LXXXIX. STUDIES OF THE COAGULATION OF THE BLOOD.

PART I. SOME PHYSICO-CHEMICAL ASPECTS OF COAGULATION.

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POSSIBLY no better example could be given of nomenclature being in advance of actual knowledge, than the literature of blood coagulation; but despite the multiplicity of alleged substances invoked and considered essential, it will be agreed, it is thought, that a complete explanation is still wanting. The purpose of this communication is to investigate how far the mechanism of blood clotting is susceptible to some of the current concepts of physical chemistry.

For convenience we can group modern theories on coagulation under four headings:

(1) *Wooldridge's theory.* Here coagulation is thought to be a reaction between the constituents of the plasma; the extra-vascular coagulant fibrin-ferment (thrombin) being a subsidiary reaction product. Wooldridge considered "lecithin" to be of great importance, but he also regarded the plasma as a unity in its behaviour in coagulation, which process he believed to be allied closely to crystallisation.

(2) "*Thrombin*" theories. Prothrombin is assumed to exist in, and to be liberated from leucocytes; on activation by calcium, thrombin is formed and is the immediate cause of coagulation [Schmidt and others, 1875]. The term thrombogen is employed by Morawitz [1904] and Fuld and Spiro [1904] in place of prothrombin; this under the combined action of thrombokinese (from tissue detritus) and calcium salts, yields thrombin. Fuld and Spiro regarded coagulation essentially as a proteoclastic digestion. To account for the normal fluidity of blood it was found necessary to postulate the existence of antithrombin [Howell, 1911]. Even then the recognised facts cannot be explained and the aid has been sought of further hypothetical substances, viz. prothrombokinese, antithrombokinese, antiprothrombin [Collingwood and Macmahon, 1912], proantithrombin [Howell and Holt, 1918], and in serum an inactive compound of thrombin and antithrombin termed by Morawitz [1904] metathrombin. Emphasis should be laid on the fact that thrombin

has not been found in normal circulating blood, but exists only in shed blood. Further, as shown by Wooldridge [1893], on massive injection it fails to induce intravascular coagulation. This latter is a crucial experiment, and unless an instantaneous production and liberation of antithrombin (so far unproved) is admitted, it is thought that doubt is cast on the theories grouped under this heading.

(3) *Theory of Nolf*. Broadly speaking, the views of Nolf [1906, 1908, 1909] may be described as based on the brilliant deductions of Wooldridge. They were fundamentally influenced, however, by his adherence to the "thrombin" theories, particularly in this author's later work. In his earlier papers, Nolf described coagulation as being essentially a mutual precipitation of colloids. He considered that this process involved a proteoclastic enzyme termed leucothrombin derived from the leucocytes and the endothelial cells of the vascular system as well as two proteins formed in the liver, hepatothrombin and fibrinogen. In the presence of calcium salts leucothrombin and hepatothrombin unite to give thrombin which in turn precipitates fibrinogen as fibrin. Excess of hepatothrombin he claimed, leads to the fluidity of normal circulating blood and of "peptone" plasma. He identified hepatothrombin with antithrombin. Nolf also maintained the existence of two forms of coagulation, that produced by thrombin in solution yielding a gel, and that caused by finely divided solid thrombin forming agglutinations. Later, to bring his work into line with that of Morawitz, he termed leucothrombin thrombozyme and hepatothrombin thrombogen. He changed his opinions also regarding the fluidity of both "peptone" plasma and normal circulating blood. The former phenomenon he ascribed to another hypothetical substance claimed to be secreted by the liver, which he called "liver antithrombin." Normal fluidity he described as due to the living cells of the blood and those lining the vascular and lymphatic systems being covered with a film of "fibrin" containing thrombozyme, thrombogen and fibrinogen. Thrombin, he stated, acts only through the medium of thromboplastic substances. The protective film having no thromboplastic action, the blood remains fluid. The preliminary act of coagulation is said to arise from the contact of either thrombogen or thrombozyme with fibrinogen. Enzyme action is claimed subsequently to occur and is termed fibrinolysis; this causing stable clotting. To round off this now somewhat complex theory one last anticoagulant substance termed antifibrinolysin is introduced. It is assumed to be formed by the liver.

(4) *The theory of Hekma*. This hypothesis is based on the close similarity of the precipitate of fibrin alkali hydrosol to fibrinogen, from which Hekma [1916, 1, 2, 3] concluded that fibrin exists in the blood and that coagulation is a colloidal precipitation occurring in two phases, (a) a change from emulsoid to suspensoid state with the appearance of ultramicroscopic crystalline-like needles (as observed by Stübel [1914]), and (b) the formation of fibrin threads by apposition of these formed elements. One may remark that Hekma does not account for the formation of thrombin in shed blood, the occurrence of

which indicates, with some precision, a chemical as well as a purely physical reaction. Further, he does not take into account the "negative phase" of blood coagulation and other seemingly anomalous phenomena.

The Negative Phase.

The delay in coagulation following slow injection of tissue extracts, first noted by Wooldridge, is one of the strongest arguments for the occurrence of antithrombin. It has been shown, however, by one of us (J. W. P.) that on injection into dogs, cats or rabbits, synthesised colloids free from phosphorus behave similarly to animal extracts. Mills [1921] has recently shown that in blood withdrawn during the "negative phase" there are no substances present which inhibit the coagulation of normal blood; this author and Gutmann [1914] suggest that the "negative phase" is due to the removal of fibrinogen from the plasma, as a diminished content of fibrinogen is found in fluid blood after establishment of the "negative phase." Mills also noted that during the "negative phase" the alkali reserve is substantially diminished. There is therefore, if this is correct, after the injection of small amounts of tissue extracts (a) a diminution of fibrinogen, (b) a lowering of alkali reserve, (c) a loss of power to clot. Bearing in mind the action of CO_2 on peptone plasma (*vide infra*), we suggest that union occurs between tissue extracts or colloids injected, those substances forming the alkali reserve, and finally with fibrinogen. The deductions of Henderson [1921] that blood is a single physico-chemical system tend rather to support this conception.

We have investigated some of the physical conditions governing the precipitation of two typical colloids, gelatin and soluble starch, by alcohol and ammonium sulphate. The amount of precipitate of either colloid depends not only on the volume of alcohol or salt added, but also on the rate at which it is added. When the precipitating agent is added slowly, drop by drop, over a long period of time (2-3 hours), the amount of precipitate formed is much less than if the same volume of the solution is added rapidly. To quote from one series of experiments: To each of two flasks containing 50 cc. of 1 % gelatin, were added 60 cc. alcohol as follows:

A. Drop by drop, time taken 2 hours.

B. As rapidly as the burette would permit.

The precipitates were collected, washed and the nitrogen content determined. The precipitates contained the following amounts:

A. 0.043 g. N.

B. 0.086 g. N.

It may be remarked that the less precipitation following the slow addition of the alcohol may be due to the colloid remaining in a high state of dispersion. Similar results were obtained with the precipitation of soluble starch by ammonium sulphate, a result in which electrical phenomena probably play a part (cf. Mines' observations referred to below). Attention may be drawn

to the fact that temperature plays an important part in this result. At relatively high room temperatures (18–22°) the difference is most marked, at low temperatures (0°) little or no difference can be detected even when the reaction is spread over long periods. Apart from the actual amount of the precipitates formed in the two cases their appearance differs. When the precipitating reagent is added slowly the precipitate is in a very fine state of aggregation, whereas when added rapidly the precipitate formed is much coarser. In this experiment we have established a condition analogous to the production of the “negative phase” under conditions which preclude the formation of an antibody. We have found also that if filtered tissue extract is added slowly, drop by drop, over a period of from 20–30 minutes to unsalted birds’ blood *in vitro* a temporary “negative phase” (or retarded positive phase) occurs in its coagulation; while an equal volume of the same tissue extract added rapidly causes rapid coagulation. The clots formed after the appearance of this temporary “negative phase” and resulting from the slow addition of tissue extract are looser and more gelatinous than those formed by its rapid addition. When the concentration of tissue extract was twice as strong we failed to obtain evidence of a negative phase even though the extract was added very slowly in small drops (*vide* protocols). These results are not without parallel in the work of other investigators. Experimenting with inorganic sols Spring [1900], Höber and Gordon [1904], Paine [1911], Galecki [1912] and others have shown that the question “as to whether a given concentration of an electrolyte will produce coagulation or not, depends, within a certain range, on how the electrolyte is added, *e.g.* slowly, drop by drop, or rapidly, with very gentle stirring or violent stirring, etc.” [Burton, 1916]. Apparently the precipitation of gelatin (due to the hydrophile action of the alcohol) obeys the same law. Dansyz [1902] noted that when ricin or diphtheria toxin was brought into contact with the corresponding antibody, the degree of neutralisation depended upon the method of preparing the mixture, in the sense that when the toxin was added to the antitoxin in two fractions with a time interval between, the resultant mixture contained a much greater amount of free toxin than if the total toxin had been added at once to the antitoxin. These results were confirmed as to diphtheria toxin by v. Dungern [1904], and were attributed by him to the formation of a supposed substance “*epitoxonoid*.” Similarly, if arsenious acid is added in just sublethal amounts to ferric hydroxide it is found [Hewlett, 1921] that to render the mixture toxic, arsenious acid must be added in amounts greater than the toxic dose of the arsenious acid alone. We submit that these phenomena are of one class and are explicable in the same manner. The precipitation of gelatin or starch and the neutralisation of arsenious acid by ferric hydroxide do not admit of any explanation other than physico-chemical, and it is improbable that any antibody is formed in birds’ blood *in vitro* by the slow addition of thromboplastic material. If this view is accepted, the necessity disappears for assuming the existence of antithrombin, antifibrinolysin, antiprothrombin, proanti-

thrombin, prothrombokinase and metathrombin, and thus a purely physico-chemical explanation of the coagulation of the blood must be sought.

So far as we are aware, all previous attempts to produce a "negative phase" *in vitro* have failed. The failures are explicable by the fact that salted plasmas were used and the salts inhibited coagulation; that attention was not paid either to the speed of delivery of the tissue extract, its concentration or to the temperature at which the reaction was carried out. Unfortunately no exact method is available for determining the actual concentration of thromboplastic material in a given solution of tissue extract, so that to obtain the negative phase referred to above, observations must be made with varying concentrations, until the correct strength is obtained.

The effect of rise of Temperature on Birds' Blood in vitro.

In the course of our experiments on birds' blood in clean glass vessels we raised the temperature to 37° thinking from our experience in the formation of a "negative phase" in the precipitation of gelatin that this phenomenon might be modified in comparison with experiments at laboratory temperatures. We found, however, that at 37° birds' blood clotted rapidly without the addition of any tissue extract. Control experiments with blood from the same animal in vessels cleaned in the same manner, but maintained at room temperature, showed the blood to remain fluid for several hours. It is well known that mammalian blood will remain fluid *in vitro* at 0° and it appears that the cooling of birds' blood to room temperatures is an essential factor in its fluidity *in vitro*. The difference in the behaviour of mammalian and birds' blood *in vitro* appears to be one of degree and to be a function of temperature.

The Fluidity of normal circulating Blood.

Much ingenuity has been expended by those who maintain the existence of platelets in circulating blood in explaining why the disintegration of these, and other formed elements, does not cause thrombosis, since in their breakdown thrombokinase is liberated into the blood stream. We suggest that parallels exist with the phenomena seen in the slow injection of tissue extracts into an animal, in the slow addition of alcohol or ammonium sulphate to a precipitable colloid and the slow addition of tissue extract to birds' blood *in vitro*, for it can be assumed justifiably that normally there never is massive disintegration of formed elements in the blood stream. We think, therefore, that this disintegration *in vivo* being equivalent to a slow injection of tissue extracts, the action would be the reverse of coagulant, and to the physical properties of these substances and of those of the plasma we may look for the protection of the plasma from intravascular coagulation. If the liberation of thromboplastic material is rapid its hydrophile properties overcome the initial inhibition produced by the introduction of small amounts of tissue extract and thrombosis occurs. We have again a parallel to the precipitation of gelatin

and starch by alcohol, for it is found that after adding slowly a small quantity of the precipitant, when a larger amount is added subsequently a precipitate is formed immediately.

The Liver and Antithrombin.

The rôle played by the liver in the production of antithrombin has been deduced largely from experiments wherein "peptone" has been employed. Gley and Pachon [1895] with impaired liver, and Hédon and Delezenne [1896] with an Eck's fistula showed that on injection peptone is without action. In this connection one can recall the experiments of Lahousse [1889] in which removal of or damage to the liver diminishes greatly the carbon dioxide content of the blood. The experiments of Wright [1894] and of Mathieu and Urbain [1874] demonstrate that a decrease of carbon dioxide tends to lessen coagulability, while an increase produces an exalted tendency to clot. The classical experiment of Wooldridge on the effect of passing carbon dioxide through peptone plasma can also be quoted, when coagulation at once takes place. From the results of these workers clearly it is evident that carbon dioxide is of utmost importance in the clotting or otherwise of plasma, and we suggest that the action of the liver in promoting the fluidity of the blood depends on the variations in the carbon dioxide content of blood arising under various conditions, normal or otherwise. Further, it would seem a simpler conception to assume a union of peptone with plasma components to take place, rather than the production of an antibody the existence of which is unproven. Regarding work directed specifically to the demonstration of the formation of antithrombin by the liver, the researches of Doyon [1912] may be quoted as typical. From this author's protocols it is evident that the clotting time of perfused blood varies with the state of the liver. When the liver is nearly normal, the coagulation time is shorter, and as the liver becomes abnormal the time is lengthened. Further, Doyon obtained antithrombin by such methods as freezing and thawing the liver, and then heating to 100° prior to extraction, and in the case of one animal (the rabbit) only by autoclaving at 120°. This technique appears to be in the highest degree unphysiological, and as evidence for the secretion of antithrombin by the *normal* liver, may be dismissed. The deduction the present authors make from Doyon's experiments is that the normal liver is less effective than the dying or dead organ in producing an anticoagulant. Attention should be drawn also to the recent work of Menten [1920], where the perfusion apparatus was maintained at 38°, but where the liver itself was at a lower temperature. The salient features of these experiments are:

- (a) Perfusion of the liver with 0.85 % NaCl yielded no anticoagulant.
- (b) Continuous perfusion of large amounts of Witte's peptone (9 % in 0.85 % NaCl) failed to produce a substance capable of diminishing the time of coagulation.
- (c) Perfusion of small amounts of peptone, separately and slowly, through

the liver gave, at first, perfusates producing immediate clotting; subsequent perfusates delayed coagulation and finally the later perfusates caused accelerated rate until coagulation was again immediate.

(d) During perfusion with peptone the acidity steadily increased throughout the experiment.

An analogy may once more be drawn between the results recorded above on the precipitation of gelatin by alcohol, and the continuous perfusion of peptone in large quantities, and when perfused in small amounts; these experiments in no way militate against the suggestion made above that the action in the liver is on the peptone rather than the formation of a specific anti-coagulant. We are at present carrying out experiments on this point.

Experiments with Blood surrounded by Oil.

It is well known that if blood is withdrawn directly into oil the length of time elapsing before coagulation is increased. In the course of observations on the thromboplastic properties of various substances obtained by extracting fibrin, this method was employed, and in some of the initial experiments what appeared to be a reversible stage in the formation of the clot was manifest. The point was investigated in some detail. Using as the test of reversibility the solubility or otherwise of the coagulating blood in tap-water, it was evident, that prior to the production of the typical clot in both mammalian (cat and rabbit) and frog's blood, there is formed a reversible gel. The duration of this phase is very short in mammalian blood, but persists for a relatively considerable time in that of the frog; the latter animal is best suited for observations on the point in question. The following are examples:

(1) Anaesthetised brown rabbit. A. C. E., paraffined cannula in carotid. Coagulation time of blood on clean glass; commencement of clot 4 minutes, clot complete 5.5 minutes. Blood received into oil in paraffined vessels; formation of gel observed after 2 hours 27 minutes, typical clot in 2 hours 37 minutes. Room temperature 15°.

(2) Anaesthetised cat. A. C. E., pithed, artificial respiration. Coagulation time on clean glass through paraffined cannula 6 minutes. Blood received into oil in paraffined vessels; formation of gel 41 minutes, typical clot in 54 minutes. Room temperature 15°.

(3) Frog pithed. Paraffined cannula in aorta. Coagulation time of blood on clean glass 15 minutes.

April 12th. Blood withdrawn into oil in paraffined vessels at 3 p.m.

„ 13th. Formation of gel noted at 3 p.m.

„ 14th. Microscopic examination at 3 p.m. showed corpuscles mobile on slight pressure of cover glass. No rouleaux.

„ 15th. Typical clot formed with entangled corpuscles and imperfect rouleaux.

When in the intermediate stage, blood transferred to oil in which thromboplastic material was dissolved clotted completely in 10 minutes.

The gel stage is soluble in tap-water and the solution after rapid centrifuging shows, in mammalian blood, usually a few fibrin filaments with the debris of corpuscles; in frog's blood no filaments are visible. Microscopic examination of this reversible stage reveals the corpuscles freely movable on slight pressure on the cover glass. There is neither formation of rouleaux nor appearance of crenation, and in good specimens the leucocytes exhibit amoeboid movement. *Pari-passu* with the formation of fibrin filaments the production of rouleaux occurs. It appears therefore that the coagulation of blood surrounded by oil is a progressive change, in which the formation of a reversible gel precedes that of a true clot. In view of the length of time for which this stage persists in shed blood of the frog it is thought that there is evidence for believing thrombin to be formed after this stage, and that coagulation commences by gelation of the colloidal aggregates of the plasma (a physical reaction), and ends in a chemical reaction in which fibrin and thrombin are formed. In this connection one may recall the fact that blood shed directly into alcohol does not yield thrombin, and that the experiments of Howell [1916] indicate that the coagulation of fibrinogen by thrombin cannot be referred to an electrical reaction between oppositely charged particles. Further, from observations on the cataphoresis of oxalated plasma, Howell has suggested that fibrinogen adsorbs both hydroxyl and hydrogen ions, the adsorption of the former leading to a structureless gel, of the latter to precipitation by aggregation. If we can regard the adsorptive changes in shed blood as similar to those occurring in oxalated plasma, it would appear that the first stage in coagulation is the adsorption of hydroxyl ions.

COMMENTS ON SOME PHENOMENA OF COAGULATION.

(1) *The action of a thread introduced into blood.* As is known this produces thrombosis in circulating blood and also starts coagulative changes in shed blood. A wetted thread becomes electro-negative; the presence of the negative charge implies the existence of an equal positive charge in the liquid in contact with it, as the other component of the Helmholtz double layer. Some of the colloidal aggregates of the plasma are electro-negative, Lusk [1912] having shown that coagulation of circulating blood takes place at the pole which accumulates negative charges, while serum is electrically inactive [Robertson, 1918]. Ions of the opposite sign to the substance precipitated act as the precipitating agent, as well as diminishing osmotic pressure and viscosity [Loeb, 1921]. The thread wetted by blood produces electrical conditions similar to those which induce precipitation, and coagulation results.

(2) *The action of some organic intravascular coagulants.* The proteins of the plasma are hydrophile and although the surface tension of hydrophile emulsoids may be lower than that of suspensoids, enormous surface tensions remain. The proteins of the plasma are also very susceptible to the action of neutral salts, the concentration of electrolytes determining their physical

state. Tissue extracts, snake venom, and such synthesised colloids as produce intravascular coagulation [Pickering, 1895, 1896], exhibit like properties in varying degrees. Their coagulant action may be explained as due to the alteration of the distribution of the electrolytes (following their injection) modifying the adsorptive unions of the salts of the plasma with the proteins of the plasma. The hydrophile qualities of those coagulants also probably play a part in the disturbance of the colloidal aggregates of the plasma and may be accompanied by the process termed by Hardy [1899] "denaturation." Tissue extracts as shown by Halliburton and Brodie [1894] and synthesised colloids [Pickering, 1896], lose their coagulant action if "purified" by repeated separation and re-solution or if kept too long in either acid or alkaline solution. The explanation of their inactivity may lie in the satisfaction of their adsorptive demands leading to more stable unions with the electrolytes, or by hydrolysis when "purified" by the acetic acid method. The viscosity of these solutions does not appear to be the coagulant factor, as they retain their opalescence and viscosity in their inactive state. Delezenne [1897] showed that tissue extracts produced coagulation *in vitro* in the unsalted plasma of birds and certain teleostean fishes, which indicates that the incoagulability of salted plasmas [Halliburton and Brodie, 1894], is due to the added electrolytes forming a stable union with the plasma and not to any intrinsic difference between circulating and shed blood. Rettger [1909] found that repeatedly washed tissue extracts failed to induce coagulation in birds' plasma *in vitro* but that the addition of either dust or feathers caused this process to take place. This investigator concluded that the so-called "kinase effect" was purely mechanical. The inactivity of washed tissue extracts observed by Rettger appears to us to be similar to the inactivity of tissue extracts and colloids when they have remained for a considerable period in solution or have been "purified." We have found that if a bird's feather be washed thoroughly and subsequently introduced into birds' blood in clean glass vessels, its coagulant action is much less than when the feather has tissue detritus and dust adhering to it. With a washed feather we have observed birds' blood to remain fluid for $1\frac{1}{2}$ hours *in vitro* at ordinary room temperature, whereas with a contaminated feather coagulation may set in in a very few minutes. A feather provides large surfaces which disturb the surface tensions of the colloids of the plasma, leading to the formation of those larger colloidal aggregates which are the precursors of coagulation. Feathers or other substances which are wetted by blood probably also behave in a similar manner to a cotton fibre introduced into the circulation, so that ions of the opposite sign to the electro-negative aggregates of the plasma aid the change from the emulsoid to the suspensoid state. The fact that a clean feather is not so potent a coagulant as thrombokinase is explicable as due to the hydrophile qualities of the latter substance accelerating the change. The relatively slow action of a clean feather in producing coagulation *in vitro* compared with the more rapid action following introduction into the circulation of material capable of being wetted by blood is explicable by

the fact that laboratory temperatures inhibit to some considerable extent the coagulation of birds' blood.

(3) *Salted plasmas*. In salted plasmas inhibition of coagulation is due to the relatively stable unions of the proteins with the added electrolytes; the withdrawal of water from active participation may be also another inhibitory factor. Directly blood is shed it tends to change towards the condition of coagulation. Both sodium chloride and sodium sulphate plasmas clot on dilution, that is, owing to a reduction in the concentration of electrolytes, a resumption of the coagulative process occurs. If excess of salt is added to these diluted plasmas precipitation takes place. We suggest that the change of state from fluid to precipitate or coagulum is a function of the concentration of the electrolytes and that the difference between the precipitate and coagulum is due to the difference in rapidity of action of the electrolytes modifying the adsorptive unions. It will be recalled that attention was drawn to the differences in aggregation of colloidal precipitates according to the rate of addition of the precipitating reagent. Magnesium sulphate plasma does not clot on dilution, as presumably the adsorptive union of this salt and the plasma is more stable than in sodium chloride or sulphate plasmas. The addition of calcium chloride to dilute sodium sulphate plasma hastens clotting and, as shown by Halliburton and Brodie [1894], this salt evokes coagulation in magnesium sulphate plasma. The injection of calcium chloride into the normal circulation increases the tendency of the blood to clot though it does not produce thrombosis. In each of the three cases the disruptive force of the ions from the kation calcium may be the coagulative factor.

Working with oxalate plasma Vines [1921] has shown that increasing amounts of calcium must be added in proportion to the period of exposure to the oxalate. This work falls into line with the fact that tissue extracts and synthesised colloids become inactive by prolonged contact with dilute neutral salts, and also with the fact that slow addition of alcohol to gelatin (which gives a relatively long exposure to the precipitant) increases, at certain temperatures and concentrations, the resistance of the colloid to precipitating action.

Cramer and Pringle [1913] found that if oxalate plasma is filtered through a Berkefeld filter it fails to clot on the addition of calcium chloride, but if the blood is withdrawn at the height of digestion, oxalated and then similarly treated, it does clot. In these and in other experiments with salted plasmas, so far as we can ascertain, the blood was not withdrawn through a paraffined or similarly treated cannula, and consequently in its passage from the animal to the oxalate coagulative changes had already commenced. The first stage in coagulation appears to be the formation of colloidal aggregates which are larger than those existing in circulating blood. The explanation of the observations of Cramer and Pringle may therefore be found in the fact that the Berkefeld filter removes these larger aggregates; this eliminates the foci from which coagulation starts under the influence of the ions arising from the

calcium chloride. In blood withdrawn at the height of digestion we have a marked increase of carbon dioxide, an accelerator of coagulation, as Wright [1894] has shown that injection of calcium chloride into an animal breathing an atmosphere surcharged with carbon dioxide produces universal intra-vascular coagulation. Moreover, in blood withdrawn at the height of digestion there occurs "blood-dust" (probably ultramicroscopic fat particles); these also may provide foci which aggregate under the influence of ions arising from the carbon dioxide and added calcium chloride.

(4) *The action of addition of small and of large volumes of water to blood.* The addition of small quantities of water to blood hastens coagulation. Howell [1912] found that while dilution with water increases the coagulability of the blood of the terrapin, dilution with an isotonic solution of sodium chloride does not alter it. Howell concluded that dilution with water weakens the action of the antithrombin. It seems to us to be more probable that dilution with small quantities of water disturbs the surface tensions of the colloidal aggregates and thus starts the change from the emulsoid to the suspensoid state, a conclusion which is borne out by the non-coagulative action of the isotonic salt solution. Dilution with large quantities of water inhibits coagulation. The mass action of excess of water not only retains the colloids of the plasma in an emulsoid form, but, we have found, so far stabilises them that on evaporation at atmospheric temperature in a desiccator to the original or even a smaller volume the property of clotting is destroyed. On evaporation to complete dryness the residuum is not in fibrillar form. The power to clot is apparently a function of the water content of the colloidal aggregates, a fact not to be lost sight of in discussing the action of inhibitors of coagulation.

(5) *Dilution with excess of calcium chloride.* Dilution with a considerable excess of this salt inhibits coagulation. Employing a 2 % solution we have found that evaporation at atmospheric temperature does not produce clotting, so far as can be observed, at any stage in the concentration of the liquid. The calcium ions appear to be unable to overcome the initial hydrolysis of the colloidal aggregates occasioned by the mass action of excess of water.

(6) *The rôle of blood platelets.* These are regarded by many physiologists as playing an important part in coagulation. Their very existence in normal circulating blood, however, has been denied. Buckmaster [1906] has pointed out that they may be produced by fixing fluids, by contact with foreign bodies and by lowering the temperature from 37° to 18° or 20°. They accumulate round a cotton fibre introduced into the circulation, and if blood is received into a glass vessel the platelets are found adherent to the glass, coagulation commencing from the walls of the vessel and spreading from without to the centre. Both the fibre and the glass are electro-negative occasioning free positive ions, and these latter being of opposite sign to at least some of the colloidal constituents of the plasma, disturb its condition. The formation of platelets appears to be analogous to the formation of spherules round a thread immersed in a solution of calcium chloride and placed in a boiled egg-white solution as observed by Hardy [1899].

Cramer and Pringle state that if mammalian blood is received into paraffined vessels, intact platelets are found and account for the fact that no coagulation is produced, by assuming that thrombokinese is not liberated. As was suggested above, even in blood remaining in a fluid condition, changes towards coagulation are already in progress. In mammalian blood the platelets appear to be the first visible sign of change and their aggregation when in contact with glass or a thread may be explained by the disturbing effect of altered surface tensions and by the disruptive activity of positive ions. We have no need to assume the liberation of any thromboplastic substance, as the physical forces at work supply a sufficient explanation. The observations of Mines [1912] on the adherence of the positively charged corpuscles to those negatively charged, when the blood of *Scyllium canicula* is mixed with certain concentrations of cerium chloride, afford an interesting comparison. When platelets are influenced by positive ions arising from the contact of the blood with glass or string, their aggregation may be due to a similar cause. We have observed in the first stages of the coagulation of frogs' blood in oil, that the corpuscles move freely in the surrounding gel on slight pressure, later, rouleaux appear, followed rapidly by complete coagulation. In the light of Mines' work, the formation of rouleaux indicates electrical change and it seems it is to electrical phenomena rather than to the liberation of thromboplastic substances that we must look for the initial development of coagulation. The divergence of opinion as to the existence or absence of platelets in normal circulating blood may possibly be explained by their formation when the formed elements of the blood are disintegrating and their absence when this process is not taking place. We have seen that a colloid may be partly precipitated by the addition of a small quantity of an electrolyte and that this precipitate may dissolve rapidly on diffusion of the electrolyte; the solution, moreover, exhibits some resistance to further precipitation. In a similar manner may not platelets dissolve under normal conditions and thus interact with the salts of the plasma to form a substance with anticoagulative properties?

(7) *The action of peptone and of lung extract.* It has been assumed that peptone or some impurity therein activates the formation of antithrombin by the liver. This organ is not, however, an essential factor in the production of fluidity in shed blood, as Pavloff [1887] found that the circulation of a mixture of defibrinated and living blood through a heart lung preparation yielded a fluid which remained uncoagulated for several days. In the following simple experiment the action of the liver was eliminated:

The aorta of a pithed cat was clamped near the heart so that blood passed only through the pulmonary and coronary circuits. Blood from the carotid prior to occlusion of the aorta withdrawn through a paraffined cannula commenced to clot in 3 minutes and was completely clotted in 5 minutes. Seven minutes after clamping the aorta blood withdrawn in the same manner commenced to clot in $9\frac{3}{4}$ minutes and was completely clotted in $14\frac{1}{4}$ minutes.

Under the conditions of this experiment therefore a short "negative phase" was produced.

Mason [1921] has recently drawn attention to the fact that a number of electro-negative substances accelerate coagulation, citing a considerable list which included carbonic acid, while Mills [1921] has stated that lung extract, a powerful intravascular coagulant, is also electro-negative in neutral solution. The experiments of Wright [1894] on the coagulability of blood during the breathing of various mixtures of carbon dioxide and oxygen, tend to show that the latter diminishes coagulability, while the former in certain concentrations increases it. In the experiment detailed above it seems probable that the great increase of oxygen and oxyhaemoglobin was the cause of the retarded coagulation. Oxygen and oxyhaemoglobin are electro-positive and presumably the free ions in the adjacent field are electro-negative, that is, of the same sign as the coagulable constituents of the plasma. We have again an apparent similarity to the phenomena of precipitation, as the presence of free ions of the same sign as that of the substances in solution tends to stability, while free ions of the opposite sign tend to change from the emulsoid to the suspensoid state.

SUMMARY.

(1) In the slow precipitation of gelatin by alcohol a condition has been found closely analogous to the "negative phase" of blood coagulation and to the "Dansyz reaction."

(2) If dilute filtered tissue extract be added drop by drop to birds' blood *in vitro* a temporary "negative phase" (or "retarded positive phase") in its coagulation is obtained. An equal volume of tissue extract added rapidly to the same volume of blood, under otherwise similar conditions, causes rapid coagulation.

(3) The concentration of tissue extract, the speed of its addition and the temperature of the reaction are factors which determine the occurrence of either a "negative phase" or rapid coagulation.

(4) In view of these *in vitro* reactions it is submitted that the necessity is obviated for assuming the existence of the following substances in discussing blood coagulation: antithrombin, proantithrombin, antiprothrombin, anti-fibrinolysin, prothrombokinase, and metathrombin.

(5) The non-occurrence of thrombosis in normal circulating plasma owing to disintegration of formed elements in the blood is a similar phenomenon to the "negative phase" following the slow injection of tissue extracts and is probably a physical process.

(6) The fluidity of birds' blood in clean glass vessels is a function of its temperature.

(7) In shed mammalian blood and in frogs' blood, the formation of a reversible gel precedes the formation of the true clot. This observation confirms the view of Wooldridge as to the formation of thrombin in coagulation.

(8) Many of the recognised facts of blood coagulation are explicable as physical phenomena.

(9) Attention is directed to the respective electrical charges of accelerators and inhibitors of blood coagulation, also to the similarity of their electrical condition to those associated respectively with precipitation and with maintenance of solution. It is suggested that coagulation of blood commences as a physical and reversible process and ends in a chemical reaction in which there are at least two products, viz. fibrin and thrombin.

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Protocols of experiments with birds' blood.

In all these experiments the blood was withdrawn through a glass cannula into glass vessels treated successively with hot alkali, hot acid, water, alcohol and ether. The tissue extract was prepared from kidney by the sodium chloride method.

(1) *Pithed fowl.* Blood in contact with tissue commenced to clot in 3 minutes 50 seconds; completely clotted in 6 minutes.

To a certain volume of blood 2.8 cc. tissue extract were added as rapidly as the burette would permit at 4.1 p.m. At 4.10 p.m. clots were observed to form. At 4.20 p.m. clotting complete.

To an equal volume of blood 2.8 cc. of the same tissue extract were added slowly from 4.5 p.m. to 4.25 p.m. At 5 p.m. the first signs of clotting were observed. Only at 5.40 p.m. was clotting complete.

An equal volume of blood without the addition of tissue extract remained fluid till observations ceased. Temperature 17°.

(2) *Pithed fowl.* Blood in contact with tissues commenced to clot in 3 minutes 50 seconds, completely clotted in 6 minutes.

To a certain volume of blood 7.3 cc. of tissue extract were added as rapidly as possible at 5.1 p.m. At 5.8 p.m. slight clots appeared. At 5.23 p.m. the clots firm and contracting.

To an equal volume of blood 7.3 cc. of tissue extract were added slowly from 3.58 p.m. to 4.17 p.m. At 4.49 p.m. first signs of clotting observed. At 5.40 p.m. clotting apparently complete.

To blood from the same animal was added a feather. Clotting appeared to commence in 35 minutes and to be complete in 45 minutes.

Blood from the same animal in clean glass had not clotted when observations were abandoned. Temperature 17°.

Repetition of these observations with tissue extract of double the concentration gave no difference between "slow" and "fast" addition of equal volumes to equal volumes of blood. The time of clotting in each case was $4\frac{1}{2}$ minutes.

(3) *Pithed fowl*. Blood in contact with tissue clotted in 7 minutes.

To a certain volume of blood 3 cc. tissue extract were added as rapidly as possible at 3.5 p.m. Clotting first observed at 3.17 p.m., apparently complete at 3.25 p.m.

To an equal volume of blood 3 cc. tissue extract were added from 2.57 p.m. to 3.27 p.m. At 3.52 p.m. first sign of clotting was observed, at 4.2 p.m. the clot was apparently complete.

Blood from the same animal remained fluid in contact with glass until observations were abandoned.

Other experiments of precisely the same nature as above have given the same results in each case.

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