STUDIES ON BLOOD COAGULATION

- by -

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THESIS

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"Studies on Blood Coagulation"

A review is presented of the various theories of blood coagulation from the time of A.Schmidt to the present day. The various factors of the coagulation process are discussed in detail. A section has been devoted to a review of anti-coagulants, and haemorrhagic diathesis from various causes is discussed in the light of recent findings.

In the preservation of blood, fibrin makes its appearance in the samples despite the presence of an anticoagulant. The experimental work, has been conducted on samples from the Blood Bank of the Biochemistry Department. It has been of an exploratory nature to determine the cause of fibrin precipitation and to find a means of completely preventing or at least delaying its appearance. The results of these investigations as well as the probable causes of fibrin precipitation are discussed.

INTRODUCTION

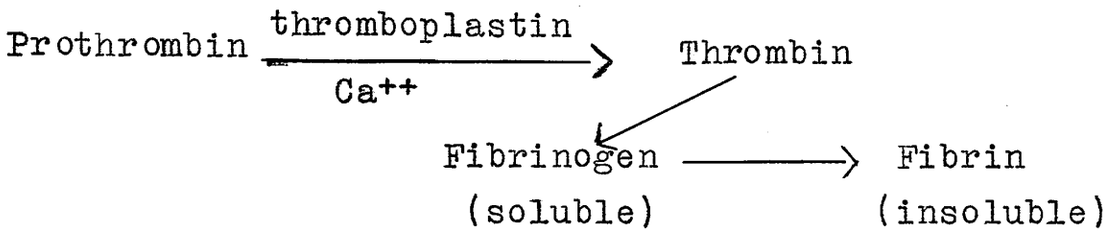
Blood is a tissue which consists of cellular elements suspended in a solution of salts and proteins. The cellular elements, known as the formed elements, are grouped into two general classes the erythrocytes and leucocytes, and apart from these we have minute cell particles known as platelets. The fluid medium, i.e. the solution of electrocytes and proteins, in which the cells are suspended is known as plasma.

In the body the blood remains fluid. If the tissues and blood vessels are injured or even if a minute capillary is damaged the blood escapes. It flows freely for a few minutes and subsequently solidifies or coagulates, and the clot which forms seals the injury. This process of coagulation is brought about by the precipitation of a plasma protein in the form of a jelly in which the various cellular elements are enmeshed. On standing a clear fluid is expressed from the clot and is known as serum. It is plasma from which fibrinogen has been removed.

It was not until the time of A. Schmidt (1862) that a systematic investigation of the clotting process was attempted. Hammarsten and Morawitz contributed much to the earlier knowledge of the problem. Even as early as 1893 Wooldridge pointed out that coagulation involves both physical and chemical factors. Among the physical factors are change in temperature, ionic equilibria and surface tension. The lowering of the surface tension produced by contact with a foreign surface such as skin, glass or cloth, is a most important factor in coagulation. In the investigation of the chemical factors of coagulation many workers have been led into erroneous interpretations. They have used material of varying degrees of purity and have worked under

poorly controlled conditions. The outcome of this has been the postulation of many contradictory theories.

At the present time the so-called "two-stage" theory of blood coagulation is generally accepted. Prothrombin is converted to thrombin by thromboplastin and calcium ions. Under the influence of thrombin fibrinogen is converted into fibrin. Represented schematically:



Until the introduction of blood banks some five years ago it was more or less accepted that a complete study of blood coagulation had been made. The preservation of human blood, however, gave rise to new problems. It was soon realized that the action of anticoagulants was not permanent and that fibrin precipitation soon took place under storage conditions. These difficulties encountered in storage together with improvements in the purification of the various coagulation factors gave new impetus to the study of the clotting process. The new investigations have resulted in the accumulation of quantitative data on the kinetics and mechanism of reaction.

THEORIES OF COAGULATION.

Although most investigators accept the two-stage theory of coagulation, some still disagree with this either in whole or in part.

Howell (1) believes that the activation of prothrombin takes place in the following way: thromboplastin replaces or neutralizes heparin, which is an antiprothrombin, and the free prothrombin then combines with calcium ions to form thrombin. This then converts fibrinogen into fibrin.

Nolf,(2) on the other hand, claims that thrombin is the result of coagulation rather than its cause. Thrombin, according to him, plays no part in coagulation, but is merely a by-product in the precipitation of fibrin. Nolf's theory has many supporters, but his fundamental thesis has been contradicted by the investigations of various workers. It has been shown that:

a) thrombin can be formed in the complete absence of fibrinogen.(3)(4)
b) factors which accelerate the formation of thrombin also accelerate coagulation.
c) coagulation follows a sudden liberation of thrombin.

More recently Mills and co-workers (5) have suggested two different mechanisms of blood coagulation. This is merely a revival of Wooldridge's old theory (6). They claim the existence of two substances, fibrinogen A and B respectively, as the active principles. The A factor is found in the tissues and is responsible for clotting in the case of wounds, etc; the B factor causes the initiation of clotting in the test tube. The latter factor does not exclude the formation of thrombin and therefore the two-stage clotting process. However, the A factor or tissue "fibrinogen", a s it is known by Mills, is capable of clotting a pure

fibrinogen solution, thus excluding the participation of thrombin. Smith, Warner and Brinkous (7) and others have shown that Mills' lung extract contained large amounts of preformed fibrinogen, prothrombin and thrombin since perfusion of the lung, prior to drying, was neglected.

Stuber, offering another mechanism of clotting, holds that the change in pH produced by glycolysis is responsible for the phenomenon. However, it should be noted that the blood is extremely well buffered and that the complete conversion of the 80 mgs. per cent. of glucose, normally present in the blood, to lactic acid would not cause a sufficient change in pH to precipitate fibrin. Moreover, Eagle and Baumberger (8) have shown that there is no demonstrable change in pH during clotting.

Hekma attempted to establish the thesis that the conversion of fibrinogen to fibrin is a reversible sol-gel transformation. He stated that fibrinogen is the alkaline hydrosol of fibrin and is regenerated when fibrin is dissolved in alkali. Others have shown that (9) an alkaline solution of fibrin is not fibrinogen since it cannot be made to clot by the action of a thrombin solution.

Since the various theories described above are mutually incompatible and are based upon false interpretations and assumptions and since the bulk of experimental evidence is opposed to them, it is quite justifiable to accept the two-stage clotting mechanism.

Normal plasma contains all the constituents necessary to cause the coagulation of blood. The various plasma factors will be discussed in great detail in a later section, but at the present a brief outline of the factors involved would not be amiss.

Prothrombin is a globulin present in circulating blood. Its formation occurs in the liver and is greatly influenced by vitamin K. Under the action of thromboplastin and calcium ion it is converted into thrombin.

Thromboplastin is the term which will be used throughout the discussion to denote the photphatid-protein complex present in platelets and tissues.

Calcium must be present to initiate clotting. There is a great deal of controversy about the form in which it must exist to bring about the formation of thrombin.

Thrombin is the factor responsible for the precipitation of fibrin in shed blood. It is formed by the interaction of the three former factors.

Fibrinogen is a soluble protein of the blood which is transformed into an insoluble form, fibrin, by the action of thrombin.

PROTHROMBIN.

Prothrombin is the protein, normally present in the blood, which,under the influence of thromboplastin and in the presence of calcium ions, is converted into thrombin. The last substance is the factor responsible for the conversion of soluble blood fibrinogen into insoluble fibrin.

Source.

The liver is now generally accepted as the site of prothrombin formation and vitamin K is believed to be a necessary principle for its manufacture.

It has been shown that hypoprothrombinemia results when the liver is damaged experimentally by specific poisons or partial hepatectomy (1,2). A deficiency of vitamin K in the diet of chicks has been shown to bring about the same change in the prothrombin level.(3,4).

The clinical counterpart of these conditions is observed in some pathological states where liver injury is prevalent, and in conditions of obstructive jaundice where the arrested bile flow results in impaired absorption and consequently a deficiency of vitamin K.(5,6,7).

Purification.

Prothrombin is associated with the globulin fraction of proteins although its exact nature has not been elucidated. The ease with which purified prothrombin disintegrates under a variety of conditions has discouraged many workers in their efforts to study this substance.

During the past year various workers have simplified the technique for the purification of prothrombin.(8,9). Seegers (9) prepares prothrombin by a ten-fold dilution of plasma with water

and adjustment of pH to 5.3 with one per cent. acetic acid. The precipitate is redissolved and $Mg(OH)_2$ suspension is added. In order to set free the prothrombin, adsorbed on the $Mg(OH)_2$ paste, CO_2 at 4 to 6 atmospheres is shaken with the paste in a metal chamber. After this elution, CO_2 is allowed to escape and $MgCO_3$ settles out. Subsequent dialysis removes any dissolved salts.

The dialysed prothrombin-containing eluate is then subjected to isoelectric fractionation. The first fraction (pH 5.6) usually contains inert protein, but at pH 5.3 a highly potent prothrombin is obtained. Even in this simplified procedure great care must be taken to use the proper amount of adsorbing substance, since an excess of $Mg(OH)_2$ will vitiate the results.

PROPERTIES.

Chemical.

Chemical analyses of the prothrombin prepared in this manner indicate that prothrombin is a carbohydrate-containing protein which would account for a nitrogen content lower than in most proteins. The activity of prothrombin is destroyed when treated with dilute solutions of nitrous acid at pH 5.5.

Solubility.

Prothrombin is very soluble both in water and normal saline at pH 7.0. In aqueous solutions low concentrations of a variety of salts, including those of Ba, Ca, Sr, and Al (approximately 0.005M) cause precipitation of prothrombin, but not of thrombin. If the salt concentration is then increased five-fold, the prothrombin precipitate redissolves. The isoelectric point of prothrombin has been determined by acidification with acetic acid. At pH 5.6 a precipitate appears which goes completely into solution at pH 3.9.

Inactivation.

Inactivation of prothrombin begins at pH 4.8 and is complete around 3.5. Prothrombin is also inactivated by heating; at 40°C there is some inactivation and this becomes complete at 60°C. Efforts to restore activity after this are unsuccessful, but in the 40° - 60° range some regeneration can be slowly brought about. (9,10).

Seegers supports the observation of Mellanby that prothrombin is non-dialysable through a collodion or cellulose acetate membrane.

PROTHROMBIN IN VARIOUS SPECIES.

Smith and co-workers (11) have studied the prothrombin content of various species by means of their two-stage prothrombin determination.(12).

They find that the prothrombin levels are far higher in mammals than in the lower vertebrates. In the dogfish plasma it is only 8 per cent. as high as in the plasma of the dog. In bony fishes, reptiles, and fowls, the values rise progressively, broadly according to their position in the evolutionary scale.

The following are the prothrombin values for various species:

guinea pig	...	185 units of prothrombin per cc.
man ...	...	295 " " " " "
rabbit	...	310 " " " " "
dog ...	...	350 " " " " "

Quick conducted similar studies by his one-stage prothrombin determination.(13,14). He made the observation that, on adding thromboplastin, rabbit plasma clots rapidly while human plasma clots slowly. He ignored the possibility that there might be a difference in the ease with which prothrombin is converted into thrombin in the

two species, and he assumed, instead, that there is a five-fold difference in the amount of prothrombin to account for the difference in clotting time. Smith has eliminated conversion time as a variable from his procedure and has shown that the prothrombin level is almost identical in the two species. He has, however, found that certain species,including man, show a slower rate of conversion of prothrombin into thrombin. Because of this sluggish conversion, these species are handicapped in the control of haemorrhage.

DETERMINATION OF PROTHROMBIN.

During recent years clinicians have found it necessary to determine the prothrombin level in the blood of patients suffering from obstructive jaundice and haemorrhagica neonatorum, and to institute treatment of these conditions in order to offset haemorrhage.

There are numerous methods available for a rough determination of prothrombin. The majority of these have the advantage of being performed on small amounts of capillary blood at the bedside.(15, 16,17). For the exact quantitative determination of prothrombin there are two principal methods available.(18,13,12). Any others are merely modifications.(19,20).

QUICK'S METHOD.

Quick employs a one-stage method.(18).

Procedure.

Nine volumes of blood obtained by venipuncture are mixed with one volume of 0.1 mol. sodium oxalate. Usually 4.5cc. of blood is a suitable amount.

In a small test tube 0.1 cc. of the plasma is mixed with 0.1 cc. of thromboplastin, prepared from rabbit brain, and recalcified with 0.1 cc. of 0.025 mol. calcium chloride. The time from the addition

of calcium to the formation of a clot is recorded on a stop-watch. The test is carried out in a water bath kept at 37.5°C. It is necessary to keep the mixture agitated by frequent gentle tiltings of the tube.

The clotting time can be directly converted to concentration of prothrombin by the following equation:

$$\text{Prothrombin concentration} = \frac{K}{CT. - a} \text{ per cent of normal}$$

CT = clotting time. K is a constant having the value 302, and A is a second constant with a value of 8.7.

SMITH'S METHOD.

Smith determines the prothrombin content by a two-stage procedure.(12).

The plasma is first treated with freshly prepared thrombin thereby converting the fibrinogen to fibrin. After a suitable period has elapsed the firm clot is removed and the plasma contains its original prothrombin, but is fibrinogen free.

Serial dilutions of this plasma are set up and $CaCl_2$ solution and thromboplastin are added. The standard unit of prothrombin is the amount which causes clotting in 15 seconds when a purified fibrinogen solution is added.

COMPARISON OF METHODS.

Smith's determination indicates the number of units of prothrombin present in a given sample of plasma. Quick expresses his results as the percentage of prothrombin present as compared with the normal.

Smith and co-workers maintain that this two-stage method should be used solely when research on prothrombin content is being carried out. In Quick's one-stage determination there are a number

of interfering factors since two processes are taking place concurrently. The conversion of prothrombin to thrombin is proceeding at the same time as the newly-formed thrombin is changing fibrinogen to fibrin. Quick's method also masks the slower conversion time of the prothrombin of various species. It was this fact that was responsible for his postulating that there is five times more prothrombin in rabbit than in human plasma.

Smith's method has admittedly the advantage of dissociation of the two clotting phases which gives specific information as to the rate of prothrombin conversion, and is independent of thromboplastin and anti-thrombin variables in the final clotting.

The two methods have been shown to give comparable results when studies of prothrombin levels have been made in cases of liver injury and obstructive jaundice.(21,22,6).

QUANTITATIVE INTERRELATIONSHIPS.

Prior to 1939 many workers had attempted to determine whether prothrombin and thromboplastin react to form thrombin, according to definite laws of proportion; however, their work had only a qualitative basis.(23,24).

With the development of new techniques in the preparation and purification of prothrombin and thromboplastin, Smith and co-workers have succeeded in obtaining potent preparations free of antithrombin and other interfering factors.(25).

By making use of these improved techniques they have been able to do work of a strictly quantitative nature. Their findings are that unlimited quantities of prothrombin are not converted into thrombin by a small amount of thromboplastin. Similarly, they observed that prothrombin definitely is limited in its capacity to

produce thrombin in the presence of large amounts of thromboplastin.

These observations finally laid to rest the old theory that thromboplastin acts enzymatically to convert unlimited amounts of prothrombin into thrombin. Thromboplastin is consumed when it reacts with prothrombin in the presence of calcium ions. Smith says that the utilization ratio of prothrombin and thromboplastin is about 261 to 1. A direct proportion has been found to exist between the amount of thrombin formed when a quantity of thromboplastin is added to an excess of prothrombin.

INACTIVATION OF PROTHROMBIN BY THROMBIN.

While Smith and co-workers were conducting the above experiments, they observed that when prothrombin, thromboplastin and calcium were incubated a substance was formed which inactivated prothrombin and subsequently caused a diminution in the yield of thrombin. They observed that this did not occur when prothrombin and thromboplastin were incubated in the absence of calcium. Nor did it occur when prothrombin and calcium were incubated without thromboplastin. The purified prothrombin and thromboplastin solutions were tested and found free of antithrombin.

The only conclusion that could be reached from these observations was that either the thrombin itself was the inhibiting factor, or some unknown substance had made its appearance in the mixture.

Through a number of experiments it was determined that thrombin was responsible for inhibiting prothrombin, its precursor.

RELATIONSHIP OF COMPLEMENT AND PROTHROMBIN.

In 1933 Fuchs suggested that the prothrombin of the blood and the complement of the immunological defense reactions were identical. Quick,(27) however, investigated this question and found that the conversion of prothrombin to thrombin has no effect on complement.

He came to the conclusion that prothrombin and complement are not identical, but appear to have some similarity in structure that makes them susceptible to the same inhibitory agents.

THE PROTHROMBIN CONTENT OF STORED BLOOD.

Various workers have investigated the prothrombin content of stored blood and their opinions are for the most part contradictory.

Rhoads and Panzer (28) using Quick's method of prothrombin determination noted that blood which was carefully preserved at 4°C lost its prothrombin within three days.

Lord and Pastore (29) using Smith's method found little diminution in the prothrombin content of stored blood.

Quick himself,(30) supports the observations of Rhoads and Panzer. He says that decalcified blood loses its prothrombin on storage and is inferior to fresh blood for transfusion of jaundiced patients.

Osterberg (21) studied the matter by using both Quick's and Smith's methods of prothrombin determination, and found a good correlation in the results. He states that there is a decrease in the prothrombin content of stored blood, but this does not take place as rapidly as Rhoads and Panzer state.

CALCIUM.

In the conventional theory of blood coagulation the presence of calcium ions is essential for the conversion of prothrombin to thrombin by the action of thromboplastin.

This fact has been admitted by all those who have interested themselves in studies of the coagulative process, but the actual role of calcium and its mode of action are still the subject of controversy.

O. Hammarsten (1875-77) noticed that non-clotting or incompletely clotted hydrocele fluids often showed the presence of fibrin if calcium chloride was added to them. J.G. Green(1887) found that calcium sulphate facilitated the clotting of diluted "salt plasma". Ringer and Saintsbury (1890) found that the delay in clotting produced by mixing blood with sodium or potassium citrate could be counteracted by the use of calcium, barium or strontium solutions.

Credit is due, however, to Arthus and Pagès (1) for conclusively demonstrating that calcium salts are essential for blood coagulation in vitro. They added to dog, ox and horse blood a) 0.1% of oxalate b) 0.15% fluorides c) 0.5% soaps of the alkali metals, and found clotting was prevented for several days. On the addition of $CaCl_2$ solution clotting immediately took place. They found that strontium, but not barium, could be substituted for calcium. Mellanby (2) has found that barium can replace calcium and that the older investigators had used too low a concentration.

C.A. Pekelharing (1891) discussed the role of calcium in relation to the other known factors of blood coagulation. In 1892 he published a monograph on "fibrin ferment" (thrombin) and

in it records the powerful anti-coagulant action of sodium citrate. He stated that "the affinity of lime for the citric acid" was responsible for preserving the fluidity of the blood.

Schmidt (1895) argued that the anticoagulant effect of sodium citrate was not due to a decalcifying effect, since precipitation of calcium citrate did not occur. A spirited battle raged between the two for several years until Sabbatini (1900-3) settled the controversy. He employed various salts which acted as anti-coagulants by inactivating the calcium factors and distinguished between those that caused calcium to precipitate out of solutions and those which did not. By his investigations he established the contention that calcium in the ionic state was necessary for coagulation.

AMOUNT OF ANTICOAGULANT NECESSARY TO INHIBIT CLOTTING.

Sabbatini stated that in vitro coagulation could be prevented by the use of three molecules of re-crystallized trisodium citrate for each atom of calcium present.

He found that four or five molecules of citrate were required to prevent clotting on intravenous injection into dogs. The necessity for using a larger amount was probably due to the oxidation of the citrate in vivo. The injection of such a large amount of citrate produced toxic effects in the dogs, but their death was described by Sabbatini as due to calcium deprivation.

Stassano and Daumas (1924) observed that a minimal coagulant concentration of calcium was between 13 and 21 mgm. per liter of blood. Lewisohn (1915-24) who pioneered in the clinical use of citrate for transfusions redetermined the minimal anti-coagulant concentration of sodium citrate to be 0.15 to 0.20 per cent.

Vines (3) (1921) pointed out that the calcium of blood existed in the combined and ionized state. He determined that the addition of oxalate, citrate or fluoride to normal blood in an amount chemically equivalent to the total calcium of the blood does not inhibit coagulation. He found that three times the equivalent of calcium must be added before clotting is inhibited. Loucks and Scott (4) (1929) confirmed Vines' observation that three or four times the amount of oxalate must be added to prevent clotting.

More recent confirmations of this fact are given by Quick(5) and Mellanby(6).

AMOUNT OF CALCIUM NECESSARY FOR CLOTTING.

It is established that the presence of calcium is required for the activation of prothrombin. Disregarding for the present the status of calcium in the reaction we turn to a quantitative consideration of its role.

Clinical and animal investigations have so far offered no grounds for believing low blood calcium to be a cause of increased coagulation time. Parathyroid tetany is a well-known condition associated with low serum calcium. Radvin and Morrison (7) report one patient with this condition whose serum calcium was 4.6 mg. per cent. and whose blood coagulation time was normal. Evidence to the contrary is cited by Kottman and Lidsky (8) who observed retarded coagulation time under similar conditions.

In cases of low serum Ca in dogs there has not been any evidence of prolonged clotting time.

Crane and Sanford (9) studied the effects of calcium variation on clotting time, and found that a practically constant clotting time resulted with concentrations of calcium from 5-20 mg.%. The

first marked prolongation of clotting time was produced with concentrations of 2.5 mg.% or less. These authors found that variations in plasma protein which are known to cause a variation in the proportion of diffusible, non-diffusible calcium, did not alter the relationship between total calcium and coagulation time. Mellanby (6) decided that 0.3 mg. per cent. calcium was required for the coagulation of fowl plasma in vitro by thromboplastin. Ransmier and McLean (10) investigated the relationship of calcium ion concentration to the coagulation of citrated plasma. They found that the minimal calcium ion concentration at which coagulation of diluted citrated plasma at pH 7.0-8.0 was observed to occur averaged 0.35 mM per liter for human plasma and 0.24 mM per liter for dog plasma. The minimal coagulation time for both human and dog plasma at pH 7.0 to 8.0 is approached above calcium ion concentrations of about 1.25 mM per liter.

Vines (3) pointed out that only 0.61 to 0.66 per cent, or one-seventeenth, of the available (total serum) calcium was needed to restore the normal coagulation time of finger-prick blood which had been prevented from clotting by the use of minimal amounts of oxalate, citrate, or fluoride. This occurred only if freshly oxalated blood was used. Within an hour the minimal calcium requirement was raised to 2.03 per cent. Vines claimed that this was due to "some process of firmer union with the oxalate" which took place during this time. Scott and Chamberlain (11) and Quick (5) have confirmed that clotting is practically instantaneous on the addition of very small amounts of calcium. There is, however, increased fibrin formation with increasing additions of calcium chloride.

EFFECT OF ADDING EXCESS CALCIUM.

Horne (1896) found that an excess of calcium inhibited clotting, an observation confirmed by Sabbatini. Later investigators, including Mellanby (1909) and Rettger (1909), also remarked on the anti-coagulant effects of an excess of calcium salts. Loucks and Scott (1929) found that Rettger's work regarding recalcification and the effects of excess addition of calcium was correct. More recently Quick (5) and Martin (16) have reported the depressing action of this ion.

DOES THROMBIN REQUIRE CALCIUM?

It has long been accepted that thrombin, once formed, is active in the presence of excess of oxalate and similar anti-coagulants. Forty years ago Hammarsten (12) failed to inactivate thrombin by oxalation and his classical experiment gave rise to the theory that ionized calcium is not necessary for the clotting process proper, i.e. the second phase of coagulation. More recently Eagle (13) has confirmed this view.

Contrary to the findings of these workers Loucks and Scott (4) tried a series of similar experiments and found that thrombin could be inactivated by means of oxalate. They stated that the phenomenon was due to the direct removal of calcium and was not a salt effect.

Ferguson (14) conducted an experiment wherein the progressive inactivation of thrombin on incubation with oxalate or citrate was shown. Tests made at various intervals from the time the thrombin mixture was a few seconds old until two hours old, invariably showed a) an immediate retarding effect of the anti-coagulant varying with the concentration; b) a progressive inactivation, confirming the work of Loucks and Scott.

On further investigation Ferguson (15),in a series of carefully controlled experiments on thrombin of various ages, came to the conclusion that an intermediary calcium complex existed during the process of coagulation.

For his experiments he used a)"fresh" thrombin (few minutes old), b)"ripe" thrombin(several hours old,) and,c) electrodialysed thrombin which had been subjected to oxalation and citration before testing clotting activity on prothrombin-free fibrinogen.

The results showed 1) prevention of thrombin formation, 2) progressive inactivation of "fresh" thrombin, 3) a characteristic immediate effect, the sole action seen in the case of "ripe" and electrodialysed thrombin.

This would indicate that a calcium-containing intermediary complex exists in the formation of thrombin. "Ripe" coagulant does not require the presence of calcium to convert fibrinogen to fibrin.

MOST RECENT THEORIES ON ROLE OF CALCIUM.

The latest theories on the role of calcium in blood coagulation are those of Martin (16) and Quick (11).

G.Martin in investigating the action of dicarboxylic acids in blood coagulation was merely completing the work of previous investigators. It was found that when the members of the oxalic acid series were administered in excess to normal rabbits there was a 100-300 per cent increase in the coagulation time. The administration of small amounts brought about a definite decrease in clotting time.

As the number of carbon atoms increased in the series so did the activity.

The same results could be produced by sodium fluoride, hence

the effect was not limited solely to oxalate. In vitro experiments gave the same results as in vivo experiments.

Martin stated that the first phase of coagulation was the one affected in this instance. He used heparinized blood to prove this point. He said that since heparin had a known anti-thrombin activity one would expect the second phase of coagulation to be inhibited. After the injection of heparin oxalic acid had no effect on coagulation time.

Martin explains the effects of the oxalate in terms of the mass action law.

An outline of the reactions involved follows:-

$$\text{Calcium Prothrombin} \rightleftharpoons Ca^{++}\text{Prothrombin} + \text{Oxalate} \rightleftharpoons \text{Calcium Oxalate}$$

$$\text{Thrombin} + \text{Fibrinogen} \xrightarrow{Ca^{++}} \text{Fibrin.}$$

Cephalin combines with and removes the anti-thrombin, heparin.

1. Add calcium ion in excess:

$$\text{Result} : \frac{(Ca^{++})(\text{Prothrombin})}{(\text{Ca Prothrombin})} > K$$

Therefore calcium ion and prothrombin combine to form calcium prothrombin until

$$\frac{(Ca^{++})\ (\text{Prothrombin})}{(\text{Ca Prothrombin})} = K$$

Leaving a minimum of free prothrombin for conversion to thrombin, and clotting times are prolonged.

2. Add small amount of oxalate ion:

$$\text{Result} : \frac{(Ca^{++}(\text{Oxalate}^{=})}{(\text{Ca Oxalate})} > K$$

Therefore calcium and oxalate ions combine to form calcium oxalate with the consequent withdrawal of calcium ions causing -

$$\frac{(Ca^{++})(\text{Prothrombin})}{(\text{Ca Prothrombin})} < K$$

Therefore calcium prothrombin dissociates into calcium and prothrombin, liberating prothrombin for conversion to thrombin and hastening coagulation.

3. Add large amount of oxalate:

$$\text{Result: } \frac{(Ca^{++})(\text{Oxalate}^{=})}{(\text{Ca Oxalate})} > K$$

Therefore calcium and oxalate ions combine to form calcium oxalate with the resultant removal of nearly all of the calcium ion. This results in complete dissociation of the calcium prothrombin combination, but the calcium ion concentration is too low to catalize the conversion of prothrombin to thrombin reaction.

Quick, also considers the mechanism as one of mass action but introduces one variation. Through his experimental work he reached the conclusion that ionized or free calcium does not take part in the conversion of prothrombin to thrombin. He found that three times the amount of oxalate required to precipitate the total calcium is necessary to prevent coagulation in the presence of an optimal concentration of thromboplastin. This finding was not new since Vines (3) Scott and Chamberlain (17) had observed this and Vines had concluded that free or ionized calcium does not enter into the coagulation mechanism.

Quick interprets his findings in the following way:

In blood Prothrombin Ca$\leftrightharpoons$Prothrombin$^{=}$ + Ca^{++}.

It is easy to understand why an equivalent amount of sodium oxalate does not inhibit coagulation for the insoluble calcium oxalate likewise dissociates:

$$\text{Ca oxalate} \leftrightharpoons Ca^{++} + \text{oxalate}^{=}.$$

Consequently, as long as there is a sufficient concentration of calcium ions, the prothrombin complex remains intact. From the law of mass action one knows that:

$$\frac{Ca^{++} \times \text{oxalate}^{=}}{\text{Ca oxalate}} = K_1 .$$

and

$$\frac{Ca^{++} \times \text{prothrombin}^{=}}{\text{Prothrombin Ca.}} = K_2.$$

an equilibrium is thus established between the two.

If an excess of oxalate is added calcium ions are depressed and at a certain concentration calcium is completely torn from the prothrombin molecule.

At this point the prothrombin is no longer able to react with thromboplastin and coagulation is inhibited.

According to Quick, calcium is already bound to prothrombin before it reacts with thromboplastin. Martin also reached the conclusion that prothrombin is a calcium compound. He, however, does not believe that this calcium prothrombin compound is converted directly to thrombin, but concludes that the compound is decalcified and ionized calcium acts upon the free prothrombin.

THROMBOPLASTIN.

Thromboplastin is the name now used for the phosphatid-protein complex responsible for the activation of prothrombin in the presence of calcium ions. This factor has in the past been known by various names and its nature has been the subject of much discussion and controversy. It appears to occur in most animal tissues, from which it is released whenever a wound is produced, thereby giving rise to extra-vascular clotting. The presence of this substance in blood platelets is most likely the cause of intravascular coagulation when the endothelium is damaged or inflamed.

In 1892 Alex Schmidt (1) discovered cellular products which were soluble in alcohol. These cell extracts contained a thermo-stable factor which he considered essential in the activation of prothrombin. The activator was called a "zymoplastic substance".

A few years later Morowitz (2) found that a similar activity was possessed by the aqueous extracts of some tissues. He considered that this substance produced its action enzymatically and consequently termed it "thrombokinase". Morowitz emphasized that this activator substance could not clot purified fibrinogen solution in the absence of prothrombin and calcium.

Fuld and Spiro (3) isolated a similar substance which they called "cytozyme". Bordet later used this term for his alcohol-soluble platelet product.

Howell (4), in his earlier work, suggested the term which is now in general use, "thromboplastin".

Nolf (5) made extracts of platelets and leucocytes and found them effective in initiating clotting. He coined the term, "thrombozyme".

A further complication was added to the already confused nomenclature when Wooldridge (6) postulated the existence of an entirely new substance. He claimed that the tissues contained a factor, different from the one found in the blood, which clotted fibrinogen directly. This substance, which acted independently of thrombin, was known as "tissue fibrinogen".

NATURE OF THROMBOPLASTIN.

The exact nature of thromboplastin was not appreciated by earlier workers, although it was generally thought to be a phospholipid. In 1893 Wooldridge isolated a phosphatide which he termed "lecithin" according to the nomenclature in use at that time. His experiments foreshadowed much of our knowledge concerning the role of lipoids in blood coagulation.

About 1910 simultaneous investigations were instituted by Zak, Bordet, Howell and McLean. The active substance appeared to be a phospholipid, and according to Howell and McLean, was "cephalin". Howell, however, postulated that in the tissues thromboplastin probably existed as a phosphatide-protein complex. In the light of contemporary investigations the phosphatide-protein nature of this substance has been confirmed. (7)

It has long been known that aqueous tissue extracts are much more potent thromboplastic agents than purified cephalin. (8) The earliest workers, principally Wooldridge, already considered this substance as a phosphatide-protein complex.

Cohen and Chargaff (7) recently have studied this subject in order to determine to which of the following factors the increased activity of the phosphatide-protein complex is due: 1) the presence of a particularly active cephalin, 2) orientation of the cephalin molecules on the surface of a specific protein, 3) the activity of the phosphatide-free protein component.

Chargaff and Cohen attempted to isolate the phospholipid from the protein complex. After many attempts at extraction by means of basic dyes, ether and chloroform, they finally succeeded in isolating the phosphatides by continuous extraction with boiling alcohol-ether. By this method all but 5 per cent. of the phosphorous of the thromboplastic protein was accounted for. The isolated phospholipid did not possess thromboplastic activity greater than that usually exhibited by an active cephalin preparation.

The protein was liberated from the lipid complex by making use of the reaction between protamines and phosphatide. A solution of the isolated protein in physiological saline was found to be without thromboplastic activity. Chemical analysis showed that it was entirely free of phosphorous. By reuniting the isolated fractions of the phospholipid-protein complex it was hoped that the thromboplastic activity would be regenerated, but such was not the case.

By means of electrophoretic experiments Chargaff and Cohen investigated the nature of the bond existing in these lipoproteins. From their results they concluded that a simple salt-like complex does not exist between the protein and phosphlipid, nor is the lipid present merely as an impurity.

Immunological experiments using the thromboplastic protein were conducted on rabbits. It was found that the thromboplastic protein was capable of producing antibodies whose specificity rested entirely with the protein moiety. The complex formed by the thromboplastic protein and its antibody was more active than the free antigen in producing coagulation.

REACTION WITH HEPARIN.

Chargaff and Cohen (10) have found that on treating the thromboplastic protein from lung with heparin the phospholipid is displaced and a heparin-protein complex results. This complex possesses marked anticoagulant properties. The activity of heparin as an anticoagulant will be discussed later, but it should be noted at this point that the protein entering the complex is not identical with the serum albumin fraction which complements the action of heparin as an antiprothrombin and antithrombin.

IS THROMBOPLASTIN AN ENZYME?

Eagle (11) has stated that the amount of thrombin generated from a mixture of prothrombin, calcium and platelet or tissue extract is independent of the amount of platelets above a certain minimum. An increase of platelets or tissue extract above this minimum amount serves merely to accelerate the rate of thrombin formation, the amount of thrombin formed being strictly dependent on the amount of prothrombin present. This would indicate that thromboplastin acted enzymatically to produce thrombin. The term thrombokinase in place of thromboplastin has been used by various authors to denote enzymic nature.

More recently, and chiefly due to the efforts of Mertz, Seegers, Smith (12-13) the enzyme theory of thromboplastic activity has been disproven. These authors have found that a definite quantitative interrelationship exists between various factors involved in the formation of thrombin. Since thromboplastin is consumed and disappears in the formation of thrombin it is evident that unlimited quantities of prothrombin are not converted into thrombin by the presence of a small amount of thromboplastin. A direct proportion exists between the quantity of thromboplastin added to an excess of prothrombin and the thrombin formed. Hence thromboplastin does

not appear to be an enzyme.

RELATION TO PROTEOLYTIC ENZYMES.

In 1909 Mellanby (14) reported that the venoms of Australian and Egyptian vipers as well as cobra venom were active in producing blood coagulation. Further investigation was conducted by various workers, among them Eagle (15), who studied the mode of action of these proteolytic enzyme-containing venoms on blood coagulation. A further observation of Eagle's (16) was that crude or crystalline trypsin could cause coagulation of citrated plasma. Eagle stated that the action of trypsin was independent of calcium or platelets and that it directly activated prothrombin.

He further suggested that since trypsin, in activating prothrombin, has qualitatively the same effect as a mixture of calcium and platelets, the latter must constitute a proteolytic enzyme similar to trypsin.

Barnes (20) has studied the enzymes present in white blood cells. Among them he identified cathepsin, trypsin, erepsin, amylase, lipase, lipozyme and adenosinase. Dialysed trypsin produced the same effect as did the crystalline trypsin of Northrop and Kunitz. Northrop and Kunitz (17), however, claimed that a trace of ionized calcium was necessary to produce this effect. Ferguson and Erickson (18) reinvestigated the question and found that trypsin, although capable of acting directly upon a prothrombin solution, was, nevertheless, more active in the presence of added calcium salt and that it was inhibited by excess citrate.

Ferguson has rather a unique idea on the mode of action of trypsin. He claims that his prothrombin solutions contain 8 to 30 mgs. per cent. of phospholipid, approximately 40 to 60 per cent. of which is cephalin. This amount of cephalin added in the free

form to a solution would be highly thromboplastic; however, it has been shown that this bound cephalin is inactive. The addition of calcium alone to a prothrombin solution fails to yield thrombin. The phospholipid is firmly held in combination by the prothrombin. Attempts to isolate the cephalin by extraction, ultrafiltration and dehydration, have been unsuccessful.

Ferguson has suggested that it is the function of the proteolytic enzyme to split off the cephalin from its combination and make it "available" for the activation of prothrombin. He writes: "Briefly their action consists in the mobilization of cephalin and calcium at the colloidal surface of the protein (prothrombin) substrate, where the close juxtaposition of the three components permits of the formation of thrombin via an 'intermediary' prothrombin-calcium-cephalin complex or compound".

MODE OF ACTION OF THROMBOPLASTIN.

There are two theories regarding the manner in which prothrombin is activated by thromboplastin. According to the conventional theory of blood coagulation which has previously been stated, prothrombin is converted into thrombin by the combined action of thromboplastin and the presence of calcium ions. The method by which this change is brought about is still the subject of investigation. The majority of workers are in agreement that thrombin results from a chemical combination of prothrombin, calcium and cephalin or thromboplastin. Ferguson has recently advanced the idea that an "intermediary complex", which is a combination of the three, exists in the formation of thrombin. "Ripe" thrombin (several hours old), however, acts independently of the presence of calcium.

Howell is the chief advocate of the theory that the function of thromboplastin is to neutralize heparin. According to him prothrombin exists in the blood stream in combination with heparin and it is the function of heparin to act as an antiprothrombase by keeping it in combination. When cephalin or thromboplastin is added to blood it neutralizes the action of heparin and prothrombin is liberated from the complex. Calcium ions present in the blood combine with the free prothrombin to produce thrombin. In his opinion thrombin is merely the combination of prothrombin and calcium.

Platelets, which are closely connected with the thromboplastin mechanism, are formed elements existing in the circulating blood. There is much controversy about their source, but the general opinion is that they are the pinched-off excresences of megarcaryocytes, the giant cells of the bone marrow.

Platelets are credited with two functions in the coagulation process. Platelet extracts were the first known thromboplastic agents of great potency. That they contain a substance necessary for coagulation has been demonstrated by Cramer and Pringle (21). They carefully collected blood in order to avoid undue contamination with tissue juice. This blood was centrifuged and the supernatant plasma passed through a Berkefeld filter, from which a platelet-free plasma resulted. The addition of calcium usually failed to induce coagulation. It could, however, be promptly brought about by addition of platelet extract. On several instances clotting took place when calcium alone was added. This, they thought, resulted from contamination with tissue juice or platelet breakdown with the liberation of sufficient extract to bring about prothrombin activation.

Tocantins (22) has studied the relation of platelets to the physical properties of blood clots. He ascribes to them a special function in the clotting process. Intact platelets converge towards the newly-formed fibrin needles and adhere to them to form large knots at their intersections. In this way they render the clot more rigid, firm and elastic, and bring about the phenomenon of synergesis or clot retraction.

THROMBIN.

Thrombin is the factor responsible for the conversion of fibrinogen into fibrin in the second stage of blood coagulation.. It has been stated elsewhere that some investigators choose to disregard the action of thrombin, crediting the effects it produces to some other factor, physical or chemical.

It has long been known that if fresh blood is allowed to clot and the serum is immediately expressed it contains a powerful thrombin which, when added in small amounts to oxalated plasma, will cause clot formation within a few seconds. It should be noted that fresh serum is used to produce this effect. If the serum is allowed to stand, even for thirty minutes, there is a rapid diminution and final disappearance of the thrombin formed. The disappearance of thrombin is due to the presence of an antithrombin in the serum. This point will be discussed later.

NATURE OF THROMBIN.

The nature of thrombin has been the subject of much controversy. Bordet (1), Fischer (2) and Mills (3), among other workers, claim that thrombin is formed by the combination of prothrombin, thromboplastin and calcium ions. Howell (4), who is one of the main dissenters against this theory, claims that thrombin is merely a prothrombin-calcium complex.

Ferguson (5) has investigated the problem and the evidence adduced points to a calcium-containing intermediary complex in the formation of thrombin. Here, as in previous cases,it has been found that the best method of studying the function and properties of a substance is to prepare it in a purified form. Purification methods have been published by Mellanby (6), Eagle (7), Seegers,Brinkous,Smith and Warner (8),Seegers (9), and Astrup and Darling (10).

Seegers (9) has prepared thrombin in a very pure form and has studied the substance quantitatively.

PREPARATION OF THROMBIN FROM PROTHROMBIN.

The preparation of prothrombin has been treated elsewhere. The conversion of prothrombin to thrombin is carried out in a 0.9% NaCl solution containing 0.15% $Ca(NO_3)_2$. The concentration of prothrombin can be allowed to vary within wide limits, but as a rule the final mixture is made up to contain 2000 to 7000 units of prothrombin per cc.

Thromboplastin, derived from lung, is consumed, apparently in stoichiometric quantities (11) during thrombin formation. Special precautions have been taken during preparation to exclude the presence of antithrombin, which would interfere with the reaction. An imidazole buffer is used and pH is adjusted to 7.3.

Thrombin formation occurs rapidly, but an hour is allowed for it to come to completion.

In order to eliminate electrolytes and to denature some of the protein impurities, the thrombin preparation is precipitated with acetone, dried with acetone and ether, and redissolved in water. (Electrodialysis would accomplish the same effect). Acetic acid is used to bring the aqueous solution to pH 5.3-5.0. A precipitate is formed containing inert protein together with some adsorbed thrombin, but most of the highly purified thrombin still remains in solution.

The thrombin can be obtained in the dried form by acetone precipitation, or by distilling off the water at or below room temperature.

The potency of the thrombin is such that less than 0.006 gamma will eventually clot 0.3 cc. of purified fibrinogen solution.

Normal human plasma contains about 325 units of prothrombin per cc., hence its potential thrombin content is less than 37 mgs. per 100 cc.

PROPERTIES OF THROMBIN.

Solubility.

Thrombin is soluble in water and in normal saline at pH.7. Unlike prothrombin it is not precipitated by low concentrations of salts. Its isoelectric point is somewhat lower than that of prothrombin. The precipitation range is pH.5.1 to 3.4.

Inactivation.

When a saline solution of thrombin is treated with acid inactivation starts at pH 4.1. This inactivation is slowly reversible as far as pH 3.5, but beyond this it is irreversible.

When treated with alkali some inactivation is shown beyond pH 10, and this becomes very marked beyond pH 11.

When an aqueous solution of thrombin is subjected to heating for thirty minutes at 40°C there is some inactivation. At 60°C the inactivation is almost complete.

Diffusibility.

Mellanby (6) reported that thrombin was diffusible through cellophane. Seegers, however, found it to be non-diffusible through cellulose acetate membranes (Visking casings). The discrepancy between the two reports is most likely due to a difference in the quality of the membranes used. The fact that thrombin is non-dialysable is an indication that it is rather a large molecule.

Chemical Analysis.

Seegers reports that thrombin is a carbohydrate-containing protein. Chemical analysis of this protein shows the following per cent. composition.

N. 13.23; C. 46.37; H. 7.35; ash 3.98.

Sulphur is also present, but further data are not available.

As with prothrombin, the activity of the thrombin preparation is dependent upon the integrity of the α-amino groups, since dilute nitrous acid destroys its activity.

Nolf (12) and Mills (13) report that thrombin preparations dissolve fibrin formed during coagulation. Seegers claims that his purified thrombin preparations do not exhibit fibrinolytic activity, and that the fibrinolytic enzyme is a distinct substance which can be eliminated by purification.

INACTIVATING ACTION OF THROMBIN ON PROTHROMBIN.

Smith and co-workers (14) observed that solutions of purified thrombin contain a substance which reacts slowly with prothrombin, causing inactivation of the latter. It has been determined that the destructive agent is not present in the calcium, prothrombin or thromboplastin solutions from which the thrombin is generated. The destructive agent is heat-labile, and the evidence points to its identity with thrombin. In destroying prothrombin present in a solution the thrombin content remains unchanged. This indicates that thrombin may act enzymatically in producing inactivation of prothrombin.

QUANTITATIVE INTERRELATIONSHIPS IN THROMBIN FORMATION.

Eagle (7) when studying the role of prothrombin and platelets in the formation of thrombin concluded that the amount of thrombin ultimately formed in a mixture of prothrombin, calcium and platelets is independent of the platelet (cephalin) and calcium used beyond a certain effective minimal concentration, and depends primarily upon the amount of prothrombin used. An increase in platelets and calcium beyond the minimum accelerated the rate of thrombin formation, but not the final amount.

On the other hand, Smith and co-workers (11) have shown that not only the rate of thrombin formation is affected by the quantity of thromboplastin used, but the quantity of thrombin produced from an excess of prothrombin, is directly proportional to the quantity of thromboplastin added to the reaction mixture.

This sharply limited capacity of thromboplastin to produce thrombin is proof that thromboplastin is consumed in the reaction. This evidence eliminates support for the old concept that thromboplastin is an enzyme.

IS THROMBIN AN ENZYME?

Eagle has discussed this problem at length(15) and in his opinion the bulk of evidence appears to indicate that thrombin is a proteolytic enzyme. The main arguments he presents are briefly outlined below:

1. An enzyme is not significantly destroyed during a reaction it accelerates. It has been found that the amount of thrombin is unchanged in a solution until the moment when fibrin is formed, when there is a decrease. This disappearance of thrombin is most likely due to its adsorption on the fibrin clot. It has been found that if a great excess of thrombin is present in a solution,more than a hundred times the amount necessary to cause clotting, disappears from solution. Further evidence for the adsorption on fibrin is Howell's finding that free thrombin can be recovered from dry fibrin.
2. The kinetics of the fibrinogen-thrombin reaction do not follow any simple equation. Similarly the

existence of a temperature coefficient and optimal pH indicate an enzymatic reaction.

3. Thrombin coagulates at least 2000 times its weight of fibrin, a circumstance which strongly suggests an enzyme reaction.
4. Eagle reports that papain can clot a fibrinogen solution over a limited range of concentrations.(16)
5. Certain snake venoms containing proteolytic enzymes also have the ability to clot fibrinogen solutions.

In the aggregate these facts constitute strong evidence for the enzymic nature of thrombin.

The evidence against the enzymic nature of thrombin is rather weak according to Eagle. He criticizes previous work of a quantitative nature which showed stoichrometric relationship between fibrinogen and thrombin. Rettger (1909) and Howell (1910) did not appreciate the presence of complicating impurities. Much work remains to be done to settle this controversy satisfactorily. The advent of highly purified preparations promises a successful solution of the difficulties. Ferguson reports that clotting time in fibrinogen thrombin mixtures is a linear function of the thrombin concentration. This would be evidence against the enzymic nature of thrombin.

ANTITHROMBIN.

The fact that there is a diminution and finally a disappearance of thrombin from fresh serum over a period of thirty minutes has been attributed to the action of an antithrombin. If the inactivated serum is added to a fresh thrombin solution, the latter is also inactivated. The nature of the antithrombin substance is unknown, but there is every reason to believe that it is present in the circulating blood.

Howell and Holt (17) isolated heparin from liver, and apart from ascribing to it an antiprothrombic role, also stated that it formed an antithrombin through its combination with some plasma precursor.

The role of heparin in destroying thrombin has received general acceptance and has been confirmed by Mellanby (18) and Quick (19).

The action of heparin will be discussed under anticoagulqnts since it is debatable whether it is of physiological significance in preventing intravascular clotting.

ANTICOAGULANTS.

When fresh blood is drawn into a vessel it loses its fluidity and within a few minutes it becomes a solid jell. This process of coagulation can be prevented and the blood preserved in a fluid state by the use of anticoagulants. These substances act by inhibiting one or more of the factors which function in blood clotting. According to the manner in which anticoagulants act they are divided into two classes:

1) So-called "decalcifying" agents which interfere with the calcium mechanism thus preventing the first stage of clotting, namely the conversion of prothrombin to thrombin.
2) Anti-prothrombins and anti-thrombins which interfere with their respective clotting factors.

Some of these substances, particularly the physiological ones, have been studied more than others. Other agents influence coagulation only when used in vivo, and although lacking practical applications, nevertheless, arouse speculation as to their mode of action.

Decalcifying Agents.

Among the decalcifying agents are sodium and potassium oxalate, citrate and fluoride. Oxalates form an insoluble salt with calcium while the other two are supposed to remove calcium by reducing it to a non-ionized state.

A brief history of the use of decalcifying substances has been given in the discussion of the role of calcium in coagulation. Arthus and Pagès(1890) conclusively demonstrated that oxalates, fluorides, and soaps of alkaline metals are effective in preventing coagulation, and that their action can be inhibited by the addition of calcium salts. In 1891 Pekelharing, who was the first to employ citrates, explained their action

on the basis of "the affinity of lime for the citric acid". The studies of Sabbatini (1901-3) on numerous anticoagulants and their division into those which precipitate calcium and those that do not, brought an end to a long-standing controversy.

Other Anticoagulants.

Dyes.

In the course of their work on capillary permeability Rous, Gilding and Smith (1) discovered that Chicago Blue 6 B, possesses a marked anticoagulant activity. In the following year Brambell and Parkes (2) successfully used this dye in perfusion experiments. Huggett and Silman (3) showed that the English equivalent of this dye - chlorazol Sky Blue FF - inhibits coagulation by acting as an anti-thromboplastin. A more intensive study on this subject was carried on by Huggett and Rowe (4) who found that a number of azo dyes can act as inhibitors. The best among these are dyes prepared by coupling tetrazotized diamines with aminoaphthol sulphonic acid.

It is interesting to note that many of the dyes possessing other pharmacological activity can also inhibit clotting. Germanin or Bayer 205, though not a diazo compound, has been shown by Stuber and Lang to possess anticoagulant properties. "Novirudin" and "Liquoid" are trade names of other compounds.

Hirudin, an anticoagulant derived from the salivary gland of the medicinal leech, acts as an antithrombin. Mellanby considers it to be an antithromboplastin.

Transfusol, the sodium salt of polyacetylene dioxysulphonic acid, derived from a resin has been used clinically as an anticoagulant in numerous cases, and apparently with success. This substance is an antithrombin.(6).

Sodium Thiosulphate (7) (8) is said to be effective at a lower

concentration than most anticoagulants.

Proteoses, Peptone. It has long been known that the injection of toxic doses of proteose and peptone render blood incoagulable. They were believed to act through depletion of fibrinogen. Recent studies show that the high antithrombin activity of "peptone" blood is due to the high content of heparin. The heparin is presumably released from the liver since injection of peptone fails to produce antithrombic activity after hepatectomy.

Heparin. The best known and most physiological anticoagulant of the antithrombin group is heparin. Its action has been studied extensively and has been the subject of much controversy. Heparin was isolated by Howell and Holt (9) in 1918 from dog's liver and other tissues. Their first preparations were somewhat crude, but they subsequently obtained them in purer form (10). Howell and Holt found that heparin prevents coagulation both in vivo and in vitro. Their well-known theory of blood coagulation is based on their interpretation of its mode of action.

Howell claims that in normal plasma prothrombin exists in combination with heparin and that thromboplastin liberates it from its complex. He also states that when heparin is added to blood or plasma it causes the formation of an antithrombin. Heparin itself is not effective as an antithrombin, but activates some precursor in the plasma. Howell calls the precursor pro-antithrombin. He identifies it as a substance which is destroyed rapidly by heating to 70°C and more slowly at 60°C. Pro-antithrombin can be precipitated unaltered from blood plasma by acetic acid or half-saturated ammonium sulphate. Howell thinks that the chief role of heparin in inhibiting coagulation is its action as anti-prothrombin, and that heparin plays a physiological role in

preventing coagulation in the circulating blood. He also thinks that pro-antithrombin constitutes a protection against the presence of small amounts of thrombin and at the same time prevents the conversion of prothrombin into thrombin.

In 1934 Mellanby (11) considered Howell's theory of the action of heparin in the light of his own experimental data. He confirmed Howell's observations that heparin is an antithrombin, but states that this possibly is due to some condition of the plasma rather than to the presence of a precursor. By dialysing oxalated plasma against distilled water, the activity of heparin is destroyed. Mellanby postulated that the presence of neutral salts, particularly NaCl, is necessary in the action of heparin.

With regard to Howell's statement that heparin is an anti-prothrombin Mellanby claims that it seemed to behave rather as an antithromboplastin than antiprothrombin. He found that heparin did not prevent the activation of a purified prothrombin solution by thromboplastin and calcium chloride. Mellanby's observations offer no support to Howell's theory of coagulation and he states that the high content of heparin in body tissues appears to indicate that its function is to prevent localized thrombosis in the vicinity of damaged tissue.

Quick (12) investigating the action of heparin on plasma and its relation to thromboplastin concluded that heparin is not strictly an antithrombin. He showed the compatability of Howell's and Mellanby's observations on the antithrombic activity of heparin. Quick stated that heparin reacts with some constituent of the plasma to form a true antithrombin, and that neutral salts are essential in this reaction. Heparin is inactive in plasma which has been dialysed against distilled water.

Quick (13) studied the nature of the normal antithrombin in the blood and its relation to heparin. He found that the antithrombin is an albumin and that its antithrombic power is intensified by heparin. His view as to its action is as follows: Fibrinogen, having a greater affinity for thrombin than albumin is converted into fibrin. Inactivation of thrombin does not take place until all the fibrin has formed. Addition of heparin increases the affinity of albumin for thrombin and the latter combines with the heparin-albumin complex without reacting with the fibrinogen.

Quick found that thromboplastin does not neutralize heparin and hence disagrees with Howell's view that heparin is an antiprothrombin.

More recently Smith and co-workers (14) have given further evidence of the dual role of heparin as described by Howell. Apart from the function of heparin as an antithrombin they found that it can act also as an antiprothrombin. The inhibition of thrombin formation requires the presence of an accessory factor present in plasma. Neither heparin nor the plasma factor alone is capable of preventing thrombin formation. The plasma factor is non-dialysable. Smith thinks that the new substance may be identical with the pro-antithrombin of Howell.

Ziff and Chargaff (15) have published a short note on their preliminary studies on the albumin fraction which increases the activity of heparin. Crystalline serum albumin is ineffective. They find that the most soluble fraction of serum albumin is the most effective potentiator. Further work on this subject, however, has to be done.

Factors Delaying Clotting.

Other factors which, while not preventing the coagulation of blood, nevertheless can retard it are temperature and the nature of the surface with which it comes in contact. At low temperature blood tends to remain fluid for a long time. This has been attributed to the greater resistance of platelets in the cold and the consequent delay in the liberation of thromboplastin. Moderate heating favours the coagulation of blood, by hastening the disintegration of platelets. Heating blood at 56°C causes coagulation by precipitation of the fibrinogen present.

The collection of blood in a vessel with a non-wettable surface, e.g. coated with paraffin, effectively delays clotting. Quick (16) has recently reported that collodion is still more effective in this respect.

HAEMORRHAGIC DIATHESIS.

Within the past seven years a new impetus has been given to the study of the bleeding tendency seen in so many pathological conditions. When discussing haemorrhagic conditions two points must be borne in mind. The first is a failure on the part of the body to arrest bleeding and secondly, increased capillary fragiliay, i.e., susceptibility to injury.

Hypoprothrombinemia.

Inability to arrest the bleeding is brought about by the absence or impaired function of some plasma factor essential in the coagulation process. It is well known that a haemorrhagic diathesis exists in hypoprothrombinemia. Before the introduction of vitamin K therapy death among newborn infants from haemorrhagic disease was not uncommon. Similarly patients with obstructive jaundice and biliary fistula were considered poor operative risks because of haemorrhagic tendency later found to be due to a deficiency in prothrombin. With the discovery of vitamin K and methods of prothrombin determination it was soon realized that a diminution of plasma prothrombin to levels of thirty per cent. of normal and even less will fail to arrest haemorrhage. The prompt administration of vitamin K, either orally or intravenously, raises this level to normal within a few hours. This increase in the prothrombin can always be produced except in cases of liver disease where synthesis of prothrombin is impaired.

Fibrinogenopenia.

Fibrinogen may be markedly decreased in severe liver diseases. In the past the bleeding tendency associated with such conditions was thought to be due to the fall of fibrinogen and was known as pseudohaemophilia. It is now recognized that the fall

in prothrombin from liver damage is a more important causative factor of the haemorrhagic diathesis. The normal values for fibrinogen vary between 0.3 and 0.75 mgs. per cent. and values in certain diseased states may be extremely low. Haemorrhage, however, occurs in fibrinogenopenia only in two cases. The first, a condition which involves the bone marrow, is caused either by tuberculosis or carcinomatous metastasis. The second is rare and is due to congenital absence of fibrinogen.

Calcium Deficiency.

In the extreme calcium deficiency of hypoparathyroidism haemorrhagic diathesis has never been seen. Serum calcium can never become low enough in the living animal to inhibit the clotting properties of blood.

Thromboplastin Deficiency.

Thromboplastin deficiency is the cause of one of the long known and obscure haemorrhagic diseases, haemophilia. The cause of haemophilia and its treatment have long been the subject of investigation. Experiments have shown that haemophilic blood contains normal amounts of fibrinogen and prothrombin. The latter substance is not qualitatively different,as some have suggested. It has been shown that haemophilic blood contains no excess of antithrombin.

The addition of thromboplastin causes the coagulation of haemophilic blood within the normal time range. A faulty mechanism associated with thromboplastin, therefore, is suspected since the other factors involved is coagulation are normal. Platelets are the source of thromboplastin in plasma and consequently it is to these that attention immediately turns. Quick (1) believes that the slow rate of liberation of thromboplastin, due to the abnormal

resistance of the platelets to lysis, is the chief cause of haemophilia. According to Quick the cure for haemophilia lies in rendering the platelets more fragile.

Ferguson (2) also believes that the thromboplastin releasing mechanism is at fault due to the absence of a specific factor. He holds that haemophilia is caused by the absence of a ferment similar to trypsin which he calls the "thromboplastic enzyme". The function of this factor is to make cephalin "available" by liberating it from combination with proteins(3). This is the status of our knowledge of haemophilia at the present time.

Thrombocytopenic Purpura.

Purpura haemorrhagica is a pathological condition associated with a great decrease in platelet count. Despite the characteristically low platelet count the coagulation time of the blood is usually within normal limits when tested in vitro. It is a well known clinical observation that patients with purpura have a prolonged bleeding time after a minute stab wound in the subcutaneous tissue. This condition may be the result of some obscure change in the capillary endothelium or of the failure of the clot to retract. This would agree with the observation of Tocantins(4) that platelets are essential for clot retraction.

Blood Coagulation following Anaphylactic Shock.

It is well established that after anaphylactic shock the blood coagulates either slowly or not at all. Eagle and co-workers(5) have found that anaphylactic shock in rabbits and dogs does not affect the fibrinogen, prothrombin, platelets or calcium, but does cause an enormous increase in the antithrombin of the plasma. In guinea pigs shock does not affect coagulation time and there is no demonstrable increase in antithrombin. Quick suggests that in human anaphylactic shock the incoagulability of the blood is caused

by the liberation of heparin. The effect, like that caused by the injection of proteose or peptone into a sensitized animal, disappears in one to three hours.

Haemostatic Action of Thrombin.

Thrombin has been reported to have a haemostatic effect in certain cases of haemorrhagic diathesis. It has been used effectively by Smith and co-workers for arresting haemorrhage, by spraying it on the bleeding surface or by dropping it into the wound (6).

PURPOSE OF THE INVESTIGATION.

In the storage of human blood it has been noticed that fibrin gradually appears in the samples. This takes place both in stored blood and in plasma. In either case it presents difficulties since the fibrin must be removed before the blood can be used for transfusion, and the purpose of the present investigation is to determine a means of preventing or retarding the precipitation of fibrin. Various workers have reported on the fall of prothrombin in preserved blood. Although there is much controversy about the rate of prothrombin diminution the controlling factor seems to be the method of preservation. It was thought advisable to investigate these reports and to correlate if possible the disappearance of prothrombin with the precipitation of fibrin during storage.

Since anticoagulants evidently do not exert a permanent effect, it leads one to speculate that this may be due to a progressive liberation of some coagulation factor, such as thromboplastin, during storage. The formation of fibrin under normal conditions is brought about by the action of thrombin and the questions which naturally arose in this investigation were:

1) is thrombin responsible?
2) is thrombin, liberated from a thrombin-antithrombin complex, responsible?
3) is the disturbance in ionic equilibria responsible?
4) are the products of the disintegration of white cells and platelets responsible?
5) are the products of haemolysis responsible?
6) is the change in temperature, p.H, surface,etc., responsible?

7) is calcium liberated from its complex with anticoagulant?

These are but a few of the various problems that demanded investigation.

EXPERIMENTAL.

PREPARATION OF MATERIALS.

(1) Preserved Blood.

Samples and whole blood and plasma were obtained from the Blood Bank of the Department of Biochemistry. The blood was taken under sterile conditions and diluted as follows:-

5 parts blood
1 part 3.2 per cent. sodium citrate.
1½ parts 5.4 per cent. glucose.

It was stored in 5 cc. rubber-stoppered tubes at 4° ± 1°C.

Plasma was also stored. Citrated blood was centrifuged at 2,000 r.p.m. for about thirty minutes and the plasma removed by aspiration. The plasma was then drawn through a fine porous filter to remove any suspended platelets and cells. Sterile technique was used throughout and the samples were stored in the same manner as whole blood.

(2) Thromboplastin.

Most workers have recommended the use of rabbit brain prepared in the following way: The rabbit is killed and the brain immediately removed. The pia and blood vessels are stripped off after the brain has been washed in saline. It is then macerated in a mortar and extracted four times with 10 cc. portions of acetone and placed to dry overnight in the oven at 37°C. The dried preparation lasts indefinitely when tightly stoppered at 0°C. A 1:20 saline suspension of this material has proven a good source of thromboplastin. It is advisable to centrifuge the suspension slowly so that only the coarsest particles collect at the bottom of the tube. It was also found advisable not to kill rabbits by

a blow on the head since this led to haemorrhage. Although rabbit brain had proved a satisfactory source of thromboplastin, it was decided to investigate the possibility of obtaining thromboplastin in greater quantities and from a less expensive source.

Calf, sheep and cat brain were processed by the method outlined above. While these extracts possessed some thromboplastic activity they were generally found to be much weaker. A saline extract of beef pituitary was tried for thromboplastic activity, but proved relatively inactive.

While cephalin is known to be less active than aqueous tissue extracts, it was thought that it might afford a purer and more uniform preparation. The method used in preparing cephalin was as follows: calf brain was ground up and extracted twice with four times its volume of acetone (to remove water, fat, sterols, pigments). It was then extracted twice with ether. From the latter solution cephalin was precipitated by adding alcohol. The cephalin precipitate was washed with acetone and dried in a desiccator under reduced pressure for 24 hours.

A saline suspension of cephalin prepared from calf brain in this manner was found to be inactive.

An extract of rabbit lung, prepared in the same way as rabbit brain, showed a high degree of activity. Since the plasma of lung tissue contains prothrombin and other coagulation factors it is necessary to perfuse the lungs with saline before removing them and this is rather a tedious operation.

An attempt to prepare thromboplastin in purer form was carried out as follows: Sheep lung was used as a source of globulin by grinding it up and extracting it with 0.25 per cent. acetic

acid. To the filtrate acetone was added to give a concentration of 54 per cent. and a white precipitate formed. Acetone was added to this filtrate to raise the content to 85 per cent.,and a fine white powder was precipitated. The latter was washed with acetone and dried in vacuo. The resulting powder is very soluble, but a saline solution prepared from it was found to be totally inactive.

Rabbit brain, therefore, appears to be the most satisfactory source of thromboplastin.

(3) Fibrinogen.

Smith's method of preparing fibrinogen was followed. Citrated beef blood was obtained from the abbatoir and precipitated by the addition of one-third its volume of a saturated ammonium sulphate solution. The precipitate was dissolved in a volume of oxalated saline equal to that of the plasma used. Precipitation was repeated twice and the final filtrate dissolved in a volume of oxalated saline equal to one-third that of the original plasma. This solution was dialysed against oxalated saline for 90 minutes. Merthiolate 1 part in 10,000 was added to prevent the growth of bacteria.

This solution was found satisfactory for over a period of two weeks when stored at 4°C.

Fibrinogen was also prepared according to Herbert's method.(1) To 30 ml. of plasma 6 ml. magnesium hydroxide suspension were added. After mixing well the magnesium hydroxide was centrifuged off carrying with it a considerable proportion of the plasma

prothrombin. 10 ml. saturated ammonium sulphate were added to 30 ml. of the supernatant plasma and mixed. The fibrinogen that precipitated was centrifuged off and the supernatant discarded. The precipitate was dissolved in 30 ml. buffered oxalated saline and 10 ml. saturated ammonium sulphate were added to precipitate the fibrinogen a second time. After centrifuging, the supernatant fluid was discarded and the fibrinogen dissolved in 10 ml. buffered oxalated saline. The fibrinogen solution was transferred to a collodion sac and dialysed for at least an hour against 400 ml. buffered oxalated saline. The external fluid was then discarded and the dialysis continued against buffered saline (without oxalate).

This fibrinogen preparation keeps one day at ordinary refrigerator temperature. It, however, lasts one week if sealed in ampoules.

(4) White Cell Extract.

Using the method of Barnes (2) a white cell suspension was prepared as follows: 250 cc. of warm sterile saline solution was injected into the peritoneal cavity of a rabbit. After three hours the remaining fluid was withdrawn from the cavity and collected in sterile citrate to prevent coagulation. The fluid was opalescent with white cells 95 per cent. of which were polymorphonuclear leucocytes. The cells were centrifuged and washed with sterile saline. The process was repeated twice and finally the cells were suspended in a small volume (10cc.) of sterile saline. Before use they were broken down by slow freezing and thawing of the suspension.

INFLUENCE OF VARIOUS SALTS AND FACTORS ON CLOTTING.

Since many substances might influence one phase or another of the clotting process, it was decided to note their influence on clotting.

Clotting Time Method.

.2cc. plasma, blood or serum.

1.4cc. normal saline or substance dissolved in saline.

.5cc. of .5 per cent. $CaCl_2$ solution.

Clotting time taken as the time from addition of calcium to the formation of a clot.

(1) Lactose.

It has been reported (3) that lactose in some unknown way, has a tendency to form a complex with calcium. Although the mechanism by which calcium is bound is based largely on inference, it was deemed of interest to see if lactose could in any way interfere with fibrin formation by removing calcium.

The addition of lactose in saline solution and $CaCl_2$ to oxalated plasma produced no variation in clotting time above the normal.

(2) Cholesterol.

Since cholesterol is a constituent of the red cell membrane it was thought that the addition of cholesterol would in some way prevent the disintegration of the red cell during storage. It was observed that the addition of cholesterol to citrated blood sometimes produced a soft clot during storage and this suggested a possible influence of this substance on fibrin formation. In a series of clotting tests performed on blood to which cholesterol had been added it was found that the presence of cholesterol did not alter the clotting time.

It should be borne in mind that these results do not indicate that cholesterol would not affect coagulation if it were present over a period of time as in storage.

(3) Action of an enzyme - Rennin.

Since rennin is an enzyme that requires the presence of Ca ions it was thought that it might be effective in inhibiting clotting by binding calcium. On several occasions it was found that the addition of rennin and calcium to oxalated plasma completely inhibited clotting. If, on the other hand, the enzyme activity was destroyed by heating to 56°C, clotting tookplace.

A repetition of this experiment two months later using rennin from the same stock as well as a purified preparation, failed to inhibit clotting. The rennin was purified by allowing it to stand in 80 per cent. alcohol for a week and then drying in vacuo.

(4) Action of ions.

From the extensive studies that have been reported on the influence of various ions on blood coagulation it was thought that the formation of fibrin during storage was due to changes in blood electrolytes, particularly potassium, which escapes from the corpuscles. The influence of potassium ions on the clotting of citrated blood by the addition of calcium ions was studied. It was found that the presence of potassium did not produce any change in the clotting time.

Similarly the use of cadmium, which belongs to the same series as potassium, did not produce any change in clotting time.

(5) Influence of temperature.

Blood was incubated at various temperatures for different periods of time and subsequently 0.2 cc. of the blood was diluted with 1 cc. saline and 0.15 cc. 5 per cent. $CaCl_2$ was added. The time from the addition of the calcium to the appearance of the clot was recorded as the clotting time.

Temperature. Incubation Period Minutes.

	5	10	15	20
22.5°C	C.T. 7.5 min.	8 min.	8 min.	8 min.
37.0°C	C.T. 8.0 "	8 "	8 "	8 "
40.0°C	C.T. 8.0 "	8 "	8 "	8 "
45.0°C	C.T. 8.5 "	8 "	8 "	8 "

There was evidently no change in the clotting time after such treatment.

(6) Heating of blood before storage.

Since prothrombin is of a protein nature and can be destroyed by heating at 60°C it was thought that a partial destruction of this factor might take place when citrated blood was heated at lower temperatures for varying periods of time. By the partial destruction of this factor it was hoped that it would be possible to inhibit fibrin precipitation or at least delay it. It was thought also that heating at 51°C or slightly above might inactivate the plasma lecithinase which according to Fahraeus gives rise to lysolecithin and haemolysis during storage.

Citrated samples were heated at 37.5°C, 40°C, 45°C, 47°C, 52°C for 5, 10, 15 and 20 minutes respectively and then stored at 4°C. Haemolysis occured more rapidly than usual in the heated samples. Fibrin appeared in these samples at the same time as in control samples and apparently to the same degree. This method of preservation, therefore, offered no advantage.

(7) Use of anticoagulants.

Since fibrin formation occurred despite the presence of citrate it was thought that by increasing the citrate concentration the matter would be remedied. If this were not the case it was thought that heparin might prove more effective in inhibiting fibrin precipitation, or better still, the combination of citrate and heparin which inhibit separate clotting factors would necessarily prevent coagulation. It was found that the use of a high concentration of citrate, such as a final concentration of one per cent. when 0.25 per cent. is customarily used, slightly retarded the formation of fibrin. The use of such a method would, however, be of little use in preservation since high citrate concentration is harmful.

Heparin in a concentration of 7.5 mg. per 100 cc. blood was not found to be any more effective than citrate. If anything, it accelerates haemolysis in stored samples.

It was found that the combination of citrate and heparin produced worse effects than citrate alone. Other anticoagulants such as thiosulphate, transfusol, etc. were found to be ineffective.

(8) Influence of dilution.

The use of high dilution has been found to be successful in retarding the precipitation of fibrin; however, excessive dilution as a method for storing blood for transfusion would not be feasible since it would dilute the blood proteins of the recipient. It should be noted that the use of sodium chloride as a diluent is not recommended. Whenever sodium chloride has been used in the

preservation of blood in this department, it has been observed that haemolysis is accelerated. This cannot be the fault of the sodium ion since sodium citrate is satisfactorily used as an anti-coagulant, therefore, the chloride ion must be responsible for the effects produced. It will be recalled that transfusol which is prepared in sodium chloride solution was found unsatisfactory as an anticoagulant in this department. It might prove more efficient if prepared in another manner.

(9) Storage under gas.

Since fibrin often appeared on the surface of stored blood and plasma in the form of a white scum it was thought that the presence of oxygen in the test tube might be the causative factor. For this reason it was decided to investigate the effects of storage under other gases.

When the gases were bubbled through it was found that fibrin was precipitated rapidly and in great quantity. This most likely was a physico-chemical phenomenon.

When blood was stored in Nesbitt flaska under carbon dioxide or nitrogen without bubbling the presence of these inert gases was found ineffective in diminishing fibrin precipitation.

Ascorbic acid is a strong reducing agent and it was thought that vitamin C would have some effect if an oxidation-reduction system were functioning. It, however, proved of little use.

(10) Influence of red blood cells.

Red cells which had been washed several times with normal saline were haemolysed by freezing and were then tested to see if they had any influence on clotting. It was hoped that by this means it would be possible to trace the appearance of fibrin to red cell breakdown, but the red cells showed no effect on clotting.

The same as was said of the other factors can be added

here. This is not a proof that red cells do not influence the appearance of fibrin, since other conditions may prevail when red cells act over a lond period.

PROTHROMBIN DETERMINATIONS.

The literature on the prothrombin content of stored blood and its rate of fall is conflicting and filled with contradictory data.

Methods:

(1) Howell's Method.

This method simply consists in adding $CaCl_2$ solution to citrated blood. The clotting time for fresh blood treated in this way is about 7 minutes. This method is still used clinically but has been abandoned by most workers since it is recognized that the amount of prothrombin and thromboplastin in a sample are variables influencing the clotting which this method does not take into account. Most investigators are interested in determining prothrombin and it is impossible by this method.

(2) Quick's Method.

Quick originated the following method by which he claims one can determine the prothrombin content in per cent. of normal. 0.1 cc. of thromboplastin solution is added to 0.1 cc. of plasma and the tube placed in a bath at 37.5°C. 0.1 cc. of a suitable $CaCl_2$ solution is added and the clotting time is determined with a stop watch. By comparing this with the clotting time of normal blood, the relative prothrombin content can be determined.

This method has received much criticism, and was reviewed in the section on prothrombin. Apart from the fundamental error in the method which does not distinguish between the two phases of clotting it has been argued that the thromboplastin.content varies with different brain extracts and also introduces a

variation. Kelley and Bray modified Quick's method by titration of the thromboplastin.

(3) Method of Kelley and Bray.

Titration of Thromboplastin:

A 1:20 suspension of thromboplastin is made in physiologic salt solution (0.9%); 0.2 gms. of the dried brain in 3.8 cc. of the salt solution is ample for the titration. After heating the suspension at 56°C for ten minutes with occasional stirring the material is centrifuged at low speed to throw down the coarse particles. The following dilutions are then made from the turbid supernatant suspension in 0.5 cc. amounts: 1:20, 1:30, 1:40, 1:60, 1:80, 1:120, 1:160. Two or more samples from normal healthy adults are used for the titration. The titrations are run in duplicate. The titration usually shows an optimum zone of low findings. For routine tests the dilution falling in the middle of this zone is used, thus giving a safe margin.

Quick's procedure is used for the remainder of the method.

(4) Herbert's Method.

Smith's method is very complicated and this modification by Herbert has been used. It gives absolute values.

Plasma is separated and a series of dilutions of the plasma is isotonic sodium chloride is prepared, giving 1 volume of plasma in 25 volumes, 1:50, 1:100 and 1:200.

From each of these the following mixtures are made:

1 ml. diluted plasma.
1 ml. glyoxaline buffer.
2 ml. brain extract (containing Ca.)

The dilutions are now 1:100, 1:200, 1:400, 1:800. These mixtures are incubated at 15-20°C and at intervals samples are

taken for clotting tests as follows:

A sample of 0.2 ml. is measured into a test tube and placed in a water bath at 37°C. Fibrinogen 0.1 ml., is rapidly added. A stop watch is started at the moment when the fibrinogen is introduced and the time taken when the mixture gels.

(5) Kato's Method.

This is a bedside method or clinical method and gives an indication of the prothrombin level.

.02 ml. plasma and.02 ml. thromboplastin are placed upon a spot plate and .02 ml. $CaCl_2$ solution added. Clotting time is recorded on a stop watch. Normal blood tested by this method gives values of 22 seconds.

RESULTS.

The method of Kelley and Bray was used for a series of blood samples.

Blood was taken on January 9,1941, and stored in the manner recommended by this department. It was decided to investigate the influence of the addition of other substance on the prothrombin level. Paraffin was used in one set to prevent oxidation at the air interface, and oestrin and cerebroside were added to two others. Oestrin was added because it had been reported to prevent haemolysis. Cerebroside being a colloid was added to see if this would prevent haemolysis. A few samples were kept at room temperature and the remainder at 4°C.

The titration of the thromboplastin using normal blood and the fresh sample gave values of 16" and 17" for a 1:40 dilution. Thromboplastin was used in this dilution throughout the series.

The values obtained follow:

Sample	DAYS OF STORAGE. 0 C.T.	13 C.T.	18 C.T.	26 C.T.	34 C.T.	40 C.T.
1. whole blood	16"	21"	20.5"	20.5"	19"	37"
2. plasma	16"	19"	19"	20"	30"	35"
3. oestrin	16"	16.5"	17"	17" *17"	20"*25	20" *25
4. cerebroside	16"	16"	16"	17" *18	20"*25	19" *20
5. paraffin	16"	18"	18"	18" *19	20"*25	19" *19

* samples kept at room temperature.

From these results it can be seen that the fall in prothrombin is very gradual and little difference except haemolysis is seen in the samples kept at room temperature.

While the values obtained in samples 3,4,5 are not highly significant they are, nevertheless, slightly below the others. It was noticed that haemolysis occurred more rapidly in these samples.

The values for prothrombin are fairly regular and the method can be recommended for its simplicity. The objections to Quick's method also apply here.

An attempt was made to follow the prothrombin content of samples, preserved by Dr. Denstedt's method. For this purpose the methods of Herbert, Quick and Kato were used. Very little agreement was shown between the three methods and this difficulty in correlating the results of one or more methods was encountered in all determinations.

Method.	DAYS OF STORAGE. 0	1	3	5	8	11	13
Herbert	100%	85%	60%	66%	-	-	-
Kato	73%	-	28%	27.5%	55%	55%	55%
Quick	41.2%	-	14.1%	13.9%	41.2%	29.4%	18.5%

CRITICISMS.

Kato's method was used at first to study prothrombin content. Great variations were introduced in identical samples of blood sometimes amounting to 3 seconds. These variations were most likely due to uncontrollable factors such as temperature and evaporation. The test is performed at room temperature which varies at different periods. Evaporation is a factor that may also contribute to variation in clotting time. The theory behind these tests is that if the same amount of thromboplastin and calcium chloride is added to the plasma then the only variable that can influence the clotting time is the prothrombin content. A constant temperature and reproducible test conditions should also be insisted upon even for rough clinical tests.

Quick's method has previously been criticized, but it should be borne in mind that even a thromboplastin variation in his test may introduce an error of a second or two. Quick reports that there is not a linear relation between prothrombin content and clotting time and consequently within a range of 5 seconds there is a 50 per cent. fall in prothrombin.

Herbert has shown that there is a linear relationship according to her method. The only criticism that can be made of her method is the time taken to prepare a fresh fibrinogen solution every twenty-four hours.

Two reasons can be offered for the unsatisfactory results on prothrombin determinations. The first is the difficulty of obtaining a constant thromboplastic extract and the second is the preparation of a lasting fibrinogen preparation. The first could be remedied by titration of each new extract with normal blood so as to obtain a standard and optimum dilution.

The difficulty with the second is the preparation of a fibrinogen solution, free from prothrombin and other coagulation factors, and which will last indefinitely so that in conducting experiments on stored blood a variation will not be introduced with each new fibrinogen preparation.

DISCUSSION.

The precipitation of fibrin in preserved blood leads one to suspect many possible causes. Probably the foremost one would be the change in temperature. Since the natural temperature of human blood is 37°C it seems that the storage of blood at 4°C may be the factor responsible for bringing about clotting; however, precipitation of fibrin occurs just as readily in samples kept at room temperature and above, and in these there is the complicating factor of rapid haemolysis.

In stored blood the formation and distribution of fibrin varies. Some samples develop the precipitate at the plasma-air interface, where it forms a white scum. The majority of samples develop the fibrin just on the surface of the red cell mass, where it presents a fuzzy appearance. All bloods do not show precipitation to the same degree, but specimens taken from the same donor and preserved in the same way behave similarly.

The site of this fuzzy layer leads one to suspect that the disintegration of the white cells and platelets, which lies above the red cells in sedimented blood, is the factor responsible for initiating clotting. White cells and platelets break down sooner than red cells and according to most investigators liberate trypsin and cephalin, which, as shown before, can convert prothrombin to thrombin. It has been observed, however, that extracts made from white cells and platelets by differential centrifugation, as well as those prepared by injection of saline into the peritoneal cavity of rabbits, do not possess marked thromboplastic activity. Thromboplastin prepared from rabbit brain was always more effective than either of the saline extracts prepared. This does not mean that under conditions of storage white cells and platelets do not possess thromboplastic activity.

Fibrin precipitation is more easily detected in stored plasma, where it collects at the bottom and sides of the tube or at the plasma-air interface. Gentle tiltings of the tube cause the appearance of numerous tiny flakes. As in the case of whole blood the presence of remaining white cells and platelets may liberate thromboplastin when destroyed. In plasma prepared by filtration through a fine porous filter to remove cellular elements the precipitation of fibrin occurs to the same or an even greater degree. This is, most likely, due to the liberation of thromboplastic substances during filtration.

A point that must be borne in mind is that while the platelets are not found to be potent in thromboplastic activity, nevertheless, what amount they do contain may eventually bring about the appearance of fibrin. That there is only a limited amount of thromboplastin liberated would appear to be true since the formation of a solid gel, such as occurs in normal clotting, has not been observed in stored blood or plasma samples.

The presence of calcium is accepted as essential in the formation of thrombin in the conventional theory of coagulation. The formation of fibrin in spite of the presence of citrate points to the possibility that calcium may be released slowly from its complex, thereby promoting thrombin formation and subsequently clotting. Quick believes that the action of citrate or oxalate is permanent once it binds calcium. The removal of calcium by citrate, however, is not immediate, but requires several minutes. Quick mentions the appearance of fibrin in stored samples and says that it is caused by contamination with tissue juice, while drawing the blood, and the action of calcium before it is removed

to form a complex with citrate or oxalate. In this connection Ferguson's observation will be recalled; that "ripe" thrombin is free of calcium although "fresh" thrombin contains calcium. The question arises, would it not be possible for the calcium to leave the "ripe" thrombin and to be available for the formation of "fresh" thrombin if sufficient thromboplastin were present?

The process of fibrin formation is not an immediate effect; it is initiated early in storage and proceeds slowly. It first becomes noticeable in plasma samples after fourteen or more days of storage. It may appear at an earlier date in samples of whole blood, but is more difficult to see. It was suspected that the variation in electrolyte distribution arising from the alteration of the red cell permeability and subsequent haemolysis was closely connected with the appearance of fibrin. Although this would not apply to plasma it is conceivable that in whole blood the presence of other ions might take the place of calcium. It is interesting to observe that haemolysis and fibrin precipitation must be related in some way. Defibrinated blood, to which gelatin or gum acacia have been added to replace fibrinogen, haemolyses more readily than normal stored specimens.

It has been observed in this department that dilution of the blood is an important factor in preventing fibrin precipitation. An effect that is due most likely to the decreased possibility of contact between ions. It, however, explains why investigators who use dilutions of 24:1 or 10:1 (blood:diluent) report the presence of clots during storage. From the present literature it can be seen that the use of a smaller proportion of blood, such as 1:$\frac{1}{2}$ to 1:1$\frac{1}{2}$ safeguards against excess fibrin precipitation.

The Russian workers use the blood of cadavers killed by accident and whose respiration ceased before the heart stopped. They report that if the blood is withdrawn up to a period of eight to ten hours after death it is still uncontaminated by bacteria and is very slow to clot. Vigorous shaking after it has clotted causes the clot to dissolve. Further work on this phenomenon of fibrinolysis remains to be done.

Since most of the work in this investigation was of an exploratory nature various other factors of a physico-chemical nature suggested themselves. During storage the buffering mechanism functions quite well and very little change in pH has been observed.

Since blood stored in an atmosphere of CO_2 or nitrogen showed fibrin precipitation the process would not appear to be initiated by oxidation.at the air interface. Many years ago Pickering and Hewitt suggested that coagulation may be brought about by a difference in potential set up at the surface of contact between the glass container and the plasma.

Whatever the factor responsible for the conversion of fibrinogen to fibrin we have found that the injection of stored blood as well as plasma into rabbits has not brought about any ill effects. This suggests that the small amount of fibrin present does not offer too great a hazard in transfusion since the blood can be filtered. If toxic substances were present in the stored blood death would ensue. Among the toxic substances that could be present are thrombin and potassium. It has been shown that the toxicity of potassium depends on its rate of injection into an animal; the same may apply for thrombin or it

may be below the dangerous concentration since no untoward symptoms were seen.

Coming to the consideration of prothrombin and its fall in stored blood it is rather hard to draw conclusions from the results obtained. The various methods of determining prothrombin have been criticized. The conflicting reports in the literature are certainly due to the use of different methods and the skill of the investigator in handling a particular method. From the various results obtained it would not appear that the prothrombin content of stored blood falls as rapidly as some investigators say. More work will have to be done on this particular aspect of the problem.

Since the prothrombin content does diminish it would appear that it is a factor functioning in the production of fibrin. There also appears to be a parallelism between fibrin formation and the progress of spontaneous haemolysis. It is very likely that a solution of anyone of these difficulties will contribute simultaneously toward a solution of the others.

One point that must be stressed is that in the preservation of blood we can never hope to duplicate the conditions under which it exists in the organism. The precipitation of fibrin in stored blood greatly disturbs us, yet this may actually happen in the body where it is rapidly removed by some physiologic process before it can accumulate. In conditions requiring vitamin K therapy it is known that a dose of this substance may almost raise the level to normal, but if the treatment is not continued, it will fall back to its previous low level within a day or two. This indicates that there is a continuous replacement of prothrombin presumably by the liver. Therefore, the fall in pro-

thrombin does not mean that a method of preservation is the cause, although it is admitted that it may bring about the fall more rapidly. Similarly, the red cells present in circulating blood are of all ages and it has been estimated that ten billion are destroyed per hour and the released haemoglobin taken up by the reticulo-endothelium system. It is not surprising then, that haemolysis takes place during storage.

Although we cannot protect the blood during storage by physiological processes such as are present in the body we hope that by using an optimum method of storage the breakdown of elements and presence of waste products will not take place more rapidly than in the human body.

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