment shows how the time of coagulation of a fibrinogen solution is affected by varying the amount of kinase.

The coagulation of fibrinogen by kinase and calcium chloride, the kinase being varied in amount.

(Fg—S)	$CaCl_2\left(\frac{N}{100}\right)$	K	H_2O	Coag. time
1 c.c.	·2	·2 c.c.	·6 c.c.	75 minutes.
		·4	·4	27 ,,
		·6	·2	8½ ,,
		·8	·0	4 ,,
		·1 (K 10)	·7	3 ,,
		·2 ,,	·6	1½ ,,
		·4 ,,	·4	1 ,,
		·8 ,,	·0	½ ,,

K 10 denotes a kinase solution ten times as strong as K.

In all the above experiments $\frac{N}{1000}$ $CaCl_2$ was present, *i.e.* one part in thirteen thousand of solution, approximately. Increasing the kinase

forty fold, *i.e.*, from ·2 K to ·8 K 10, diminished the coagulation time from seventy-five minutes to half a minute.

The relation of kinase and calcium salts to one another in the generation of fibrin ferment from prothrombin has been previously discussed. The question now under consideration is whether, if the active molecule of viper venom be postulated to contain kinase and calcium salts in combination with one another, the relation of these constituents is such that in addition to rendering the combined calcium much more active than the uncombined calcium, the combination possesses a maximum efficiency, *i.e.* if the coagulating activity of viper venom is not increased by adding more calcium or more kinase to it.

The following experiments show the influence of calcium chloride on the coagulation of fibrinogen by the venom of the Australian viper.

(Fg—S)	N. V.	H_2O	$CaCl_2\left(\frac{N}{10}\right)$	Coag. time
1 c.c.	·1 c.c.	·9 c.c.	·0 c.c.	11 minutes.
	·1	·7	·2	6½ ,,
1 c.c.	·2	·8	·0	8 minutes.
	·2	·6	·2	4 ,,
1 c.c.	·4	·6	·0	6 minutes.
	·4	·4	·2	4 ,,

In this experiment the adjuvant influence of $CaCl_2\left(\frac{N}{100}\right)$ on the coagulation of fibrinogen by this venom is well shown—the addition of this quantity of calcium chloride approximately doubled the efficiency of a given quantity of snake venom as a coagulant for a solution of fibrinogen.

A similar experiment showed a similar action of calcium chloride on the venom of the Indian viper.

(Fg—S)	E. V.	H_2O	$CaCl_2\left(\frac{N}{10}\right)$	Coag. time
1 c.c.	·2 c.c.	·8 c.c.	·0 c.c.	15 minutes.
	·2	·6	·2	4½ ,,
	·4	·6	·0	12 minutes.
	·4	·4	·2	3½ ,,
	·1 (E. V.) 10	·9	·0	6 minutes.
	·1 ,,	·7	·2	1½ ,,
	·2 ,,	·8	·0	4 minutes.
	·2 ,,	·6	·2	1½ ,,

1 c.c. (E. V.) 10=10 c.c. of E. V.

From the above it may be seen that $CaCl_2\left(\frac{N}{100}\right)$ has a more marked adjuvant action on the venom of the Indian viper than on that of the Australian viper.

In these experiments a constant quantity of calcium chloride was added in each case, the amount of venom alone being variable. But in the following experiment the venom was kept constant and the amount of calcium added was varied.

(Fg—S)	E. V.	$CaCl_2\left(\frac{N}{100}\right)$	H_2O	Coag. time
1 c.c.	·4 c.c.	·0 c.c.	·6 c.c.	14 minutes.
		·2	·4	5 ,,
		·4	·2	5 ,,
		·6	·0	4½ ,,

This experiment showed the very small quantity of calcium chloride required to markedly diminish the coagulation time of a fibrinogen solution by the venom of the Indian viper. The addition of $CaCl_2\left(\frac{N}{1000}\right)$ diminished the coagulation time from fifteen to five minutes.

From the above results it appeared that the efficiency of the active coagulating agent contained in viper venom was increased by the concomitant presence of calcium chloride. The result might have been due to the added calcium chloride assisting the action of a fibrin ferment contained in the venom although from the absence of any influence of small quantities of calcium chloride upon fibrin ferment obtained from blood such a mode of action seemed improbable. To prove that the influence of the calcium chloride was exerted on a kinase-like substance contained in the venom appropriate experiments were made. Venom and calcium chloride were added to solutions of prothrombin and the increase in ferment activity of the resultant solution was determined. In these experiments the results were compared with similar experiments to which no calcium chloride had been added. The action of the venom of the Australian viper on prothrombin only was first determined.

The following mixture of prothrombin and venom was made up.

Pro.	N. V.	H_2O	
1 c.c.	·2 c.c.	·8 c.c.	= Pro. N.

After various times the ferment activity of this solution was determined by adding ·1 c.c. of it to 1·9 c.c. of fibrinogen solution. The following figures show the rate of

development of ferment activity. The original prothrombin solution contained no appreciable quantity of fibrin ferment.

(Fg—S)	Pro. N.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	30 minutes	10 minutes.
			20 ,,	30 ,,
			15 ,,	50 ,,
			12 ,,	70 ,,
			10 ,,	90 ,,

The development of ferment activity in the prothrombin solution was slow but progressive. A similar prothrombin venom solution containing $CaCl_2\left(\frac{N}{100}\right)$ was made. The rate of development of ferment activity in this solution was so rapid that in ten minutes ·1 c.c. of the solution coagulated 2 c.c. of fibrinogen solution in two minutes. The addition of $CaCl_2\left(\frac{N}{100}\right)$ had increased to a very considerable degree the activity of the venom as a generator of fibrin ferment from prothrombin.

The following experiment shows the rate of development of fibrin ferment in the same prothrombin solution containing $CaCl_2\left(\frac{N}{100}\right)$ but only one half the quantity of venom of the previous two solutions.

Pro.	N.V.	$CaCl_2\left(\frac{N}{100}\right)$	H_2O		
1 c.c.	·1 c.c.	·2 c.c.	·7 c.c.	=	Pro. N. Ca.

The activity of this solution after various times as a coagulant for fibrinogen is shown in the following figures.

(Fg—S)	Pro. N. Ca	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	9 minutes	5 minutes.
			5 ,,	8 ,,
			3 ,,	11 ,,
			2½ ,,	15 ,,
			2 ,,	20 ,,

From the above experiments it is clear that calcium chloride markedly increased the efficiency of the venom of the Australian viper as a coagulant for fibrinogen solutions, and that this effect of the added calcium chloride was due to its adjuvant action on the kinase in generating fibrin ferment from the prothrombin associated with the fibrinogen rather than in assisting the action of a body similar to fibrin ferment contained in the venom.

The same results were obtained with equal clearness when the influence of calcium chloride on the generation of fibrin ferment from prothrombin by the venom of the Indian viper was considered.

The venom of the Indian viper was added to a prothrombin solution in the following proportions:

Pro.	E. V.	H_2O		
1 c.c.	·4 c.c.	·8 c.c.	=	Pro. E.

After various times the ferment activity of this solution was tested on a fibrinogen solution.

(Fg—S)	Pro. E.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	70 minutes	60 minutes.
			34 ,,	90 ,,
			17 ,,	120 ,,
			9 ,,	150 ,,

From the above figures it is clear that the rate of development of ferment activity in the prothrombin solution under the influence of the venom was small. The following experiment shows how this rate was increased under the influence of $CaCl_2\left(\frac{N}{100}\right)$.

Pro.	E. V.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
1 c.c.	·4 c.c.	·2 c.c.	·4 c.c.	=	Pro. E. Ca.

The rate of development of ferment activity in this solution is shown in the following figures:

(Fg—S)	Pro. E. Ca	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	11 minutes	10 minutes.
			1½ ,,	20 ,,

The addition of $CaCl_2\left(\frac{N}{100}\right)$ to the prothrombin venom solution diminished the time in which sufficient ferment was generated for ·1 c.c. of the mixture to coagulate 2 c.c. of fibrinogen in ten minutes from 2½ hours to ten minutes.

These experiments clearly show that although viper venoms alone can generate fibrin ferment from prothrombin yet their actions are greatly assisted by the concomitant presence of calcium salts. The facts are capable of two explanations (*a*) that only a portion of kinase in viper venom is combined with calcium and that the remainder requires extraneous calcium to be added before it can produce fibrin ferment from prothrombin, or (*b*) that the calcium combined in the kinase molecule is too small in amount to permit the kinase exercising its full activity on prothrombin solutions when the venom is present in minimal quantities, and that the addition of calcium salts to these solutions permits this more powerful action. Of these two hypotheses probably the latter affords a more accurate interpretation of the phenomena observed.

The influence of potassium oxalate on the coagulation of fibrinogen solutions by viper venom. The analysis of the effects produced by calcium salts on the coagulation of fibrinogen solutions by viper venoms suggested an inquiry into the influence of potassium oxalate on these processes. If the active coagulating agent in such a venom consists of a body containing kinase and a calcium salt in close union with one another it appeared probable that the activity of such a body would be influenced by the presence of potassium oxalate. The effects of potassium oxalate on the coagulation of plasma and fibrinogen and on the generation of fibrin ferment from prothrombin by snake venom were therefore determined.

The following experiment shows the effect of varying quantities of potassium oxalate on the coagulation of plasma by the venom of the Australian viper.

Pl.	$K_2\bar{O}$ $(\frac{N}{10})$	$N_{,,}V_{,,}$	H_2O	Coag. time
1 c.c.	·0 c.c.	·2 c.c.	·8 c.c.	4 minutes.
	·2		·6	8 ,,
	·4		·4	11 ,,
	·6		·2	14 ,,
	·8		·0	28 ,,

(1 c.c. $N_{,,}V_{,,}$ = ·1 c.c. of N.V.)

From the above it is evident that potassium oxalate diminishes the coagulating activity of the venom of the Australian viper on plasma but does not stop it. The result is in accordance with the previous observations—that this venom can generate fibrin ferment from prothrombin only but that this action is assisted by the presence of calcium salts. The added oxalate precipitates the adjuvant calcium salts but does not stop the venom working. It may be stated that the effects noted are more marked than would be exerted by the same quantities of potassium oxalate on the coagulation of plasma by fibrin ferment.

Similar results were obtained when oxalated plasma was coagulated with the venom of the Indian viper.

Pl.	$K_2\bar{O}$ $(\frac{N}{10})$	E.V.	H_2O	Coag. time
1 c.c.	·0 c.c.	·4 c.c.	·6 c.c.	2 minutes.
	·1		·5	$2\frac{1}{2}$,,
	·2		·4	3 ,,
	·3		·3	$4\frac{1}{2}$,,
	·4		·2	5 ,,
	·5		·1	30 ,,
	·6		·0	35 ,,
	·2 (N)		·4	80 ,,
	·4 ,,		·2	3 hours.

In the above figures the noteworthy point is the large increase in the coagulation time when the potassium oxalate was increased from $\frac{N}{50}$ to $\frac{N}{40}\left(K_2\bar{O}\left(\frac{N}{40}\right)\right.$ corresponds approximately to ·2 % $\left.K_2\bar{O}\right)$. With this quantity of potassium oxalate all the calcium salts in the plasma were precipitated and there remained an excess of oxalate in the plasma.

The more marked influence of oxalate on the coagulation of plasma by Indian viper venom compared with that noted on the coagulation effected by Australian venom suggested that in the former case the oxalate in addition to precipitating the adjuvant calcium salts inhibited the activity of the venom also.

But an examination of the influence of potassium oxalate on the coagulation of plasma by viper venom afforded only an indication of the ultimate action of this salt on the active principle in the venom. To determine this action more accurately the effect of potassium oxalate on the coagulation of fibrinogen solutions by venoms was determined.

The following comparative experiments show the influence of potassium oxalate on the coagulation of fibrinogen by the venom of the Australian viper.

(Fg—S)	N.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$	H_2O	Coag. time
1 c.c.	·5 c.c.	·0 c.c.	·5 c.c.	6 minutes.
		·1	·4	2½ ,,
		·2	·3	2 ,,
		·3	·2	1½ ,,
		·4	·1	1¼ ,,
		·5	·0	1¼ ,,

The figures show the somewhat unexpected result that potassium oxalate assisted the coagulation of fibrinogen by this venom. This effect was shown more markedly in the following comparative experiments. The amounts of venom and fibrinogen were the same in the two experiments the only difference being that in the one case no potassium oxalate was present and in the other case $K_2\bar{O}\left(\frac{N}{50}\right)$ had been added.

Amount of venom solution present in 2 c.c. of fibrinogen solution	Coagulation times	
	No $K_2\bar{O}$ present	$K_2\bar{O}\left(\frac{N}{10}\right)$ present
·1 c.c.	13 minutes	2½ minutes
·2	11 ,,	2¼ ,,
·3	8 ,,	2 ,,
·4	7 ,,	1¾ ,,
·5	6 ,,	1½ ,,

Similar experiments were made on the venom of the Indian viper.

(Fg—S)	E.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$	H_2O	Coag. time
1 c.c.	·1 c.c.	·0 c.c.	·9 c.c.	18 minutes.
	·3	·0	·7	11 ,,
	·5	·0	·5	8 ,,
1 c.c.	·1 c.c.	·4 c.c.	·5 c.c.	12 minutes.
	·3	·4	·3	10 ,,
	·5	·4	·1	7 ,,

In the case of this venom it may be seen that the adjuvant effects of the oxalate were not so marked as in the case of the Australian venom.

The interesting problem now arose whether this paradoxical influence of the potassium oxalate was exerted on the fibrinogen, prothrombin, or the venom itself. The influence of potassium oxalate on the generation of fibrin ferment from prothrombin by these venoms was therefore determined. The following experiment shows the effect of potassium oxalate on the production of fibrin ferment from prothrombin by the Australian venom.

The activity of the venom alone in generating fibrin ferment was first determined. The following mixture of venom and prothrombin was made.

Pro.	N.V.	H_2O		
1 c.c.	·5 c.c.	·5 c.c.	=	Pro. N.

After various times the activity of this fluid was tested on a fibrinogen solution.

(Fg—S)	Pro. N.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	15 minutes	10 minutes.
			12 ,,	20 ,,
			10 ,,	30 ,,
			9 ,,	45 ,,
			7 ,,	60 ,,
			5 ,,	120 ,,

The same quantity of venom was now added to the same prothrombin solution but in this case $K_2\bar{O}\left(\frac{N}{40}\right)$ was also present.

Pro.	N.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$
1 c.c.	·5 c.c.	·5 c.c.

After ten minutes ·1 c.c. of this solution coagulated 2 c.c. of fibrinogen in two minutes. The rate of generation of fibrin ferment from the prothrombin by the venom had been enormously increased by the potassium oxalate.

The same experiment was repeated with the difference that only ·2 c.c. instead of ·5 c.c. of the venom was added to the prothrombin solution.

Pro.	N.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$	H_2O		
1 c.c.	·2 c.c.	·5 c.c.	·3 c.c.	=	Pro. N'.

The activity of this fluid after various times is shown below.

(Fg—S)	Pro. N'.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	3½ minutes	10 minutes.
			2 ,,	15 ,,
			1½ ,,	30 ,,

The influence of the potassium oxalate was such that with the smaller amount of venom the rate of formation of fibrin ferment from the prothrombin was greater than with the larger quantity of venom only.

Similar experiments were made with the venom of the Indian viper but in this case the added oxalate exercised a slightly depressing influence on the activity of the venom.

The following solution of prothrombin and Indian venom was made up.

Pro.	E.V.	H_2O		
1 c.c.	·5 c.c.	·5 c.c.	=	Pro. E.

The activity of this solution after various times is given below.

(Fg—S)	Pro. E.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	23 minutes	15 minutes.
			12 ,,	30 ,,
			9 ,,	45 ,,
			7 ,,	60 ,,
			5½ ,,	90 ,,
			4½ ,,	120 ,,

The same solution of venom and prothrombin was made up but potassium oxalate to the extent of $\frac{N}{40}$ was also added.

Pro.	E.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$		
1 c.c.	·5 c.c.	·5 c.c.	=	Pro. E.

The following figures show the activity of this venom after various times.

(Fg—S)	Pro. E.	H_2O	Coag. time	Tested after
1 c.c.	·1 c.c.	·9 c.c.	28 minutes	15 minutes
			25 ,,	30 ,,
			17 ,,	60 ,,
			13 ,,	120 ,,

If these two experiments be compared it will be seen that potassium oxalate slightly depressed the activity of the Indian venom when generating fibrin ferment from prothrombin. The relation of this result to that previously given showing the slightly adjuvant effect of potassium oxalate on the coagulation of fibrinogen by the venom of the Indian viper will be discussed later.

The accelerating influence of potassium oxalate on the generation

of fibrin ferment from prothrombin by the venom of the Australian viper did not permit a decision to be reached as to the ultimate site of action of the oxalate. From these results it was probable that the oxalate did not exert any marked influence on the fibrinogen. But the problem still remained whether the oxalate affected the venom only or the prothrombin only or both. To elucidate this point the potassium oxalate was first added to the venom and the venom was afterwards added to fibrinogen and prothrombin solutions to determine if any change had taken place in it. The following experiment shows the influence of adding potassium oxalate to the venom of the Australian viper thirty minutes before its activity was tested on a fibrinogen solution.

A mixture of venom and potassium oxalate was made and this was allowed to remain at 30° C. for 30 minutes before being tested.

$$5 \text{ c.c. } K_2\bar{O}\left(\frac{N}{10}\right) + 5 \text{ c.c. N.V.} = (\text{N.V. } K_2\bar{O}).$$

The activity of this solution was then compared with that of similar quantities of venom and oxalate which were added direct to the fibrinogen solution.

	(Fg—S)	N.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$	H_2O	(N.V. $K_2\bar{O}$)	Coag. time
A	1 c.c.	·5 c.c.	·0 c.c.	·5 c.c.	·0 c.c.	13 minutes.
	1	·5	·5	·0	·0	4 ,,
	1	·0	·0	·0	·1	2 ,,
B	1	·4	·0	·6	·0	40 ,,
	1	·4	·4	·2	·0	5½ ,,
	1	·0	·0	·2	·8	2 ,,
C	1	·3	·3	·4	·0	7 ,,
	1	·0	·0	·4	·6	2½ ,,
D	1	·2	·2	·6	·0	10 ,,
	1	·0	·0	·6	·4	3 ,,
E	1	·1	·1	·8	·0	20 ,,
	1	·0	·0	·8	·2	5 ,,

In A and B above the influence of adding oxalate to the fibrinogen mixture is well shown on comparing the times of coagulation of A 1 and B 1 with A 2, 3, and B 2, 3. All the results of A, B, C, D, E, especially of C, D, E, show the greatly enhanced effect produced by first adding the oxalate to the venom solution and allowing this mixture to incubate for thirty minutes. From the results it appeared that the adjuvant action of the oxalate was due primarily to the effect it produced on the venom itself. This inference was borne out by the following experiments.

Solutions containing a constant quantity of venom and variable quantities of potassium oxalate were made up.

N.V.	$K_2\bar{O}\left(\frac{N}{10}\right)$	H_2O			
4·5 c.c.	·1 c.c.	·4	= A	(contains	$\frac{2N}{100}$ $K_2\bar{O}$)
	·2	·3	= B	(,,	$\frac{4N}{100}$,,)
	·3	·2	= C	(,,	$\frac{6N}{100}$,,)
	·4	·1	= D	(,,	$\frac{8N}{100}$,,)
	·5	·0	= E	(,,	$\frac{10N}{100}$,,)

These mixtures were allowed to incubate for an hour and their activities on a fibrinogen solution were determined.

(Fg—S)	H_2O		Coag. time
1 c.c.	·8 c.c.	·2 A	4 minutes.
		·2 B	3 ,,
		·2 C	$2\frac{1}{2}$,,
		·2 D	$2\frac{1}{2}$,,
		·2 E	$2\frac{1}{2}$,,

From these results it appears that $\frac{5N}{1000}$ $K_2\bar{O}$ produced an optimum increase in the activity of the venom of the Australian viper.

In the following experiment the above results are compared with those produced by adding potassium oxalate to a venom fibrinogen solution direct.

(Fg—S)	$K_2\bar{O}\left(\frac{N}{100}\right)$	H_2O	N.V.	Coag. time	$K_2\bar{O}$ present
1 c.c.	·0 c.c.	·8 c.c.	·2 c.c.	15 minutes	0
	·2	·6	·2	10 ,,	$\frac{N}{1000}$
	·4	·4	·2	8 ,,	$\frac{2N}{1000}$
	·6	·2	·2	6 ,,	$\frac{4N}{1000}$
	·8	·0	·2	4 ,,	$\frac{6N}{1000}$

By comparing the results of these two experiments it will be seen that if the oxalate were first added to the venom the increase in the coagulating efficiency took place with much smaller quantities of oxalate than if the oxalate and venom were added simultaneously to the fibrinogen.

The same results were obtained when the activity of the oxalated venom of the Australian viper on a solution of prothrombin was compared with that of a similar solution of venom which had not been treated with oxalate.

The following solution of venom and potassium oxalate was made up and the mixture was kept at 30° C.

9 c.c. N.V. + ·1 c.c. $K_2\bar{O}$ + ·9 c.c. H_2O = N.V. $K_2\bar{O}$.

After an hour this oxalated venom was added to a prothrombin solution in the following proportions.

4·5 c.c. Pro. + ·1 c.c. N.V. $K_2\bar{O}$ + ·4 c.c. H_2O = (X).

A similar solution of prothrombin and venom only was made = (Y). After two hours the activities of (X) and (Y) were such that ·2 c.c. of (X) coagulated 2 c.c. of fibrinogen solution in nine minutes whilst the same quantity of (Y) coagulated the same quantity of fibrinogen in 30 minutes.

All the experiments therefore definitely showed that the adjuvant action of potassium oxalate was exerted on the venom and not on the fibrinogen or prothrombin.

In the case of the venom of the Indian viper it has been seen that the simultaneous addition of venom and oxalate to a fibrinogen solution showed a slight increase in the coagulating power of the venom, whilst the addition of similar quantities of venom and oxalate to a prothrombin solution showed a retarding action of the oxalate on the generation of fibrin ferment. But when potassium oxalate was first added to the solution of venom and the mixture was allowed to incubate for an hour at 30° C. the activity of the venom was always increased both as a coagulant for a fibrinogen solution and as a generator of fibrin ferment from prothrombin. These results are shown in the following experiment.

Potassium oxalate was added to a solution of venom of the Indian viper in the following proportions:

9 c.c. E.V. + ·1 c.c. $K_2\bar{O}N$ + ·9 c.c. H_2O = E.V. $K_2\bar{O}$.

This solution of venom contained therefore $\frac{N}{100}$ potassium oxalate. After the mixture had remained at 30° C. for one hour its activity on fibrinogen and prothrombin was determined and compared with that of a solution of venom of the same strength to which no oxalate had been added. ·2 c.c. of the oxalated venom solution coagulated 2 c.c. of fibrinogen solution in 14 minutes. ·2 c.c. of the pure venom solution coagulated the same quantity of fibrinogen solution in 19 minutes.

A similar adjuvant influence of the potassium oxalate on the venom was seen on testing its action on a prothrombin solution.

The following mixtures of prothrombin with oxalated venom (E.V. K_2O) and pure venom (E.V.) were made.

4·5 c.c. Pro. + ·1 c.c. E.V. $K_2\bar{O}$ + ·4 c.c. H_2O = (X)′,
4·5 c.c. Pro. + ·1 c.c. E.V. + ·4 c.c. H_2O = (Y)′.

The activities of these solutions (X)′ and (Y)′ after various times were as follows.

(a)	(X)′	H_2O	Coag. time	Tested after
1 c.c.	·2 c.c.	·8 c.c.	4 minutes	2½ hours.
			2 ,,	4 ,,
	(Y)′			
1	·2	·8	10 ,,	2½ ,,
			5 ,,	4 ,,

These experiments on prothrombin solutions also showed a distinct increase in the activity of the Indian viper venom when previously submitted to the influence of potassium oxalate. Therefore so far as the action of potassium oxalate on the venom itself was concerned the effect of this salt on the venom of the Indian viper was the same as that exerted by it on the venom of the Australian viper. The contradictory results obtained when oxalate and Indian venom were added simultaneously to fibrinogen and prothrombin solutions will be discussed when all the experiments dealing with these two venoms are considered.

The influence of potassium chloride, potassium sulphate and calcium chloride on the coagulating activity of viper venom. In the previous pages the adjuvant influence of calcium chloride on the coagulation of fibrinogen by venom, when the venom and calcium chloride were added simultaneously to a fibrinogen solution, has been described and discussed. In the following experiments the above salts were added to the venom solution and the salt venom mixtures were allowed to remain for some time at 30° C. before their activities were tested. It has already been seen that venom treated in such a way with potassium oxalate showed the unexpected effect of increased activity. A similar paradoxical result was obtained when the venom of the Australian viper was submitted to the action of calcium chloride.

The following mixtures of the venom of the Australian viper and the above salts were made.

9 c.c. N.V. + ·1 c.c. K_2O (N) + ·9 c.c. H_2O = (A),
9 c.c. N.V. + ·1 c.c. KCl (N) + ·9 c.c. H_2O = (B),
9 c.c. N.V. + ·2 c.c. $K_2SO_4\left(\frac{N}{2}\right)$ + ·8 c.c. H_2O = (C),
9 c.c. N.V. + ·5 c.c. $CaCl_2\left(\frac{N}{5}\right)$ + ·5 c.c. H_2O = (D),
9 c.c. N.V. + 1 c.c. H_2O = (E).

All the above venom salt solutions contained salt to the extent of $\frac{N}{100}$. They were allowed to remain at 30° C. for various times before being tested. The activities of these mixtures tested on fibrinogen solution gave the following results.

(Fg—S)	H_2O		Coag. time tested after 1 hour	Coag. time tested after 3 hours
1 c.c.	·8 c.c.	·2 c.c. E	14 minutes	20 minutes.
		·2 A	5 ,,	5½ ,,
		·2 B	15 ,,	25 ,,
		·2 C	15 ,,	20 ,,
		·2 D	16 ,,	45 ,,

From the coagulation times of the fibrinogen solution after the venom salt mixtures had been allowed to incubate for one hour at 30° C. it would appear that potassium chloride, potassium sulphate and calcium chloride were without action on the venom, whilst potassium oxalate displayed its usual accelerating action. But after the venom salt mixtures had remained at 30° C. for three hours the effects of the salts were more marked. During this time the activity of the venom solution itself had deteriorated as evidenced by the coagulation time of the fibrinogen being extended from fourteen minutes to twenty minutes. Potassium sulphate had apparently exercised no deleterious action; whilst potassium chloride had influenced to some extent the coagulating activity of the venom. But the most marked effect may be noted in the case of calcium chloride. The influence of this salt on the venom was such that after three hours the coagulation time of the fibrinogen solution was increased from sixteen to forty-five minutes. Calcium chloride added to a fibrinogen solution assists the coagulating action of the venom; but calcium chloride added to a venom solution actively destroys the coagulating principle.

Experiments similar to the above were made with the venom of the Indian viper. Similar results were obtained with the salts, potassium oxalate, potassium chloride and potassium sulphate. But calcium chloride, contrary to its action on the venom of the Australian viper, showed a marked accelerating action on this venom.

The figures show the time of coagulation of 2 c.c. of fibrinogen produced by ·2 c.c. of the following solutions:

(1) venom alone, (2) venom + $K_2\bar{O}\left(\frac{N}{100}\right)$, (3) venom + $CaCl_2\left(\frac{N}{100}\right)$.

These solutions were kept at 30° C. for an hour before being tested.

Venom alone ... ... Coag. time 19 minutes.

Venom + $K_2\bar{O}\left(\frac{N}{100}\right)$ ... ... Coag. time 14 minutes.

Venom + $CaCl_2\left(\frac{N}{100}\right)$ ... ... Coag. time 5 minutes.

The addition of calcium chloride in this quantity had the same influence on the venom when its activity was tested on a prothrombin solution. It appeared probable therefore that calcium chloride had an adjuvant action on the venom of the Indian viper contrary to the influence it exerted on the venom of the Australian viper. It was possible that the calcium chloride added to the Indian venom did not influence the venom itself but worked in conjunction with it on the prothrombin attached to the fibrinogen when the venom calcium mixture was tested on this solution. The possibility seemed somewhat remote in view of the extremely small quantity of calcium which would be present in the fibrinogen solution to which the venom calcium mixture was added. But the following experimental results proved its accuracy.

(Fg—S)	E.V.	$CaCl_2\left(\frac{N}{100}\right)$	H_2O	Coag. time
1 c.c.	·2 c.c.	·0 c.c.	·8 c.c.	14 minutes.
		·2	·6	5 ,,
		·4	·4	5 ,,
		·6	·2	$4\frac{1}{2}$,,
		·8	·0	4 ,,

The addition of $CaCl_2\left(\frac{N}{1000}\right)$ diminished the coagulation time of a fibrinogen venom solution from fifteen to five minutes. This quantity of calcium chloride was present when ·2 c.c. of the venom calcium chloride mixture was added to 2 c.c. of fibrinogen. It was probable therefore that the addition of calcium chloride to a solution of Indian venom did not exert an adjuvant action on the venom *per se*, but that the accelerating influence noted when the venom calcium mixture was added to a fibrinogen solution was due to the calcium acting in conjunction with the venom on the prothrombin attached to the fibrinogen. This influence of minute traces of calcium chloride on the coagulation of fibrinogen by the venom of the Indian viper would probably obscure any deleterious influences which the calcium chloride exerted on the venom itself. The results obtained, therefore, on adding calcium chloride to the venoms of the Australian viper and the Indian viper, cannot be asserted to be due to the contrary action of this salt on the two venoms but may be explained by the very small quantities of calcium salts which are required to accelerate the coagulating action of the Indian viper venom on fibrinogen.

The nature of the active coagulating principles.

The experiments detailed in the first portion of this paper on the coagulation of fibrinogen and the generation of fibrin ferment from prothrombin by viper venoms received an adequate explanation on the assumption that these venoms contained an active principle possessing the properties of a mixture of tissue kinase and calcium salt. This analogous action suggested that the active principle of these venoms was composed of kinase combined with calcium salts. The adjuvant influences exerted on these venoms by small quantities of calcium salts added to the fibrinogen or prothrombin solutions were shown to be similar to the effects produced when small quantities of calcium salts were added to fibrinogen kinase solutions in which the kinase was present in minimal quantities. The dilution of a venom solution in which one principle contained both the kinase and the calcium salt entailed that this dilution diminished at the same time both the active elements and it has been shown that a reciprocal relation apparently exists between the kinase and the calcium salt—the diminution of one factor demanding a proportionate increase of the other factor to keep the time of coagulation at a certain value. Therefore the accelerating influence exercised by the addition of calcium to fibrinogen or prothrombin solutions on the action of these venoms did not demand a modification of the hypothesis that the coagulating principle contained in them was composed of kinase combined with calcium salt.

But the question naturally arose whether this hypothesis of the constitution of the active principle in these venoms was capable of explaining the actions exerted on them by neutral salts. The salient facts discovered in the actions of neutral salts on these venoms were that potassium oxalate when added to the venoms exercised a marked adjuvant influence on their coagulating properties whilst calcium chloride added to the venom of the Australian viper progressively destroyed it. The paradoxical influence of the calcium salt on the Australian venom may possibly be due to the action of the divalent calcium ion on the electro-negative venom. This venom is markedly acidic to litmus paper and the observed relations of positive ions and negative colloids make the explanation feasible. But the assumption that the active principles of these venoms consist of kinase and calcium in combination offers no adequate basis for the explanation of the influence of potassium oxalate on them. On this hypothesis the

natural expectation was that the addition of potassium oxalate to them would diminish their coagulating property since the calcium contained in the active principle would require to be comparatively loosely bound with the kinase in order to exercise its ionic effects, and such a loose combination would tend to be broken down by the added oxalate in a quantity dependant upon the relative affinities of the kinase and oxalate for the combined calcium. This fundamental difficulty suggested a modification of the original hypothesis of the constitution of the active coagulating principle in viper venom. This modified hypothesis is that the coagulating principle in these venoms consists of pure kinase only, and that the calcium salt required to work in conjunction with it in the generation of fibrin ferment from prothrombin is associated with the protein contained in the fibrinogen or prothrombin solution.

No direct proof has been obtained that the active calcium is associated with the fibrinogen rather than with the venom. And in the absence of this direct proof the above suggestion that the coagulating principle of snake venom consists of kinase only must remain a matter of conjecture. Hammarsten[4] proved that fibrinogen contained calcium to the amount of ·055 %. That this calcium can be influenced so as to effect the coagulation of the fibrinogen with which it is associated may be assumed from the action of potassium oxalate on fibrinogen solutions. The addition of potassium oxalate to a fibrinogen solution so that ·08 % of this salt was present usually induced coagulation in the course of four to twelve hours. Such a fibrinogen solution invariably coagulated in the course of one or two hours when ·05 % calcium chloride was added to it and from what has been previously said it probably contained kinase. The action of the oxalate in causing coagulation would seem to depend, therefore, upon first splitting some of the adsorbed calcium from the fibrinogen aggregates, and the calcium oxalate thus split off being partially soluble in water (the very minute quantities of calcium required to coagulate fibrinogen solutions in the course of a few hours has been shown above) working in conjunction with the kinase present would ultimately generate sufficient fibrin ferment to produce coagulation.

No method has been devised by means of which pure kinase can be made from tissue extracts and experiments with these tissue extracts themselves are of little value as the following observations show. The addition of kinase to a fibrinogen solution containing $\frac{N}{1000}$ calcium

chloride produced coagulation in five minutes. Twenty times this quantity of tissue extract produced coagulation in the same quantity of fibrinogen solution free from calcium in fifteen minutes. In this second case the calcium must have been contained either in the fibrinogen solution or in the tissue extract. From the comparative times of coagulation of the two fibrinogen solutions the amount of calcium present in the kinase fibrinogen mixture would not be more than $\frac{N}{5000}$ or one part of calcium chloride in one hundred thousand parts of solution. From these numbers it is possible that the necessary calcium was contained in the crude testis extract. The conjectures that fibrinogen solutions contain calcium salts associated with the protein which are able to work in conjunction with pure kinase and that kinase is always contained in fibrinogen solutions (since such solutions coagulate on adding calcium salts only to them) raise the question why fibrinogen solutions do not spontaneously coagulate. The answer to this question is that fibrinogen solutions prepared from the most stable blood always coagulated spontaneously in the course of a few days if kept at 30° C. But whether this coagulation was due to the action of the kinase and calcium associated with the fibrinogen or to a trace of fibrin ferment contained in the fibrinogen solution cannot be determined.

The very small quantities of venom which are required to coagulate fibrinogen solutions might be taken as an indication that the active principle of the venom consisted of kinase only and that the active calcium was derived from the fibrinogen solutions. A minimal amount of calcium required to coagulate a fibrinogen solution to which tissue extract has been added is about one part in twenty thousand. The activity of the venom of the Australian viper is such that the addition of one part of this venom to twenty million parts of fibrinogen solutions produces coagulation in a few minutes. On the assumption that calcium is required to produce coagulation of a fibrinogen solution in every case the quantity contained in the venom would appear too small to be able to exert any evident action.

In the following pages the action of cobra venom on the processes which occur in the coagulation of blood is discussed. From the experiments there detailed it is probable that this venom contains an anti-kinase only. On the assumption that the venoms of the Indian viper and the Australian viper contain kinase it was interesting to determine whether cobra venom neutralised viper venom. It was found that no influence was exerted by the cobra venom on the viper venom.

These observations indicated that the coagulating principle of viper venom did not consist of kinase, or that the capacity of cobra venom to neutralise tissue kinase depended upon the union of the venom with a substance to which the tissue kinase was associated rather than to the kinase itself. The experiments did not offer any satisfactory proof of the constitution of the coagulating principle in viper venom.

From the above discussion it is clear that no certain proof has been adduced that the coagulating principles contained in the venoms of the Indian viper and Australian viper consist of kinase only, the comparative purity of this kinase enabling it to work in conjunction with the calcium salts associated with the protein in fibrinogen or prothrombin solutions in the generation of fibrin ferment. On the other hand certain indications have been observed that such a constitution of the active principle in viper venom is possible. The question now arises whether the observed action of potassium oxalate on these venoms receives a reasonable explanation on this hypothesis. The main fact observed was that potassium oxalate added in small quantities to the venoms of the Australian viper and the Indian viper markedly increased their action. It has been seen that the assumption that the active principles in these venoms are composed of kinase in union with calcium salts is entirely at variance with this action of potassium oxalate. But on the above hypothesis the following explanation of the mode of action of potassium oxalate appears feasible. The molecules of potassium oxalate become associated with the kinase molecules so that the active coagulating principles become molecular aggregates of kinase and potassium oxalate. By virtue of the associated potassium oxalate these molecular aggregates have an affinity for the calcium attached to the protein molecules stronger than that previously possessed by the kinase only. From this it follows that more calcium will be attracted to the kinase molecules, the relative amounts distributed between the kinase and fibrinogen at any time being a function of the relative affinities of these two substances for the calcium salt present in the mixture. This increase in the quantity of calcium transferred to the sphere of influence of the kinase molecules will result in a larger generation of fibrin ferment from the prothrombin present in a given space of time just as an addition of calcium to a venom prothrombin solution results in a more rapid production of ferment. The addition of potassium oxalate to a fibrinogen or prothrombin solution always increases the action of the venom of the Australian viper. The affinity of this venom therefore for potassium oxalate must be great. But in the case of the venom of the Indian

viper the previous addition of the potassium oxalate to a fibrinogen or prothrombin solution leads to variable results. Sometimes the action of the venom is accelerated, at other times it is depressed. On the hypothesis at present under discussion this variable action may be explained by assuming that in addition to the oxalate combining with the venom molecules it also splits off the calcium salt from the protein and precipitates it as an insoluble oxalate. These two actions of the potassium oxalate are antagonistic to one another so far as the generation of fibrin ferment is concerned and the relative amounts of each action will determine in any case whether the coagulation process is hastened or delayed.

When potassium oxalate was added to plasma it was found that in the case of both these venoms the coagulation process was delayed—the influence of the oxalate being more marked in the case of the venom of the Indian viper. This effect of the oxalate was clearly due to the precipitation of the ionised calcium salts in the plasma, the addition of a calcium salt to a fibrinogen solution having been shown to markedly accelerate the coagulating actions of both these venoms. The more marked inhibitory influence of the oxalate on the venom of the Indian viper receives the same explanation that has been given above to account for the relative effects of the previous addition of potassium oxalate to a fibrinogen or prothrombin solution on the activities of these venoms.

The results of the above discussion may be briefly stated. The addition of the venom of the Indian viper or the Australian viper to a fibrinogen or prothrombin solution causes a production of fibrin ferment. Therefore on the present theory of the coagulation of blood the prothrombin venom mixture contains kinase and calcium salts. The venom clearly contains the kinase. The question then arises as to which constituent of the mixture contains the calcium salts. From general considerations and more particularly from the action of potassium oxalate on these venoms the hypothesis has been advanced that the calcium salt is not associated with the kinase in the venom molecule but is present in the fibrinogen or prothrombin solution adsorbed by the protein aggregates.

The positive and negative phases in blood coagulation—intravascular coagulation.

In 1886 Wooldridge[5] described a series of experiments in which he produced intravascular coagulation in living animals by the

injection of tissue fibrinogen. On the present theory of the coagulation of blood the tissue fibrinogen owed its coagulating properties to the kinase contained within it. It may therefore be assumed that the phenomena which he described are similar to those which would be observed after the intravenous injection of varying quantities of kinase. The main facts described by Wooldridge were that the injection of small quantities of tissue fibrinogen led to an increase in the coagulability of the blood, whilst larger quantities often produced intravascular coagulation—the degree of coagulation varying from a slight thrombosis in the portal area to extensive clotting throughout the body. If after the injection of moderate quantities of tissue fibrinogen the intravascular coagulation was small in amount or absent then the remaining blood showed little tendency to coagulate (the so-called negative phase in blood coagulation). The production of this negative phase conferred a temporary immunity on the animal against the injection of additional large quantities of tissue fibrinogen, the blood drawn from the animal after this second injection showing no tendency whatever to coagulate. Wooldridge considered this negative phase blood to be different to peptone blood.

The similarity of peptone blood and negative phase blood and the identity of the conditions under which both could be produced led Wright[6] to state that they were identical. Lilienfeld[7] assumed that the stable blood obtained after injecting tissue fibrinogen owed its properties to an excess of histon within it.

The researches of C. J. Martin[2] on the effects produced on the blood of animals by the intravenous injection of various snake venoms and the similarity of the results obtained with those described by Wooldridge after the injection of tissue fibrinogen have already been mentioned. From these experiments it was clear that if the effects produced by the injection of tissue fibrinogen were due to the presence of kinase then snake venom contained a substance possessing properties similar to tissue kinase. Wooldridge injected about two grams of tissue fibrinogen into a dog to produce intravascular coagulation; the quantity of venom used by Martin to produce a similar effect was about two milligrams. The relative weights of the two substances emphasises the purity of the snake venom. In the previous pages the actions of the venoms of the Australian viper and the Indian viper on plasma and fibrinogen have been described. In the following pages the effects produced on blood after the intravenous injection of the venoms into living animals are described.

The details of the experiments published by Martin render it a comparatively easy matter to produce intravascular coagulation or fluid blood by the intravenous injection of snake venom into animals. In the case of cats the rapid injection of two milligrams of venom leads to intravascular coagulation—the slow injection of the same quantity (the injection extending over thirty minutes) leads to the production of fluid blood. Smaller quantities of the venom of the Australian viper than the Indian viper are required to produce these effects—a relation which has been previously observed on the coagulation of fibrinogen by these venoms. The following is a description of an experiment in which the venom of the Australian viper was injected into the jugular vein of a cat (weight 3 kilos).

The cat was anaesthetised, and a tracheotomy operation performed. The left jugular vein and the right carotid artery were isolated and cannulae were tied into them. ·5 mgrm. of venom dissolved in 30 c.c. of Ringer solution were slowly injected into the jugular vein. This injection occupied twenty minutes. At the end of this time a sample of blood taken from the carotid artery clotted in ten seconds—the blood showed a well marked positive phase of coagulation. After the lapse of five minutes ·3 mgrms. of venom dissolved in 5 c.c. of Ringer solution were slowly injected into the jugular vein. This injection occupied three minutes. After the lapse of five minutes the animal was bled to death. The blood was non-coagulable. It showed no sign of coagulation after the lapse of twenty-four hours. 60 c.c. of this fluid blood were centrifuged and the plasma syphoned off. The plasma was slightly red owing to a small amount of haemolysis having taken place. The following experiments were done on this plasma.

Fibrinogen, fibrin ferment, and kinase were prepared from a cock. The activities of the fibrin ferment and the kinase are shown below.

	(Fg—S)	H_2O	Ff.	Coag. time
(I)	1 c.c.	·9 c.c.	·1 c.c.	½ minute.

From this it follows that the ferment solution was very active.

	(Fg—S)	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O	Coag. time
(II)	1 c.c.	·2 c.c.	·2 c.c.	·6 c.c.	1½ minutes.

Therefore the kinase solution was active and the fibrinogen solution contained prothrombin.

(III) 1 c.c. of fibrin ferment was added to 1 c.c. of fluid cats' plasma—no coagulation occurred. Therefore the cats' plasma contained no fibrinogen, or an excess of anti-fibrin-ferment, or some other inhibitory body.

(IV) To determine whether all the fibrin ferment disappeared in (III), the following experiment was done.

1 c.c. (Fg—S) + ·2 c.c. (III)	...	...	Coag. in 20 minutes.
1 c.c. (Fg—S) + 1 c.c. (III)	...	...	Coag. in 5 minutes.

Therefore the mixture (III) (originally composed of equal parts of cats' plasma and cocks' fibrin ferment) contained after the lapse of 30 minutes something which would coagulate cocks' fibrinogen.

(V) The effect of kinase on this fluid cats' plasma was now tried.

Cats' plasma	K.	$CaCl_2\left(\frac{N}{10}\right)$	Coag. time
1 c.c.	·8 c.c.	·2 c.c.	No coag. occurred.

Although a large quantity of kinase was added in the above experiment the absence of coagulation might have been due to the presence of an anti-kinase in the cats' plasma.

(VI) But

1 c.c. (Fg—S) + ·2 c.c. (V) + ·2 c.c. $CaCl_2\left(\frac{N}{10}\right)$ + ·6 c.c. H_2O—Coag. in 5 minutes.

Therefore, from the above experiments, it was clear that the cats' plasma obtained after injecting the venom of the Australian viper would not coagulate on adding cocks' fibrin ferment or kinase although the subsequent examination of portions of cats' plasma so treated showed that they contained an excess of free fibrin ferment and kinase. Similarly Martin has shown that such fluid blood obtained from dogs will not coagulate on adding more venom to it. The only conclusion to be drawn from these results was that the cats' plasma contained no fibrinogen. This conclusion was confirmed by the following experiments.

(VII) 1 c.c. of cocks' fibrinogen solution was added to 1 c.c. of fluid cats' plasma. Coagulation occurred in 2½ minutes. Therefore the cats' plasma contained those constituents necessary to coagulate fibrinogen but did not coagulate itself. From this it follows that fibrinogen must have been absent in the plasma.

(VIII) There was a possibility that cocks' fibrin ferment would not coagulate normal cats' plasma. But this was disproved by adding this fibrin ferment to oxalated plasma obtained from another cat.

1 c.c. oxalate cats' plasma + ·9 c.c. H_2O + ·1 c.c. Ferment—Coag. in 1 minute.

The fibrin ferment obtained from the cock was an efficient coagulant of the oxalated plasma obtained from a cat. The effect of cocks' kinase on cats' plasma could not be determined since oxalated cats' plasma contained sufficient kinase to clot spontaneously on adding sufficient calcium chloride to it.

From all the above experiments it follows that blood in the negative phase obtained after injecting the venom of the Australian viper is non-coagulable since it contains no fibrinogen. The direct proof that it contained no fibrinogen was obtained by precipitating the globulin from such a plasma by dilution with distilled water and neutralisation of the

diluted mixture with a little acetic acid. The globulin so obtained when dissolved in tenth normal sodium chloride would not coagulate on adding fibrin ferment or kinase and calcium chloride to it. Therefore it was composed of serum globulin only and contained no fibrinogen. In the case of this experiment the possible presence of inhibitory factors was excluded by preparing the globulin free from all the other constituents of the plasma.

The fact that negative phase blood owes its fluidity to the absence of fibrinogen offers an easy explanation of the phenomena which are observed after the injection of tissue fibrinogens into animals. The injection of a small amount of such a substance increases the quantity of kinase in the blood. Consequently the blood becomes more coagulable and the positive phase of blood coagulation becomes evident. Whether intravascular coagulation or fluid blood results on the further injection of kinase depends on the rate at which it is injected. If the kinase is quickly injected there is a rapid formation of fibrin, the molecules of fibrin when formed coalesce, filaments are produced and intravascular coagulation results. If the kinase is slowly injected there is a correspondingly slow formation of fibrin, the molecules of fibrin when formed are removed by the tissue cells and finally all the fibrinogen is formed into fibrin, the fibrin being removed from the blood before it can form filaments. Whether intravascular coagulation or fluid blood results on the injection of kinase into animals the effect of the kinase is the same in the two cases—the generation of fibrin ferment and the formation of fibrin from fibrinogen. The diverse final effects observed depend only upon the rate of formation of the fibrin. If the rate of formation of the fibrin is too great for the tissue cells to deal with it intravascular coagulation results; if the rate is sufficiently small to allow the tissue cells to work then fluid blood ultimately results.

In the above description the experiments have dealt with blood in which perfect fluidity was obtained. By a smaller injection of venom blood which was spontaneously coagulable in a longer or shorter time could have been obtained. Such blood is necessarily the result of injecting a quantity of venom too small to effect the transference of the whole of the fibrinogen into fibrin. The time of coagulation of this blood will necessarily be hastened by such factors as influence the coagulation of peptone blood since these procedures shorten the time of coagulation of normal blood. The extended times of coagulation of "partial" negative phase blood depend upon the concomitant diminution

of two factors concerned in coagulation—the fibrinogen and associated prothrombin. For this reason a sample of blood containing only a small quantity of fibrinogen may take days to clot spontaneously and in fact may not coagulate spontaneously if the quantity of fibrinogen contained in it is so small that the generation of fibrin ferment from the prothrombin is sufficiently slow to permit of its neutralisation by the anti-fibrin-ferment contained in the plasma.

The temporary immunity observed after the injection of tissue fibrinogen also receives a ready explanation. The first injection of the kinase causes the disappearance of the fibrinogen from the blood; the second injection is therefore not able to produce intravascular coagulation until the fibrinogen has been restored to the blood, *i.e.* within the next twenty-four hours during which time the immunity is stated to last.

The analysis of the phenomena which have been observed on injecting snake venom into the blood stream of animals throws an interesting light on the relation of the blood to the tissues in general. It may be assumed that all tissues contain kinase in greater or less amounts. The tissues are continually dying or being injured. Consequently kinase must get into the blood stream. This kinase in conjunction with the calcium salts in the blood generates some fibrin ferment, and the production of ferment in molecular continuity with fibrinogen necessitates that this fibrinogen will be coagulated. There is no evident quantity of anti-kinase in the blood. Therefore if the organism could not deal with a slow formation of fibrin thrombosis would be a continual occurrence in the life history of animals. Probably the same cells which are concerned in the removal of small quantities of fibrin from the blood stream are also concerned in the removal of any products of cell disintegration containing kinase. That the body is continually occupied with the formation of fibrinogen is evident from the observation that after the production of fluid blood by the injection of kinase the blood regains its normal coagulability within the next twenty-four hours. It is not conceivable that the fibrinogen would return so rapidly to the blood if the tissues were not continually concerned in the manufacture of this protein.

Wright's statement that negative phase blood is similar to peptone blood has been mentioned above. It may be stated, in conclusion, that a dog which had developed peptone immunity as the result of a peptone injection, gave negative phase blood after the injection of the venom of the Australian viper, although the blood before the injection of this

venom was normally coagulable. The result of this experiment conclusively differentiates the two classes of blood. But the properties of peptone blood will be discussed in a subsequent section.

COBRA VENOM.

In contradistinction to the properties of the two viper venoms previously discussed the venom of the cobra always produces non-coagulable blood whether it is injected intravenously into an animal or added to blood *in vitro*. Morawitz[8], as the result of a series of experiments on this venom, came to the conclusion that it contained an anti-kinase. The coagulation of blood by thrombin was not hindered by its presence, but a quantitative relation was found to exist between the venom and thrombo-kinase. This deduction of Morawitz was confirmed by the results of the following experiments.

The influence of cobra venom on the coagulation of fibrinogen by fibrin ferment.

2 mgrms. of cobra venom were dissolved in 5 c.c. water. This solution is designated by the letters C.V. It may be noted that this solution of cobra venom was about one hundred times as strong as the solutions of the venoms of the Indian viper and Australian viper previously used to coagulate fibrinogen solutions. The following experiment shows the influence of cobra venom on the coagulation of fibrinogen by fibrin ferment.

(Fg—S)	C.V.	Ff.	H_2O	Coag. time
2 c.c.	·0 c.c.	·2 c.c.	·8 c.c.	1 minute.
2	·2	·2	·6	1 ,,
2	·3	·0	·7	No coagulation.

From the above it is clear that cobra venom alone does not coagulate fibrinogen, nor does it prevent the action of fibrin ferment on this protein.

Cobra venom was added directly to a fibrin ferment solution as follows.

1 c.c. C.V. + 1 c.c. Ff. = C.V.F.

After ten minutes the activity of this solution was tested on a fibrinogen solution.

(Fg—S)	C.V.F.	H_2O	Coag. time
2 c.c.	·4 c.c.	·6 c.c.	1 minute.

From the time of coagulation it is evident that the cobra venom exercised no influence on the fibrin ferment.

The influence of cobra venom on kinase.

The effect of cobra venom on the generation of fibrin ferment from prothrombin by kinase and calcium salts was now determined. This influence was estimated in two ways: (*a*) by adding the venom to a fibrinogen solution and determining the change in the coagulation time induced by definite amounts of kinase and calcium chloride, and (*b*) adding the venom to a prothrombin solution and determining the rate of change in the production of fibrin ferment by given quantities of kinase and calcium salt. The following experiment shows the influence of cobra venom on the coagulation of fibrinogen by kinase and calcium chloride.

(Fg—S)	C.V.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O	Coag. time
2 c.c.	·0 c.c.	·2 c.c.	·2 c.c.	·6 c.c.	3 minutes.
	·1	·2	·2	·5	4½ ,,
	·2	·2	·2	·4	8 ,,
	·3	·2	·2	·3	11 ,,
	·4	·2	·2	·2	14 ,,
	·5	·2	·2	·1	16 ,,
2 c.c.	·5	·2	·2	·1	6 ,,

In the first six experiments above the cobra venom was added to the fibrinogen solution immediately before the kinase and calcium chloride. In the last experiment the venom was first added to the fibrinogen and the mixture was allowed to incubate for fifteen minutes before the kinase and calcium chloride were added. The preliminary incubation of the venom with the fibrinogen diminished the coagulation time by ten minutes, *i.e.* from sixteen minutes to six minutes. It was clear therefore that the fibrinogen took up the cobra venom and so protected the kinase subsequently added.

The influence of cobra venom on fibrinogen may be seen if a large quantity of venom is added to a fibrinogen solution and after an interval the coagulation time of this solution by fibrin ferment be compared with the time taken by the same quantity of ferment to coagulate a fibrinogen solution free from venom. This influence is shown in the following experiment.

(Fg—S)	Ff.	C.V.	H_2O	Coag. time
2 c.c.	·1 c.c.	·0 c.c.	·9 c.c.	1½ minutes.
2	·1	·5	·4	3½ ,,

The preliminary incubation of the venom with the fibrinogen had made this fibrinogen more resistant to coagulation by fibrin ferment—

the increased resistance being shown by a two minutes extension of the coagulation time.

The influence of cobra venom on the generation of fibrin ferment from prothrombin by kinase and calcium chloride is shown very clearly in the following experiments.

The rate of generation of fibrin ferment from the prothrombin solution by definite quantities of kinase and calcium chloride was shown thus:

	Pro.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
(I)	2 c.c.	·1 c.c.	·2 c.c.	·7 c.c.	=	P'.

After definite times the ferment activity of this solution was determined.

(Fg—S)	P'.	H_2O	Coag. time	Tested after
2 c.c.	·2 c.c.	·8 c.c.	110 minutes	3 minutes.
			20 ,,	6 ,,
			9 ,,	12 ,,
			4 ,,	15 ,,
			2 ,,	21 ,,

The same quantities of prothrombin, kinase and calcium chloride were made as in (I) above, but ·1 c.c. of C.V. was added to the mixture thus:

	Pro.	K	$CaCl_2\left(\frac{N}{10}\right)$	C.V.	H_2O	
(II)	2 c.c.	·1 c.c.	·2 c.c.	·1 c.c.	·6 c.c.	= P.V.

The ferment activity of this solution may be determined from the following results.

(Fg—S)	P.V.	H_2O	Coag. time	Tested after
2 c.c.	·2 c.c.	·8 c.c.	Trace of coag. in 100 mins.	20 mins.
	·2	·8	Small coag. in 1 hr. 33 mins.	40 ,,
	·4	·6	,, ,, ,,	40 ,,
	·4	·6	15 mins.	60 ,,
	·4	·6	6 ,,	80 ,,

On comparing the rates of generation of fibrin ferment in (I) and (II) above the inhibitory effect of the cobra venom may be readily seen.

In the third experiment given below the relative amounts of prothrombin, cobra venom and calcium chloride were the same as in (II), but the kinase was doubled in quantity thus:

	Pro.	K.	$CaCl_2\left(\frac{N}{10}\right)$	C.V.	H_2O	
(III)	2 c.c.	·2 c.c.	·2 c.c..	·1 c.c.	·5 c.c.	= P'. V'.

The ferment activity of this mixture was tested after various times.

(Fg—S)	P'.V'.	H_2O	Coag. time	Tested after
2 c.c.	·2 c.c.	·8 c.c.	15 minutes	20 minutes.
			4 ,,	40 ,,
			3 ,,	60 ,,

By comparing the results in (II) and (III), it may be seen that doubling the amount of kinase very materially diminished the inhibitory influence exerted by a constant quantity of cobra venom.

The influence of cobra venom on the venoms of the Australian viper and Indian viper.

The absence of any neutralising effect of cobra venom on the active coagulating principles contained in the venoms of the Australian viper and the Indian viper has been alluded to in the section dealing with the properties of these venoms. The following experiment shows the times of coagulation of birds' plasma by the venom of the Australian viper in the presence and absence of cobra venom.

Plasma	C.V.	N.V.	H_2O	Coag. time
1 c.c.	·0 c.c.	·1 c.c.	·9 c.c.	1½ minutes.
	·1	·1	·8	2½ ,,
	·5	·1	·4	2½ ,,

The addition of cobra venom to the plasma produced a small delay in the coagulation time, but the delay was negligible compared to that produced by similar quantities of this venom on thrombo-kinase. There was no increased or diminished effect on adding the cobra venom to the plasma and allowing the plasma venom mixture to incubate for ten minutes before adding the Notechis venom.

The following experiment shows that the influence of cobra venom on the venom of the Indian viper was the same as that exerted on the Australian venom.

Plasma	C.V.	E.V.	H_2O	Coag. time
1 c.c.	·0 c.c.	·3 c.c.	·7 c.c.	1½ minutes.
1	·5	·3	·2	2 ,,

All the above experiments with cobra venom, fibrinogen, fibrin ferment, and kinase, showed conclusively that this venom contains an anti-kinase. The diminished inhibitory effect exerted by the venom on kinase after the preliminary incubation of the venom with a fibrinogen solution proved that the venom was taken up by the fibrinogen and so removed from the sphere of action of the kinase. The negligible influence of cobra venom on the venoms of the Australian and Indian vipers indicated that these latter venoms did not contain kinase if the assumption that cobra venom contained an anti-kinase were correct. But Martin[3] has shown that the serum of

a horse immunised against the venom of the Australian viper neutralised upwards of 10,000 times the minimal coagulating quantity of venom for 1 c.c. of plasma and yet was without effect on the venom of the Indian viper. The active coagulating principles contained in the Australian and Indian vipers have been seen to possess similar physiological properties. Consequently Martin's experiments appear to denote that identity of physiological action by no means implies a similarity of those properties on which the neutralisation of a substance by its anti-body depends.

PEPTONE BLOOD.

Introduction. Since the discovery by Schmidt-Mulheim[9] in 1882 that the intravenous injection of peptone into certain animals caused the blood to remain fluid for a considerable time after removal from the body, peptone blood has been the subject of many researches. But despite all the work that has been done on the subject the cause of the stability of this blood still remains obscure.

After the promulgation of the present theory of the coagulation of blood Fuld and Spiro[10] and Morawitz[11] investigated this problem. These observers came to the conclusion that a large quantity of anti-thrombin was present in peptone blood. But Morawitz definitely recognised that this anti-thrombin (which he stated was produced in large quantities after the injection of the peptone) was not the main cause but only an adjuvant factor in the preservation of the fluidity of peptone blood. He stated that peptone plasma contained prothrombin since Wooldridge and Hewlett[12] showed that it coagulated on adding tissue extracts to it. Further that it contained kinase since the precipitate obtained by Wooldridge on cooling peptone plasma contained this substance. Also that it contained calcium. Therefore it contained all the elements necessary for the formation of fibrin ferment and yet there was some factor present which prevented any production of ferment. This factor he stated to be the important cause of the preservation of the fluid state of peptone blood.

Many properties of peptone blood have been described but the most important methods of producing coagulation in it were discovered by Fano[13]. He found that peptone blood could always be coagulated by neutralisation with an acid, passage of carbon-dioxide, dilution with water, and the addition of neutral salts. Schmidt-Mulheim

stated that it could be coagulated by fibrin ferment. But Fano and Wooldridge contradicted this statement. Pekelharing however showed that peptone blood would coagulate on the addition of fibrin ferment and Dastre and Floresco[14] proved that it did contain fibrin ferment since the addition of peptone plasma to the pericardial fluid of a horse produced coagulation.

Preparation of peptone plasma.

The many diverse statements which have been made with regard to peptone blood appeared to indicate that a detailed investigation of the subject would be of interest.

A fasting dog was anaesthetised and a tracheotomy operation performed. Cannulae were inserted into the left jugular vein and the right carotid artery. The animal was first bled from the carotid artery to the extent of 100 c.c. into a tube containing potassium oxalate, the final quantity of oxalate in the blood being about ·15 %. A 5 % solution of Witte's peptone dissolved in Ringer's fluid was quickly injected into the jugular vein to the extent of ·3 grams of peptone per kilo of body weight of the animal. Five minutes after the injection of the peptone 200 c.c. of blood were obtained from the carotid artery. After the lapse of three hours the blood in the animal regained its normal coagulability but a subsequent injection of peptone was unable to render the blood non-coagulable—the animal showed the well recognised peptone immunity. A small quantity of the venom of the Australian viper was now slowly injected and stable fluid blood (*i.e.* blood in the negative phase of coagulation) was obtained. The last portion of the experiment has been mentioned in the section dealing with intravascular coagulation. The results showed in a striking manner that different factors were involved in the production of peptone blood and negative phase blood. The oxalate blood and peptone blood were centrifuged and the supernatant specimens of plasma were syphoned off.

The coagulation of dogs' peptone plasma by birds' fibrin ferment and kinase.

Fibrinogen, kinase and fibrin ferment were obtained from a bird in the way previously described. The following results show the strengths of the birds' kinase and fibrin ferment used in the experiments on the dogs' peptone plasma and oxalate plasma.

Strength of kinase.

(Fg—S)	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O	Coag. time
2 c.c.	·2 c.c.	·2 c.c.	·6 c.c.	3 minutes.
2	·1	·2	·7	4½ ,,

Strength of ferment.

(Fg—S)	Ff.	H_2O	Coag. time
2 c.c.	·2 c.c.	·8 c.c.	1½ minutes.

The efficiency of this ferment solution on the dogs' oxalated plasma (= Pl. $\bar{O}$) was determined.

Birds' ferment on dogs' oxalate plasma.

Pl. $\bar{O}$	Ff.	H_2O	Coag. time
2 c.c.	·2 c.c.	·8 c.c.	4 minutes.
2	·4	·6	1½ ,,

From this experiment it was evident that birds' fibrin ferment was an efficient coagulant for dogs' oxalate plasma. The effect of adding birds' kinase to dogs' oxalate plasma could not be tried since this plasma normally coagulated on adding calcium chloride to it in a quantity chemically in excess of the oxalate. But the effect of adding birds' kinase and fibrin ferment on peptone plasma are shown in the following experiments.

Birds' fibrin ferment on peptone plasma. (= Pl. P.)

Pl. P.	Ff.	H_2O	Coag. time
2 c.c.	·2 c.c.	·8 c.c.	No coag. in 30 minutes.
2	·4	·6	3 minutes.

The coagulation of peptone plasma could be produced by moderate quantities of birds' fibrin ferment. But there was clearly something present in the plasma which inhibited the action of the ferment.

Birds' kinase on peptone plasma.

Pl. P.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O	Coag. time
2 c.c.	·2 c.c.	·0 c.c.	·8 c.c.	6 minutes.
	·2	·2	·6	4 ,,

Coagulation in this plasma could also be produced by adding the extract of a cock's testis to it, and this action was increased by the addition of $CaCl_2\left(\frac{N}{150}\right)$.

From the above experiments it followed that dogs' peptone plasma could be coagulated by birds' fibrin ferment and kinase but that there was some factor present in the plasma which inhibited the actions of these substances.

The globulin present in 50 c.c. of peptone plasma was precipitated in the usual way by dilution with ten volumes of distilled water and neutralisation of the mixture with dilute acetic acid. The globulin so obtained was dissolved in ·75 % NaCl (= a′). There was a fair quantity of precipitate which did not dissolve in the sodium chloride and aggregated like fibrin.

The coagulation of (a′) *by birds' kinase and calcium chloride.*

	(a′)	K.	$CaCl_2$	H_2O	Coag. time
(A)	2 c.c.	·2 c.c.	·2 c.c.	·6 c.c.	3 minutes.

The coagulation of (a′) *by birds' fibrin ferment.*

	(a′)	Ff.	H_2O	Coag. time
(B)	2 c.c.	·2 c.c.	·8 c.c.	3 minutes.
		·6	·4	¾ minute.

After 30 minutes the fluid was expressed from (A) above and tested for fibrin ferment.

	(a′)	(A) fluid	H_2O	Coag. time
(C)	2 c.c.	·2 c.c.	·8 c.c.	2 minutes.

From (A) and (B) above it followed that the peptone plasma contained fibrinogen which could be coagulated by kinase and calcium chloride or by fibrin ferment. From the experiment (C) it was evident that a fair quantity of prothrombin was associated with the fibrinogen. The fibrinogen solution (a′) coagulated spontaneously within an hour proving that the fibrinogen was associated with a small quantity of fibrin ferment. The readiness with which the fibrinogen prepared from peptone plasma could be coagulated indicated that the inhibitory factor present in this plasma did not accompany the fibrinogen in its precipitations.

The inhibitory factor in peptone plasma.

The problem which now remained for solution was the nature of this inhibitory factor. It has been stated that the general consensus of opinion is that the stability of peptone blood is due to the presence in it of an anti-thrombin, this anti-thrombin being excreted into the blood under the stimulus of the injected peptone. But it has been

previously shown that normal blood contains anti-thrombin, the quantity of this substance usually present being largely in excess of that necessary to neutralise all the ferment which can be formed in the blood. The reason why this anti-thrombin does not prevent normal blood from spontaneously coagulating was shown to be due to two factors (*a*) that fibrin ferment is produced from prothrombin in molecular proximity to the fibrinogen, and (*b*) that a definite time interval is involved in the neutralisation of fibrin ferment by anti-thrombin. Also it was shown that the function of anti-thrombin was not to prevent coagulation taking place at the site of the lesion (*i.e.* where the kinase was liberated) but to prevent the formation of an extending coagulum due to the passage of free fibrin ferment in the blood stream to places remote from the lesion. These facts rendered it improbable that the fluidity of peptone blood was due to the presence in it of a large quantity of anti-thrombin. The conjecture was submitted to the test of experiment.

·2 c.c. of dogs' oxalate serum were added to 1 c.c. of birds' fibrin ferment = A.

·2 c.c. of dogs' peptone plasma were added to 1 c.c. of birds' fibrin ferment (an instant coagulum was produced) = B.

After twenty minutes the activities of A and B were tested on birds' fibrinogen.

(Fg—S)	A	H_2O	Coag. time
2 c.c.	·2 c.c.	·8 c.c.	8 minutes.
2	·4	·6	2 ,,
	B		
2 c.c.	·2 c.c.	·8 c.c.	5 minutes.
2	·4	·6	1½ ,,

From the above experiments it followed that dogs' peptone plasma did not contain a quantity of anti-fibrin-ferment in excess of that contained in the same animals' oxalate plasma. The results conclusively proved that dogs' peptone blood does not owe its stability to the anti-fibrin-ferment contained within it.

The problem still remained as to the cause of the stability of peptone plasma. It has been shown that this plasma coagulates on adding to it moderate quantities of ferment or kinase, that it contains fibrinogen and prothrombin, and that it does not contain an excess of anti-thrombin. Further it contains small quantities of kinase since peptone plasma invariably coagulates on keeping (a week's interval may sometimes elapse before coagulation is produced). This coagulation can be considerably hastened by adding birds' fibrinogen to it as the following result shows.

2 c.c. of birds' fibrinogen were added to 2 c.c. of peptone plasma. The birds' fibrinogen was instantly dissolved and coagulation occurred in five minutes. Similar experiments were made with other samples of peptone plasma with the same result—coagulation always ensued in a longer or shorter time, the length of time which elapsed before coagulation occurred being a function of the amount of fibrinogen added, a larger quantity of fibrinogen causing more rapid coagulation. Therefore the peptone plasma contained kinase and calcium salts capable of generating fibrin ferment from prothrombin.

The influence of alkali on the coagulation of oxalated dogs' plasma and birds' plasma.

But all these results follow directly from the original observations of Fano, that peptone blood quickly coagulates when carbon-dioxide is passed through it or its alkalinity is neutralised by an acid. And these experiments give the clue to the cause of the stability of peptone blood—its excessive alkalinity. This explanation is so obvious that it has been previously advanced but has been rejected since various experimenters have stated that peptone blood is not more alkaline than normal blood. Thus Dastre and Floresco[14] definitely came to the conclusion that peptone blood owed its stability to the presence of an excess of alkali in it and showed that fibrin ferment was present in this blood since it coagulated the pericardial fluid of a horse (it may be mentioned that the presence of kinase and calcium salts in it would have effected the same result). But no great reliability can be placed on determinations of the alkalinity of blood owing to the protein in it combining with the alkali and so obscuring any determinations of its real value. The essential proof that peptone blood owes its stability to an excess of alkali within it rests on a comparison of the effects noted on adding alkali to normal blood and those found in experiments on peptone blood. The following series of experiments shows this comparison.

The coagulation of dogs' peptone plasma (Pl. P) *by birds' fibrin ferment.*

Pl. P	Ff.	H_2O	Coag. time
2 c.c.	·2 c.c.	·8 c.c.	24 minutes.
	·3	·7	2 ,,
	·4	·6	1½ ,,
	·5	·5	¾ minute.

The influence of alkali on the coagulation of oxalated dogs' plasma (Pl. Ō) *by birds' fibrin ferment.*

Pl. Ō	Ff.	NaOH $\left(\frac{N}{10}\right)$	H_2O	Coag. time
2 c.c.	·1 c.c.	·3 c.c.	·6 c.c.	30 minutes.
		·2	·7	3 ,,
		·1	·8	1 minute
		·0	·9	1 ,,

By comparing these two experiments it may be seen that the addition of sodium hydroxide to oxalated dogs' plasma caused precisely the same effects so far as coagulation by fibrin ferment was concerned as was shown on the addition of ferment to dogs' peptone plasma. The similarity between peptone plasma and plasma to which alkali had been added was still more clearly brought out in the following experiments.

The coagulation of oxalate plasma to which varying quantities of alkali were added by fibrin ferment.

Pl. Ō	Ff.	NaOH $\left(\frac{N}{8}\right)$	H_2O	Coag. time
2 c.c.	·1 c.c.	·0 c.c.	·9 c.c.	1½ minutes.
	·1	·1	·8	3½ ,,
	·1	·2	·7	No coag. in 1 hour.
	·2	·2	·6	45 minutes (A).
	·3	·2	·5	10 ,,

In the above results the balance between the added alkali and fibrin ferment is shown—the greater the quantity of alkali present the more fibrin ferment must be added to produce coagulation in a given time.

Two tubes containing plasma etc., similar to (A) above were made. At the end of five minutes the contents of one tube were diluted ten times and the diluted mixture was divided into two portions (*a*), (*b*). CO_2 was passed through (*a*) at once—a granular precipitate of globulin and a few filaments of fibrin were produced. Therefore at the end of five minutes only a small change of fibrinogen into fibrin had occurred. The second portion (*b*) was allowed to remain at 30° C. for an additional 15 minutes. At the end of this time the passage of CO_2 through it produced an instant production of fibrin although no apparent coagulation had previously taken place. CO_2 was passed through the second tube above after ten minutes—an instant coagulum formed. From this it follows that in experiment A above the change of fibrinogen into fibrin had taken place in ten minutes although the fibrin did not reach a state of insolubility so as to form a coagulum until 45 minutes had elapsed.

Therefore the addition of sodium hydroxide to plasma affects coagulation by fibrin ferment in two ways (1) it delays the action

of the ferment on the fibrinogen, and (2) it retains the newly formed fibrin in solution. The influence of salts on the retention of newly formed fibrin in solution has been previously mentioned. The influence of alkali appears to be very marked in this respect. The results of the above experiments clearly show the parallelism which exists between peptone plasma and oxalate plasma to which alkali has been added, so far as coagulation by fibrin ferment is concerned.

Similar results were obtained when the actions of kinase on peptone plasma and plasma to which alkali has been added were compared. It has been previously shown that peptone plasma contains a small amount of kinase but that some factor in the plasma inhibits the action of this kinase. Also that dogs' peptone plasma coagulates readily on adding birds' kinase to it. The following experiment shows the influence of alkali on the coagulation of birds' plasma by kinase. It is obvious that in this case dogs' oxalate plasma cannot be used, since this plasma quickly coagulates on adding calcium chloride to it.

The influence of sodium hydroxide on the coagulation of birds' plasma by kinase.

Plasma	K.	NaOH $\left(\frac{N}{8}\right)$	H_2O	Coag. time
1 c.c.	·05 c.c.	·0 c.c.	·95 c.c.	3½ minutes.
	·1	·0	·9	2½ ,,
	·2	·0	·8	2 ,,
	·05	·05	·9	20 ,,
	·1	·05	·85	7 ,,
	·2	·05	·75	4½ ,,
	·3	·05	·65	3¼ ,,
	·1	·1	·8	3 hours. (B).
	·2	·1	·7	100 minutes. (C).
	·3	·1	·6	17 ,,
	·5	·1	·4	13 ,,

These three series of experiments show the influence of making birds' plasma alkaline to the extent of NaOH $\left(\frac{N}{320}\right)$ and NaOH $\left(\frac{N}{160}\right)$ on its coagulation by varying quantities of kinase. It may be stated that the original plasma was quite stable. The coagulation times with varying quantities of alkali and a constant quantity of kinase are precisely similar to those obtained with peptone plasma. In fact this experiment reflects even more faithfully than the experiments with ferment on alkaline plasma the conditions which arise in peptone

plasma, since before coagulation can spontaneously occur in this plasma ferment must be produced from the prothrombin by the kinase and calcium salts within it. Before coagulation occurred in tubes (B) and (C) the condition of the fibrinogen was determined. In the case of (B) at the end of thirty minutes CO_2 was passed through a portion of it—coagulation occurred in two minutes. At the end of sixty minutes a portion of it was added to an excess of water—no precipitate of fibrin was produced. Thus in the case of the mixture in tube (B) neutralisation of the alkali after thirty minutes caused coagulation to occur in two minutes. But previous to this no formation of fibrin had occurred since even after sixty minutes dilution showed an absence of fibrin. From this it is evident that in the case of plasma to which alkali has been added the main reason for the delay in coagulation on adding kinase is the inhibitory action of the alkali on the production of fibrin ferment from prothrombin. And this is probably the main reason for the maintenance of the fluid state in peptone blood, the influence of the alkali on the coagulation of the fibrinogen by the ferment which is ultimately formed being of secondary importance to the delay in the production of the ferment by the kinase. In the case of experiment (C) although coagulation occurred only after a hundred minutes yet the passage of CO_2 through a portion of the liquid after sixty minutes produced a coagulum in thirty seconds.

From the close analogy which existed between the phenomena observed in the coagulation of peptone plasma and the coagulation of plasma to which alkali had been added by kinase and by fibrin ferment it appeared legitimate to assume that peptone plasma owed its stability to the excess of alkali contained in it. The assumption was the more justified by the previous experiments showing that there was no excess of anti-thrombin present in this plasma. The question naturally arose as to the quantity of alkali which must be present in plasma to inhibit the formation of ferment and to prevent this ferment when formed from coagulating the fibrinogen. Probably the nearest approximation to this value is given in the experiments above showing the coagulation of birds' plasma, to which alkali had been added, by varying quantities of kinase. In these results it may be seen that ·1 c.c. of kinase solution coagulated the normal plasma in $2\frac{1}{2}$ minutes, but required three hours to coagulate the same plasma to which sodium hydroxide to the extent of $\frac{N}{160}$ had been added. Therefore $NaOH\left(\frac{N}{160}\right)$ may be regarded as a measure of

the amount of alkali which must be added to blood when peptone is injected into the circulation of a fasting dog. But this alkali does not exist in a free state in the plasma. The greater part of it is taken up by the proteins present in the blood as the following experiment showing the influence of alkali on the coagulation of fibrinogen by fibrin ferment proves.

The influence of sodium hydroxide on the coagulation of fibrinogen by fibrin ferment.

(Fg—S)	Ff.	NaOH $\left(\frac{N}{80}\right)$	H_2O	Coag. time
2 c.c.	·1 c.c.	·0 c.c.	·7 c.c.	1 minute.
	·1	·5	·4	6 minutes.
	·1	·7	·2	45 ,, (D).

In (D) above after five minutes a portion was diluted with five volumes of water—no precipitate occurred but after the lapse of an additional five minutes the whole of the diluted fluid had coagulated. CO_2 was passed through a second portion of (D) after eight minutes—an instant coagulum was produced.

From the above figures it may be seen that NaOH $\left(\frac{N}{340}\right)$ produced a marked delay in the coagulation of fibrinogen by fibrin ferment. From a consideration of the avidity of alkalies for globulin it is probable that a part of the added sodium hydroxide combined with the fibrinogen and that not more than NaOH $\left(\frac{N}{500}\right)$ remained free in solution. Therefore it may be assumed that plasma to which sodium hydroxide has been added to the extent of $\frac{N}{160}$ (a quantity of alkali sufficient to prevent the coagulation of the plasma by small quantities of kinase or fibrin ferment) does not contain more than NaOH $\left(\frac{N}{500}\right)$ free alkali. This consideration renders it very doubtful whether any determinations of the alkalinity of peptone plasma would be sufficiently accurate to prove that this plasma was not more alkaline than normal plasma. At any rate the very small excess of free alkali required to be present would seem to be of little use in preserving the fluidity of peptone blood if the results of actual experiments on plasma to which alkali had been added *in vitro* were not at hand to enable comparisons to be made.

Peptone immunity.

The deduction that peptone blood owes its stability to the excess of alkali contained within it affords an easy explanation of the speedy restoration of the coagulable state when the blood is left in the animal, and the so-called peptone immunity results. The blood of an animal which has been made incoagulable by the injection of peptone usually regains its condition of coagulability about three hours after the injection has been made. This restoration of the power of coagulation is clearly due to the excretion of the excess of alkali from the blood into the tissues or urine. After the blood has become coagulable a second injection of peptone within the next 24 hours produces no effect on the coagulability of the blood (peptone immunity). Many experimenters (notably Delezenne[15]) have shown that the liver is the chief organ concerned in the production of that body, which, under the influence of the injected peptone, is excreted into the blood and produces the condition of non-coagulability. On the theory at present under discussion the intravenous injection of peptone into an animal causes the liver to excrete alkali into the blood. The blood in turn gets rid of this excess of alkali into the tissues and urine and so regains its normal coagulability. A second injection of peptone now finds the liver without any excess of alkali stored up in it. Consequently no more can be excreted into the blood, and no change in coagulability results. The lapse of 24 hours permits the liver to regain its normal quantity of alkali and so the peptone immunity passes off in this time. It is evident that if peptone blood owed its stability to a body such as anti-thrombin the phenomena observed in peptone immunity would be unique. Briefly stated then—the close analogy between the properties of peptone plasma and plasma to which alkali has been added justifies the assumption that peptone blood owes its stability to the excess of alkali contained within it. The very small amount of free alkali which requires to be present vitiates any argument which may be brought against this hypothesis on the ground that peptone blood is not more alkaline than normal blood. The speedy restoration of coagulability to peptone blood *in vivo* is due to the excretion of the excess of alkali into the urine and tissues, and peptone immunity is due to the absence from the liver of that alkali which the first peptone injection caused it to pour into the blood. The termination of peptone immunity depends upon the accession to the liver of a further supply of alkali similar to that which it previously excreted under the peptone stimulus.

HIRUDIN. (LEECH EXTRACT.)

In 1882, Haykraft discovered that leech extract inhibited the coagulation of blood. Haykraft[16] considered that the active principle was an anti-ferment. But at that time the present theory of the coagulation of blood had not been put forward and it was necessary to reinvestigate the subject in the light of this theory. Morawitz[17] considered the question from this point of view and also came to the conclusion that the active principle of leech extract was an anti-fibrin-ferment. In the following pages the actions of hirudin—the active principle of leech extract—on plasma, fibrinogen, fibrin ferment, and kinase are considered.

Hirudin was injected into the jugular vein of an anaesthetised rabbit and blood was obtained from the carotid artery. Non-coagulable plasma was obtained by centrifuging this blood. A small quantity of the blood was diluted with an equal volume of distilled water. This portion clotted after an hour—thus confirming an observation of Pekelharing[18].

Coagulation of hirudin plasma by kinase.

40 c.c. of plasma were diluted to 100 c.c. with distilled water. No precipitation or change took place in the plasma. The influence of varying amounts of kinase and hirudin on the coagulation of this plasma was determined.

	Plasma	(K.)	H_2O	Coag. time
(A)	2 c.c.	·1 c.c.	·9 c.c.	70 minutes.
		·2	·8	18 ,,
		·3	·7	6 ,,
		·4	·6	4½ ,,
		·5	·5	3½ ,,

The above figures give the coagulation times of the original hirudinised plasma on adding kinase to it. To the remainder of the plasma varying amounts of hirudin were added and the coagulation times on adding kinase were again determined.

·03 % hirudin = H.

	Plasma	(K.)	H_2O	(H.)	Coag. time
(B)	2 c.c.	·3 c.c.	·6 c.c.	·1 c.c.	13 minutes.
		·4	·5		7½ ,,
		·5	·4		4½ ,,
		·6	·3		3 ,,

	Plasma	(K.)	H_2O	(H.)	Coag. time
(C)	2 c.c.	·4 c.c.	·4 c.c.	·2 c.c.	9 minutes.
		·5	·3		5½ ,,
		·6	·2		4 ,,
		·8	·0		2½ ,,
(D)	2	·4	·3	·3	11 ,,
		·5	·2		7 ,,
		·6	·1		5 ,,
		·7	·0		3 ,,
(E)	2	·4	·2	·4	15 ,,
		·5	·1		9 ,,
		·6	·0		6 ,,
(F)	2	·5	·0	·5	12 ,,

In the series of experiments (A)—(F) the hirudin present in the plasma was increased uniformly by ·1 c.c. of ·03% hirudin to each 2 c.c. of plasma. From a comparison of the coagulation times of these specimens of plasma with varying amounts of kinase it seemed possible that a clue to the nature of hirudin might be obtained. Since the coagulation curves obtained on varying the amounts of kinase have no regular form an accurate comparison could only be obtained by determining how much kinase coagulated the various portions of hirudinised plasma in equal times. The following table gives this comparison.

Experiment	Amount of hirudin added *in vitro*	Amount of kinase added
A	Original plasma	·2 c.c. +
B	O. Pl + ·1 c.c. H	·3 c.c.
C	O. Pl + ·2 c.c. H	·4 c.c. −
D	O. Pl + ·3 c.c. H	·4 c.c.
E	O. Pl + ·4 c.c. H	·4 c.c. +
F	O. Pl + ·5 c.c. H	·5 c.c.

In all the above experiments the approximate time of coagulation was twelve minutes. The + sign indicates that from the series of experiments shown a little more than the indicated amount of kinase was required to produce coagulation in twelve minutes; and the − sign that a little less kinase was required. The conclusion drawn from the above table was that hirudin was an anti-kinase. Every addition of ·1 c.c. of ·03% hirudin to 2 c.c. of plasma required the addition of about ·05 c.c. of kinase to produce coagulation in the same time—in fact the kinase solution was just twice as potent as the hirudin solution as determined by their respective capacities to coagulate or prevent the coagulation of plasma. Such a conception of the nature of kinase

would agree with the obvious function of hirudin. The initial cause of the coagulation of blood is the excretion of kinase into it from some injured tissue. If then hirudin contained an anti-kinase, the kinase liberated from the tissues bitten by the leech would be neutralised and so coagulation of the withdrawn blood would not occur. But it is clear that if hirudin contained anti-kinase only, then it should have no effect on the coagulation of fibrinogen by fibrin ferment. The following experiments show the effect of hirudin on the coagulation of fibrinogen by fibrin ferment.

The influence of hirudin on the coagulation of fibrinogen by fibrin ferment.

·05 % hirudin = H.

I.	No hirudin.				
	(Fg—S)	Ff.	H_2O	H.	Coag. time
	2 c.c.	·1 c.c.	·9 c.c.	·0 c.c.	3 minutes.
		·2	·8		1½ ,,
		·3	·7		1 minute.
II.	·1 c.c. of hirudin.				
	2	·1	·8	·1	90 minutes.
		·2	·7		17 ,,
		·3	·6		8 ,,
III.	·2 c.c. hirudin.				
	2	·1	·7	·2	Partially in 16 hours.
		·2	·6		120 minutes.
		·3	·5		60 ,,
IV.	·3 c.c. hirudin.				
	2	·1	·6	·3	Partially in 16 hours.
		·2	·5		180 minutes.
		·3	·4		120 ,,

An analysis of the above results showed that the amounts of fibrin ferment required to coagulate the fibrinogen solutions to which varying amounts of hirudin had been added in two hours were:

I.	No hirudin.	About ·01 c.c. ferment.
II.	·1 c.c. hirudin.	About ·1 c.c. ,,
III.	·2 c.c. hirudin.	About ·2 c.c. ,,
IV.	·3 c.c. hirudin.	About ·3 c.c. ,,

The conclusion drawn from these figures was that hirudin contained an anti-fibrin-ferment. They showed that hirudin affected other factors concerned in the coagulation of blood beyond the kinase. But that the hirudin did not neutralise the ferment according to the law of

chemical equivalents was clear from the figures showing the times of coagulation of fibrinogen to which ·1 c.c. of hirudin had been added. From the general consideration of the results above it appeared that ·1 c.c. of hirudin was equivalent to ·1 c.c. of ferment. But in table II, we see that even with ·3 c.c. of ferment the addition of ·1 c.c. of hirudin extended the time of coagulation to eight minutes. If the ·1 c.c. of hirudin neutralised only ·1 c.c. of ferment the coagulation time should have been 1½ minutes. Similar results may be seen on examining tables III and IV.

The adsorption of hirudin by fibrinogen.

There was a further possibility that the action of the hirudin was not exerted on the kinase or ferment but upon the fibrinogen. This idea was put forward by Bodong[19], but has not yet been confirmed. Such a conception would explain the apparent anti-kinase and anti-fibrin-ferment nature of hirudin since its effect would be exerted on the ultimate cause of coagulation—the fibrinogen. To determine whether hirudin exerted its action on fibrinogen rather than on fibrin ferment or kinase the following experiment was done.

The activity of the fibrin ferment.

(Fg—S)	Ff.	H_2O	Coag. time
2 c.c.	·1 c.c.	·9 c.c.	1½ minutes.
	·2	·8	1 minute.

·2 c.c. of this ferment and ·2 c.c. of a ·1 % solution of hirudin were added simultaneously to the fibrinogen solution thus:

(Fg—S)	Ff.	H.	H_2O	Coag. time
2 c.c.	·2 c.c.	·2 c.c.	·6 c.c.	40 minutes.

This coagulation time was contrasted with the time taken by the same quantity of ferment to coagulate the same fibrinogen to which the hirudin had been added ten minutes previously. In this experiment the time of coagulation was ten minutes only. From this result it was clear that the preliminary incubation of the hirudin with the fibrinogen protected the subsequently added fibrin ferment from its action—in fact so far as the fibrin ferment was concerned the fibrinogen removed the hirudin from its sphere of action. This result was fully confirmed by adding the hirudin directly to the ferment solution and allowing the mixture to incubate at 30° C. for fifteen minutes before testing its activity on a fibrinogen solution.

The following mixture of ferment and hirudin was made:

1 c.c. Ff. + ·2 c.c. H. = Ff.H.

After fifteen minutes the ferment activity of this mixture was estimated.

	(Fg—S)	Ff. H.	H_2O	Coag. time
(X)	2 c.c.	·1 c.c.	·9 c.c.	15 minutes.
		·2	·8	12 ,,
		·4	·6	5 ,,

The simultaneous addition of ferment and hirudin to a fibrinogen solution in the same quantities as in experiment (X) above produced coagulation in two minutes. Therefore from these experiments it was evident that fibrinogen removed hirudin from the sphere of action of fibrin ferment, this action of the fibrinogen obscuring the anti-fibrin-ferment contained in the hirudin when the hirudin and ferment were simultaneously added to a fibrinogen solution. This result was diametrically opposite to that stated by Bodong as to the anti-coagulant action of leech extract. He stated that the delay in the coagulation of blood caused by adding hirudin to it was due to the hirudin altering the fibrinogen and so making it more resistant to the coagulation process.

The influence of hirudin on the generation of fibrin ferment from prothrombin.

From the above experiments showing the influence of hirudin on the coagulation of fibrinogen by fibrin ferment it appeared probable that the apparent anti-kinase action of this substance shown in the experiments on hirudinised rabbits' plasma was due to the anti-ferment contained in it. But such a conclusion was opposed to the experimental results obtained when determining the properties and functions of anti-fibrin-ferment contained in normal blood. It has been repeatedly pointed out that this normal anti-ferment is incapable of preventing coagulation in the presence of kinase. If then the anti-coagulant properties of hirudin were solely due to the anti-fibrin-ferment contained in it the properties of this anti-ferment would need to be very different to those of the anti-ferment contained in normal blood. A series of experiments were therefore made to determine whether hirudin contained an anti-kinase. In these experiments the influence of hirudin on the generation of fibrin ferment from prothrombin by kinase in the presence of calcium salts was determined.

Four solutions containing the same quantity of prothrombin but varying amounts of kinase and hirudin were made up and the activities of the resultant solutions determined after various times.

I. Kinase and $CaCl_2\left(\frac{N}{10}\right)$ alone.

Pro.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
2 c.c.	·1 c.c.	·2 c.c.	·7 c.c.	=	A.

(Fg—S)	A	H_2O	Coag. time	Tested after
2 c.c.	·1 c.c.	·9 c.c.	1 minute	15 minutes.

II. Hirudin : Kinase :: 1 : 1.

Pro.	H.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
2 c.c.	·1 c.c.	·1 c.c.	·2 c.c.	·6 c.c.	=	B.

(Fg—S)	B	H_2O	Coag. time	Tested after
2 c.c.	·1 c.c.	·9 c.c.	2 minutes	15 minutes.

III. Hirudin : Kinase :: 2 : 1.

Pro.	H.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
2 c.c.	·2 c.c.	·1 c.c.	·2 c.c.	·5 c.c.	=	C.

(Fg—S)	C	H_2O	Coag. time	Tested after
2 c.c.	·1 c.c.	·9 c.c.	120 minutes	15 minutes.
			55 ,,	30 ,,
			30 ,,	45 ,,
			20 ,,	60 ,,
			15 ,,	150 ,,
			10 ,,	270 ,,

IV. Hirudin : Kinase :: 2 : 2.

Pro.	H.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
2 c.c.	·2 c.c.	·2 c.c.	·2 c.c.	·4 c.c.	=	D.

(Fg—S)	D	H_2O	Coag. time	Tested after
2 c.c.	·1 c.c.	·9 c.c.	35 minutes	15 minutes.
			15 ,,	30 ,,
			10 ,,	45 ,,
			7 ,,	120 ,,

V. Hirudin : Kinase :: 2 : 3.

Pro.	H.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
2 c.c.	·2 c.c.	·3 c.c.	·2 c.c.	·3 c.c.	=	E.

(Fg—S)	E	H_2O	Coag. time	Tested after
2 c.c.	·1 c.c.	·9 c.c.	10 minutes	15 minutes.
			6 ,,	30 ,,
			3 ,,	45 ,,

From these results it was evident that hirudin contained a very energetic anti-body for kinase. The difficulties of an exact estimate of the quantity of anti-kinase contained in the hirudin were increased

by the fact that this substance also contains an anti-ferment so that the first formed ferment was neutralised. In experiment I, the amount of hirudin was too small to diminish to any marked degree the kinase and ferment. But in experiments II, III, IV, V, the action of the hirudin on the kinase and subsequently produced ferment caused the rate of production of an excess of ferment to be very slow and consequently the times of coagulation were correspondingly influenced. But that the main delay in all the above experiments was due to the anti-kinase action of the hirudin rather than to the anti-ferment contained in it may be seen from the rates of generation of ferment activity in experiments III, IV and V. In these experiments the amount of hirudin was constant, the kinase only being varied. Therefore the same amounts of ferment were neutralised in every case by the hirudin anti-ferment and the varying rates of production of fibrin ferment from the prothrombin depended only on the amount of kinase in excess of the anti-kinase contained in the hirudin. The following results show the comparatively small part played by the hirudin anti-ferment in the delay in the generation of ferment activity in this prothrombin solution.

The ferment was generated in the prothrombin solution by kinase and calcium chloride thus:

Pro.	K.	$CaCl_2\left(\frac{N}{10}\right)$	H_2O		
2 c.c.	·1 c.c.	·2 c.c.	·7 c.c.	=	Ff.

After 20 minutes ·2 c.c. of H. were added to the above solution. After an hour the activity of this hirudinised solution was such that it coagulated 3 c.c. of fibrinogen in 2½ minutes, *i.e.* the solution still contained a large amount of fibrin ferment in excess of the anti-ferment contained in the hirudin.

The results of all the above experiments prove that leech extract owes its anti-coagulating properties to two principles—an anti-kinase and an anti-ferment. Of these two principles the anti-kinase is the more potent and is probably more concerned in the preservation of the fluidity of blood to which hirudin has been added. But the large quantity of anti-ferment present in hirudin indicates that it also plays a part in the anti-coagulant action. The adsorption of hirudin by fibrinogen and the consequent removal of this hirudin from the action of ferment or kinase subsequently added is similar to the phenomena observed with fibrinogen and cobra venom.

Concluding Remarks.

All the experiments made to analyse the phenomena observed in the coagulation of blood have received an adequate explanation on the Morawitz-Fuld theory: that the coagulation of blood is ultimately due to the action of fibrin ferment on fibrinogen, and that this fibrin ferment does not circulate in the blood but is generated from prothrombin by the combined action of kinase and calcium salts.

But in the past few years papers have been published by Nolf[20] in which he has advanced a theory of the coagulation of blood of a nature somewhat similar to that previously put forward by Wooldridge and essentially different to that stated above. This theory, briefly stated, is that the coagulation of blood is due to the union of three colloids—leucothrombin, hepatothrombin and fibrinogen[1]. Leucothrombin is a proteolytic ferment derived from the leucocytes: hepatothrombin and fibrinogen are proteins derived from the liver. In vertebrates fibrin ferment plays no part in the coagulation process except that it may be produced when fibrin is formed. Also calcium salts are not essential but merely act as zymoplastic substances, *i.e.* assist coagulation.

The essential difference between this theory and that of Morawitz is that in the former theory fibrin ferment is of no importance whilst in the latter it is absolutely necessary to the coagulation process. The question then arises whether fibrin ferment is or is not an important factor in coagulation. Many experiments detailed in my previous paper were concerned with the properties of fibrin ferment and fibrinogen and with the ability of the properties observed to elucidate the mechanism of the coagulation of blood; and all the experiments given in this paper have been made on assumption that the Morawitz-Fuld theory of blood coagulation is essentially accurate. The capacity of this theory to explain all the phenomena observed affords very strong evidence of its truth. It is in the highest degree improbable that fibrin ferment which can be produced from blood in such a quantity that the amount generated from a definite volume of blood is able to coagulate thirty times that quantity of blood in less than a minute at 30° C. is a subsidiary factor in blood coagulation. The reason why fibrin ferment is practically absent from serum has been previously explained and it is only necessary to say that blood plasma contains a very considerable quantity of anti-fibrin-ferment which neutralises free fibrin ferment. No indication has

[1] These three colloids are also called thrombozyme, thrombogène and fibrinogène.

been observed in the coagulation of birds' plasma or fibrinogen that a proteolytic ferment is concerned in the process. Such a proteolytic ferment would manifest its presence by the occurrence of fibrinolysis of the clot. No such fibrinolysis occurs in coagula produced from stable birds' plasma or fibrinogen prepared from such plasma. Certainly fibrinolysis occurs in fibrinogen prepared from oxalated horse plasma. But in this plasma there has always been a breakdown of leucocytes and the occurrence of a powerful proteolytic ferment in leucocytes has long been recognised. The association of this ferment with the precipitated fibrinogen illustrates the property of proteolytic ferments in general to attach themselves to the substances on which they act. Also the fibrinolysis observed in coagula prepared from horse fibrinogen is largely due to the comparative solubility of this fibrin when first formed. The presence of an excess of salts may exert so powerful a solvent action on the first formed fibrin that the transference of fibrinogen into fibrin may not be evidenced by the appearance of a coagulum. The function of calcium in the blood coagulation process has been fully dealt with and need not be further discussed. Although Nolf considers that fibrin ferment is a secondary factor in blood coagulation yet he states that anti-fibrin-ferment occurs in peptone blood. It is difficult to appreciate the necessity for this substance on his theory of blood coagulation. Finally it may be stated that all Nolf's experimental results can be adequately explained on the Morawitz-Fuld theory of blood coagulation. But it would be difficult on Nolf's theory to account for the gradual production from a prothrombin solution of a substance which can coagulate blood in a few seconds, the rate of production of which may be controlled by adding varying quantities of tissue extract or calcium salts.

The substances whose properties have been examined in this paper afford an admirable illustration of the ways in which the coagulation of blood may be prevented or delayed. Briefly stated negative phase blood owes its fluidity to the absence of fibrinogen; cobra blood to the presence of an anti-kinase; peptone blood to the presence of an excess of alkali; and hirudin blood to the presence of an anti-kinase and an anti-ferment.

No experiments have been made on the one blood of clinical importance—the blood of a person suffering from haemophilia. Apparently the blood of such a person always coagulates. It therefore contains fibrinogen and prothrombin. The slow onset of coagulation in such blood may be due to: (*a*) the occurrence in it of a small quantity of

fibrinogen and a corresponding small quantity of prothrombin; or (*b*) a relative stability of the tissues so that kinase is only slowly liberated; or (*c*) the presence in it of an excess of alkali as in peptone blood. From a purely hypothetical point of view it is probable that haemophilic blood owes its stability to its alkalinity in the same way as peptone blood. It is also probable that birds' blood owes its comparative stability to its alkaline property. This alkaline property exerts its influence in several ways: it depresses the formation of ferment from prothrombin; it inhibits the action of the ferment on the fibrinogen; and it assists in the preservation of the corpuscles and so prevents the liberation of kinase. The plasma prepared from a bird is very alkaline compared with that obtained from a horse. In the latter case a ten fold dilution with distilled water produces a copious precipitate of globulin but in the former case such a procedure produces no trace of precipitate and a fair quantity of acetic acid must be added before precipitation occurs. In the case of plasma prepared from a goose the alkalinity played an obvious part in the preservation of the fluid state. The plasma did not coagulate in ten days but the fibrinogen prepared from the plasma coagulated spontaneously in two hours. Also the properties of peptone plasma are similar in many respects to those of birds' plasma, the main difference being that in the former case more kinase is usually present than in the latter case and correspondingly the excess of alkali needs to be greater to preserve the fluidity. If the blood of people suffering from haemophilia owes its stability to the same causes which operate in birds' blood, and generally in the blood of animals containing nucleated red blood corpuscles, the cases show an interesting reversion to type.

But whatever the ultimate cause of the stability of haemophilic blood may be, there is no doubt about the treatment which should be adopted to coagulate such blood. The coagulation of blood is due to the action of fibrin ferment on fibrinogen. Haemophilic blood contains fibrinogen. Therefore in order to coagulate it fibrin ferment should be applied. There are many arguments why tissue extracts (kinase) should not be used, the most important of which is that the injudicious use of kinase may lead to the formation of an extensive intravascular thrombosis. The small quantities of viper venom required to produce intravascular coagulation afford strong evidence of the dangers which may arise from the use of tissue extracts as a styptic. But with fibrin ferment the danger of intravascular coagulation is negligible owing to the presence in the blood of a large quantity of anti-fibrin-ferment which quickly neutralises free fibrin ferment. The safety of fibrin

ferment as a styptic is clear from the extreme difficulty of producing intravascular coagulation in an animal by the intravenous injection of fibrin ferment. A quantity of birds' fibrin ferment sufficient to coagulate two litres of blood in less than a minute was injected into an anaesthetised cat. After the injection the blood of the animal only showed a well marked positive phase of blood coagulation. In addition to the safety of fibrin ferment as a styptic its use may be recommended since a fairly strong solution of ferment produces coagulation in a few seconds.

The snake venoms used in the experiments described in this paper were given to me by Dr C. J. Martin. I wish to record my thanks to him for his generous gift.

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SUMMARY.

(1) The venoms of an Australian viper (*Notechis scutatus*) and an Indian viper (*Echis carinata*) contain principles which generate fibrin ferment from prothrombin. They therefore coagulate any fibrinogen solution to which they may be added. Consequently their active principles are physiologically equivalent to a mixture of tissue kinase and calcium salt and by analogy may be assumed to consist of substances containing kinase and calcium salt in union with one another. But owing to the paradoxical influence of potassium oxalate on these venoms (this salt largely increases their coagulating activities) the hypothesis is advanced that the active blood coagulating agents contained in them consist of pure kinase and that the calcium salt necessary to work in conjunction with this kinase is present in the prothrombin or fibrinogen solution adsorbed by the protein.

(2) The rapid injection of small quantities of these venoms into the vascular system of an animal results in extensive intravascular coagulation. This intravascular coagulation is due to the rapid production of fibrin ferment by the kinase contained in the injected venom and the consequent rapid production of fibrin.

(3) The slow injection of small quantities of these venoms results in the production of fluid blood (negative phase blood). Venom slowly injected causes a slow production of ferment and a correspondingly slow production of fibrin. The blood and tissue cells generally are capable of dealing with a slow formation of fibrin and consequently general

coagulation does not result. Negative phase blood owes its stability to the absence of fibrinogen. The disappearance of the negative phase is due to the restoration of fibrinogen to it.

(4) Cobra venom prevents the coagulation of blood by means of an anti-kinase. This anti-kinase is rapidly adsorbed by fibrinogen so that a preliminary incubation of the venom with a fibrinogen solution diminishes the capacity of the venom to neutralise any kinase subsequently added. Cobra venom does not contain an anti-fibrin-ferment.

(5) Peptone blood coagulates on adding to it birds' fibrin ferment, kinase, or an adequate quantity of fibrinogen. It does not contain a greater quantity of anti-fibrin-ferment than normal blood derived from the same animal. The similarity between the properties of peptone blood and blood to which alkali has been added indicates that the stability of peptone blood is due to the excretion of an excess of alkali into it by the liver under the influence of the injected peptone stimulus. On this theory the renewed capacity of peptone blood, left in the animal, to coagulate is due to the excretion of the excess of alkali from it into the urine and tissues; and peptone immunity is caused by the temporary disappearance from the liver of the excess of alkali which it excreted into the blood under the toxic peptone stimulus.

(6) Hirudin contains both an anti-kinase and an anti-fibrin-ferment. The former substance appears to be the more largely concerned in the preservation of the fluid state of hirudin blood. Hirudin is adsorbed by fibrinogen and so removed from the action of subsequently added ferment or kinase in a way similar to that exercised by fibrinogen on cobra venom.

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