THE VISCOUS METAMORPHOSIS OF THE BLOOD PLATELETS.

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PLATE 37.

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The phenomenon of agglutination and fusion of the blood platelets into glassy masses and strands, as observed in films of wet blood between a slide and cover-glass, was first described by Hayem (1) in 1878. Some years later, it was more exactly described by Bizzozero (2) and named "viscous metamorphosis" by Eberth and Schimmelbusch (3). It occurs during the process of coagulation and is well marked at the time of the first appearance of the fibrin threads. It does not take place if sodium oxalate or certain other salts have been added to the blood during the bleeding in sufficient amount to prevent coagulation and if other conditions are favorable. Mosen (4) in 1893 was the first to show that platelets from such a non-coagulating blood, freed from blood elements by centrifuging, do not fuse with one another, but retain their separate identity. They appear when in suspension in isotonic salt solution and observed with medium powers between a cover-glass and slide, as refringent, glistening granules or round bodies of a diameter varying approximately from one-fourth to one-half that of a red blood corpuscle (Fig. 1); they are not clumped but uniformly distributed and remain so until they disintegrate. We have found, however, that such platelets normally have not lost the ability of metamorphosis, but that they undergo the characteristic changes within a few minutes if serum or certain more or less closely related substances are added to the suspension. As observed under the conditions just mentioned, the platelets begin their metamorphosis by aggregating and agglutinating in masses and strands which increase in size up to a certain point within a few minutes. Then within a few more minutes, the plate-

lets in these masses and strands lose their high refrangibility, seem to fuse one with another, and their separate identity becomes lost or obscure. The masses and strands assume thereby the appearance of a more or less homogeneous, glassy, refringent substance in which refringent granules are usually present. During this transformation or viscous metamorphosis some contraction of the volume of the masses and strands may occur and by this contraction in volume, bands and strings of hyaline material may be produced, which unite masses of platelets in the process of transformation or already transformed. The peripheral or marginal portions of the masses and strands are usually more hyaline than the central parts and the marginal outlines are finely wavy and denticulate.

This metamorphosis of the isolated platelets in the presence of serum has been briefly described in recent papers by Lee and Robertson (5) and by Minot and Lee (6) in which also some results of experiments with other substances are noted. The present paper is based on further experiments on this line in the endeavor to determine the nature of the substance in the serum which causes or permits the metamorphosis to take place.

It should be noted that this metamorphosis is to be distinguished from simple agglutination of platelets without fusion, which may be caused by certain substances. The microscopic appearances of a weak simple agglutination are shown in Fig. 2. Here the agglutinated platelets retain their separate identity.

Figs. 3, 4, and 5 show the appearances of suspensions of platelets similar to that shown in Fig. 1; the suspensions have undergone the viscous metamorphosis.

Lee and Robertson (5) have shown, and it may be emphasized here, that this phenomenon of agglutination and fusion of platelets associated with coagulation is to be distinguished from agglutination and lysis of platelets caused by antiplatelet serum.

The phenomenon seems to begin when the first signs of clotting are seen and is thus perhaps the first demonstrable sign of the clotting process. This has been our experience with not only normal blood, but blood with delayed coagulation time, as from cases of hemophilia, pneumonia, and jaundice, no matter whether observed in whole blood in glass tubes, or from cloudy plasma (plasma relatively rich

in platelets) obtained from centrifugalized whole blood in iced paraffined tubes. In the abnormally slow coagulating cloudy plasma one may observe that clotting begins near the edge of the tube and microscopic preparations from this portion of the plasma will show the fusion of the platelets, while material obtained at the same time from the still fluid portion will show the platelets free.

Method.

Rabbit and human platelets were used. The blood was drawn from the rabbit's ventricle or from the veins of man by means of a needle and syringe. Before drawing the blood, a sufficient quantity of freshly prepared 1 per cent solution of sodium oxalate in 0.9 per cent sodium chloride solution was placed in the barrel of the syringe to constitute at least one part to nine parts of blood. Sometimes the needle and barrel of the syringe were coated with paraffin. In order to obtain platelets that are unaltered and suitable for studying the viscous metamorphosis, it is important that the needle enter the ventricle or the vein at the first thrust so as to avoid contamination with the tissue juice as much as possible, and that the blood should flow quickly and freely into the syringe and be rapidly mixed with the oxalate solution. If these conditions are not observed, the platelets are likely to undergo more or less fusion before isolation or to be otherwise altered so that they will not undergo the characteristic metamorphosis. Slight alteration in them, inappreciable in any change in microscopic appearance and often impossible to control, seems to render them incapable of undergoing the changes. Many times we have isolated platelets in apparently intact and unaltered condition which showed themselves but feebly active or inert.

On the other hand, platelets may be quite grossly altered without essentially affecting their activity as cytozyme (Bordet and Delange (7)) or as thromboplastin (Howell (8)). Even after they have undergone their metamorphosis we have found them capable of acting efficiently as factors in coagulation of the blood.

The platelets were separated from the other elements of the blood by centrifuging at low and high speeds, and were at least twice sus-

pended and thrown down in 0.9 per cent sodium chloride solution. Unaltered platelets, thus isolated and washed, form a white, semi-fluid, somewhat sticky sediment at the bottom of the centrifuge tube and they are readily miscible with salt solution by agitation with the pipette. If the sediment of the platelets forms an elastic coherent mass or masses, some metamorphosis has already occurred and such platelets are not suitable for use.

The isolated platelets in salt solution retain their ability to undergo the metamorphosis for a period varying from usually a few hours to rarely 2 or 3 days. This ability seemed to be retained longer at room temperature than in the ice box or incubator, and also if a very small amount of oxalate had been added to the salt solution.

The blood elements used in studying this phenomenon of agglutination and fusion of the blood platelets were usually obtained from rabbits. Most of the experiments were also done with human material. No differences in the results were noted when the platelets came from man or rabbit and the various other substances came from the same animal or a different animal of the same or different species. It was found rather more difficult to obtain suitable platelets from the arm veins of normal men than from the hearts of normal rabbits.

In testing the effect of the various fluids and solutions on the isolated platelets a thick suspension in 0.9 per cent sodium chloride solution was found best to use. This was prepared usually by mixing the sediment of platelets at the bottom of the centrifuge tube with three or four drops of the solution. It was found important that the platelets should be well mixed with this salt solution so as to be separated from one another and that they should be used in a very thick suspension as described. A very thin suspension of platelets was found undesirable for it seemed possible for the platelets to go through their metamorphosis in but tiny clumps or singly, so that the phenomenon was not clearly observable. Again, very compact masses of platelets were not used both on account of a possibility of an excess of platelets altering the results and also because the substance affecting the platelets may not penetrate to the center of the mass so that the metamorphosis will be observed only about the edge of the mass.

After a satisfactory suspension of blood platelets was obtained the action of various substances upon them was observed by mixing from one to three small drops of the materials in question with a very small drop (obtained from capillary glass tubing) of the very thick platelet suspension on a glass slide, covering with a cover-glass, and observing microscopically.

Effect of Serum, Thrombin, Calcium, and Antithrombin and Thromboplastin.

Serum was capable of producing well marked metamorphosis of the platelets usually in 2 to 7 minutes. Sometimes the change did not occur until after longer periods, up to 20 minutes or more. The time required varied both with different lots of platelets and with different sera. It was not clearly dependent on the age of the serum, although it may be said that sera a few hours old often seemed more effective than when very fresh, or sera 10 or more hours old.[1]

This power of serum was found present on some occasions in dilutions (made with 0.9 per cent salt solution) of 1: 10, on other occasions in dilutions as high as 1: 50. The greater the dilution the longer it took for the metamorphosis to occur. When calcium was added to the serum dilutions so that they contained 0.05 per cent calcium chloride it accelerated the metamorphosis and caused it to appear with those dilutions in which only slight agglutination had occurred. It is to be noted that with the higher dilutions that caused metamorphosis relatively fewer platelets seemed to be transformed than with the lower dilutions, suggesting a quantitative action of the substance that metamorphoses platelets.

Table I is a typical protocol showing the effect of dilution of serum with and without calcium.

With serum this phenomenon can be demonstrated macroscopically. If several small drops of a thick platelet suspension are added to 1 cc. of serum they will be at first evenly distributed through the serum giving it a cloudy appearance. When the metamorphosis has occurred they will be found clumped in the bottom of the tube and

[1] Rarely a serum produced no effect.

TABLE I.

Serum dilution with 0.9 per cent sodium chloride solution.	One drop of 0.9 per cent sodium chloride solution.		One drop of 0.5 per cent calcium chloride solution in 0.9 per cent sodium chloride solution.	
		min.		*min.*
0	F*	2	F	2
1 : 6	F	5	F	2
1 : 15	F	8	F	4
1 : 25	F	13	F	6
1 : 40	F	20	F	12
1 : 60	A	15	F	18
1 : 80	A?	20+	F	20
1 : 100	0		F	25
1 : 120	0		A	20
1 : 150	0		A?	25
1 : 200	0		0	

* The figures in the table give the time in minutes for definite fusion (F) (viscous metamorphosis) or slight agglutination (A) of the platelets to take place under the given conditions.

upon shaking the tube the platelets will be seen to appear as small granular masses in a clear serum.

When platelets were treated with distilled water some portion of them went into solution and they lost their glistening appearance. Such a solution has definite thromboplastic (cytozymic) action in contrast to salt solution in which platelets have stood and been removed. The remaining shells of the platelets found after the platelets had been exposed to water were slightly clumped by serum, but a true metamorphosis did not take place.

Serum heated to 56°C. for half an hour or serum heated up to 60°C., at which temperatures thrombin is destroyed, caused no metamorphosis of platelets but did permit an agglutination of the platelets, and on some occasions seemed to allow a very slight lysis of the platelets. Platelets so treated, however, could still be metamorphosed by unheated serum, though possibly not so actively as when added directly to serum. This heated serum contained, as far as elements of coagulation are concerned, essentially only antithrombin and calcium.

From the above experiments we were inclined to believe that the viscous metamorphosis of platelets was due essentially to thrombin

aided by calcium. This, however, does not seem to be true, not only because of the results with "serozyme" (7), egg white, etc., described later, but also because repeatedly solutions of very active pure crystallized thrombin, prepared by Howell's method (9), did not produce metamorphosis of the platelets; although a slight slow clumping sometimes occurred there was never any fusion. If, however, calcium was added to the pure thrombin solution the platelets were often quite rapidly agglutinated into little ball-like masses but there was no metamorphosis.

Centrifuged, clear, oxalated plasma heated to 60°C. contains antithrombin but essentially no other coagulating element. Such antithrombin produced usually a very slight agglutination but no metamorphosis of the platelets. If, however, calcium was added, an agglutination, and possibly on some occasions, slight lysis occurred exactly as occurred but to a greater degree with the heated serum which contained the same two elements, antithrombin and calcium. When thrombin and antithrombin were mixed and allowed to stand 1 minute or 1 hour, the effect of the mixture on the platelets was the same as with thrombin or antithrombin alone. Antithrombin incubated with serum for a longer or shorter period did not have any definite effect on the ability of the serum to metamorphose platelets.

When pure thrombin and antithrombin from serum heated to 60°C. were mixed for 1 minute or 1 hour and calcium was added before or after the substances had remained in contact, the action of the mixture on the platelets was the same. Such a mixture produced a marked clumping of the platelets, faster and more marked than with thrombin and calcium or antithrombin and calcium. Occasionally this mixture also slowly caused a true metamorphosis of the platelets. Usually this was found not to be the case, but rather that besides the agglutination, a pseudo- or abortive metamorphosis occurred. This pseudo- or abortive metamorphosis consisted simply of a very marked agglutination with apparently a slight lysis of the platelets. On staining with Wright's blood stain the individual platelets in these masses were distinct and fairly sharply outlined and did not tend to form a homogeneous fused mass as was seen when true viscous metamorphosis occurred. Platelets so altered or platelets simply agglutinated by antithrombin and calcium or thrombin and

calcium were still capable of being metamorphosed with serum, but apparently not quite so effectively as unaltered platelets. This indicates that the process of agglutination of the platelets is to be distinguished from the viscous metamorphosis, though agglutination of some type is an integral part of the viscous phenomenon.

When mixtures of pure thrombin and calcium with antithrombin from oxalated plasma rather than from serum were mixed with platelets we never observed a true metamorphosis. Such a mixture did allow definite agglutination and at times a weak pseudo- or abortive metamorphosis.

Thromboplastin in various forms (fresh tissue juice, cephalin, or coagulen[2]) did not apparently influence the platelets either alone or when added in reasonable amount to any of the various substances or mixtures in the experiments outlined above or below.

Effect of Serozyme, Oxalated Plasma Plus Thrombin, and Fibrinogen.

Oxalated plasma whether dialyzed or not had, of course, no effect on the platelets, and when oxalate was added to any of the substances referred to above or below, it either prevented or weakened (if relatively little was added) their action on platelets. When the amount of calcium that clotted most quickly a given oxalated plasma was mixed with it and platelets were added at once to the mixture, which was then observed under the microscope, the platelets were seen to undergo their typical transformation. They began to do so as the first signs of coagulation appeared. In such a mixture, however, they completed their metamorphosis more slowly than under more favorable conditions. This we attributed to the presence of oxalate, for when a plasma had been dialyzed to remove the excess of oxalate, and calcium was then added to it with platelets, the latter elements underwent their metamorphosis more rapidly than when the control undialyzed oxalated plasma was used. This was true whether fresh plasma or dried plasma a year old, but freshly dissolved, was used.

The fluid resulting from the defibrination of a clot, formed with oxalated plasma and an optimal amount of calcium, is called by

[2] Commercial preparation made by the Society of Chemical Industry, Basle, Switzerland.

Bordet and Delange (7) "serozyme." When this is freshly prepared it often contains free thrombin. On standing some of the thrombin seems to combine with antithrombin and other portions of the thrombin become perhaps altered in some manner so that free thrombin cannot be demonstrated. A good serozyme, *i.e.*, one without free thrombin, as evidenced by its inability to clot fibrinogen in 24 hours, is to be obtained from a very clear rather than a cloudy plasma. Serozyme and cytozyme (tissue juice, thromboplastic substance) and calcium form thrombin in Bordet and Delange's terminology as is shown by the fact that after this mixture has stood for about 6 minutes it clots fibrinogen in 1 to 2 minutes, while suitable controls form no thrombin. Serozyme was found to be usually as active in causing the metamorphosis of platelets as serum, and its activity increased if a mere trace of calcium was added to it. It seemed to make little difference whether the serozyme contained free thrombin or not. Though rather definite in some instances but not in all, a serozyme containing free thrombin, prepared from cloudy plasma, seemed to be not quite so active in causing the platelet metamorphosis as a serozyme that had no free thrombin. This would suggest that the substance causing platelet metamorphosis had been partly used up in transforming the platelets in the cloudy plasma.

The following observations on the action of a mixture of Howell's pure thrombin and oxalated plasma were made. When these two substances, which form a clot in about 3 minutes, were mixed, and platelets added at once, the typical viscous metamorphosis of the platelets occurred usually in 2 to 3 minutes, provided apparently that the oxalated plasma was not oxalated to contain above about 0.1 per cent of oxalate. On some occasions, usually if the oxalation was excessive, only a rather marked agglutination or pseudometamorphosis of the platelets occurred about the fibrin strands, which suggests, as do the experiments with the mixture of thrombin, serum antithrombin, and calcium, that this phenomenon is an abortive type of true metamorphosis.

When thrombin and dialyzed oxalated plasma were used the platelets were always metamorphosed.

We may note that presumably all the substances mentioned before which produced this change in the platelets contained at least

traces of ionized calcium. Perhaps, with the rearrangement of the colloidal substances which occurs when thrombin clots the fibrinogen of the oxalated plasma, some calcium is temporarily set free. That some substance, as well as possibly calcium, is set free in this reaction which metamorphoses the platelets, is suggested by the above experiments, and that this substance then rapidly becomes relatively inactive is suggested by the following observations.

The fluid resulting from the complete clotting of oxalated plasma with thrombin seldom permitted the viscous metamorphosis of the platelets; sometimes a very slow and poor but true transformation occurred. This result was probably due to the oxalate, because the fluid resulting from thrombin and dialyzed plasma did permit, though always slowly (20 minutes) the transformation of the platelets. Such delayed reactions are in contrast to the relatively rapid effect on platelets during the clotting of plasma with thrombin. If, however, calcium was added to either of the fluids resulting from clotting dialyzed or undialyzed oxalated plasma with thrombin, they then not only always permitted a metamorphosis of the platelets, but also a rapid one (5 minutes). This may have been partially due to the effect of calcium on the oxalate, besides its evident accelerating action, as well as perhaps to some change that occurred in these fluids when the calcium acted on the prothrombin present in them. That these fluids contained prothrombin is evident, because this substance was in the oxalated plasma and there had been no calcium added to transform it to thrombin.

Magnesium sulfate plasma and thrombin acted essentially like oxalated plasma and thrombin.

Solutions of fibrinogen, prepared according to a modification of Hammarsten's method, were found to have no effect on the platelets. These fibrinogen solutions unfortunately were not absolutely free of prothrombin, but contained enough to clot in 3 hours with the optimal amount of calcium. The results of experiments with thrombin and this fibrinogen were essentially like those with thrombin and dialyzed plasma, except that the metamorphosis of the platelets was never marked even when calcium was added to the serum from the coagulation of this fibrinogen by thrombin. It would seem that, during the process of clotting fibrinogen there occurred, with the

rearrangement of the colloidal particles, a setting free of some substance allied to serozyme which was very active on the platelets, and, with the completion of such coagulation, relatively little substance was left free, though addition of calcium, perhaps by its property of aggregating colloidal particles, would permit the substance causing platelet metamorphosis to become more active.

Absorption from Serum of the Substance Causing Metamorphosis.

Experiments were conducted to determine whether one could absorb from serum the substance that transforms platelets. Barium sulfate absorbs serozyme-like substances as shown by Bordet and Delange and Lee and Vincent (10), but it does not seem to affect formed thrombin in Bordet and Delange's sense. We found that when 1 cc. of serum was treated with 0.4 cc. of washed barium sulfate for 2 hours and then centrifuged clear, that the serum had lost its power of metamorphosing platelets even after calcium was added.

The effects of adding large amounts of cephalin to serum were next studied. A relatively large amount of cephalin in solid form was added to serum so that some of it dissolved in the serum. When this mixture was then centrifuged, so that the resulting fluid was clear, it was found to have lost its ability to metamorphose platelets, though sera so treated were able to clot fibrinogen. When calcium was added to such serum it did not restore the ability to metamorphose platelets. This effect of cephalin was obtained in but a third of the numerous times it was tried on 6 different days. It seemed that if the sera could be centrifuged so that they were clear after treatment with cephalin, this substance was much more apt to be effective than if the centrifuged sera remained at all cloudy. Sera, when somewhat diluted with salt solution to which thick suspensions of platelets were added and then centrifuged clear after the platelets were fully agglutinated and fused, sometimes lost their ability to further metamorphose platelets, as was the case with sera treated with cephalin. Undiluted sera could not be affected in this way, probably because too few platelets were added. Similar results were obtained upon treating sera with cholesterolized syphilitic antigen as with cephalin and platelets. We have found no explanation for these inconstant results.

It was thought that the power of syphilitic serum to metamorphose platelets might be more easily exhausted with syphilitic antigen than normal serum, in view of the inhibitory effect of syphilitic serum in Bordet and Delange's method of forming thrombin as shown by Hirschfeld and Klinger (11). No such difference, however, could be detected.

Starches of various kinds, agar, gelatin, gold chloride, cholesterol, and chloroform in various strengths, and various other substances did not, after being mixed with serum for 2 hours, alter its ability to produce this metamorphosis. Neither did these substances alone metamorphose the platelets.

Ability of Serum Globulin and Egg White to Metamorphose Platelets.

In order to determine whether the power of serum to metamorphose platelets resided in the globulin or albumin fraction, the following experiment was made. Serum was dialyzed against distilled water. When a small amount of precipitate had appeared at the bottom of the dialysis sac, the serum was still active. Later, when a considerable amount of precipitate occurred in the dialysis sac, the fluid with or without calcium was inactive on the platelets. The dialysate was also inactive. The precipitated globulin, when redissolved in salt solution with the aid of a trace of dilute alkali, which of itself did not affect the platelets, caused a feeble metamorphosis of the platelets. However, when calcium was added to this solution a definite active metamorphosis of the platelets was produced with it.

Egg white alone or shaken with isotonic salt solution had a marked effect on the platelets. It caused them to be rapidly metamorphosed and often seemed to act more efficiently than serum. This result suggested that egg white contains a serozyme-like element. Experiments showed this to be the case, for it was found that it could take the place of serozyme in the formation of thrombin by the method of Bordet and Delange. Hirschfeld and Klinger (12) have recently also detected a serozyme-like action of egg white. Egg white alone apparently does not clot fibrinogen and therefore does not contain formed thrombin.

Numerous pure proteins,[3] lactalbumin, gliadin, legumin, vignin, edestin, and cottonseed globulin, and also milk with or without calcium had no effect on the platelets.

Metamorphosis of Platelets from Abnormal Bloods.

The platelets from cases of hemophilia, pneumonia, purpura hæmorrhagica, purpura rheumatica, and obstructive jaundice were transformed by either their serum or normal serum in the same way and essentially in the same time as normal platelets. In this connection it is to be noted that hemophilic platelets are, however, defective in their cytozymic action and that they do not begin to fuse until the blood shows visible signs of clotting, as has been shown by Minot and Lee (6). On this account the metamorphosis of hemophilic platelets and normal platelets was studied with those substances causing metamorphosis of normal platelets, derived from normal and hemophilic blood, but no real differences in the character or rate of metamorphosis could be detected.

SUMMARY AND CONCLUSIONS.

From the experiments above described it is evident that the viscous metamorphosis of the blood platelets in shed blood is intimately associated with the early stages of coagulation and that the presence of calcium is a very important element, though perhaps not absolutely necessary. No constant differences in this phenomenon could be detected with platelets or coagulating elements from the blood of normal or diseased individuals or from man or rabbits.

All the substances, with the exception of egg white, causing this phenomenon were derived from the blood either during the alteration of fibrinogen or after it had been acted on by thrombin. It seems that the substances or mixtures capable of producing the metamorphosis are especially those associated with the early stages of coagulation or capable in the test-tube of forming or liberating that active coagulating element known as thrombin.

The substance in serum that is capable of metamorphosing platelets seems to be attached to the globulin fraction rather than the

[3] These proteins were kindly furnished by Dr. W. Denis.

albumin fraction and is destroyed by heat at temperatures which destroy prothrombin, thrombin, and serozyme, and precipitate fibrinogen.

The reaction is not caused by pure thrombin or a mixture of pure thrombin and calcium, though substances causing the metamorphosis are intimately related to thrombin. The metamorphosis seems to be caused by serozyme-like substances as shown both by the fact that barium sulfate absorbs the power of serum to cause the reaction and that a serozyme-like substance is probably to be recognized in all the substances or mixtures, including egg white, causing this change in the platelets, except perhaps when thrombin and fibrinogen are reacting. It is to be noted that in serozyme are contained antithrombin, calcium, and potential thrombin, and that a combination of these isolated factors mixed together occasionally allowed the viscous metamorphosis to occur and not infrequently an abortive metamorphosis.

The pseudo- or abortive metamorphosis caused by the mixture of pure thrombin, antithrombin, and calcium may be interpreted on the supposition of a close approximation but not a real reproduction of the colloidal state known as serozyme. That such a reaction is related to real viscous metamorphosis is suggested, because it sometimes occurred with the above mixture and when thrombin and heavily oxalated plasmas were reacting, rather than a real metamorphosis.

Simple agglutination of the platelets may occur independently of a viscous metamorphosis, though an agglutination of platelets is to be considered an integral part of the viscous metamorphosis phenomenon.

The inconstant results seen with the cephalin-treated sera are probably due to the fact that exactly the same mixtures were not obtained. The results with cephalin-soaked and platelet-soaked sera and with dilutions of sera suggest that the reaction of viscous metamorphosis of the platelets is quantitative.

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EXPLANATION OF PLATE 37.[4]

FIG. 1. Suspension of blood platelets in isotonic salt solution. × 300.

FIG. 2. Simple, weak agglutination of platelets. There are fewer platelets in this preparation than in those shown in the other figures. × 300.

FIGS. 3, 4, and 5. Viscous metamorphosis of platelets. Essentially the same number of platelets was in these preparations as in the preparation shown in Fig. 1. × 300.

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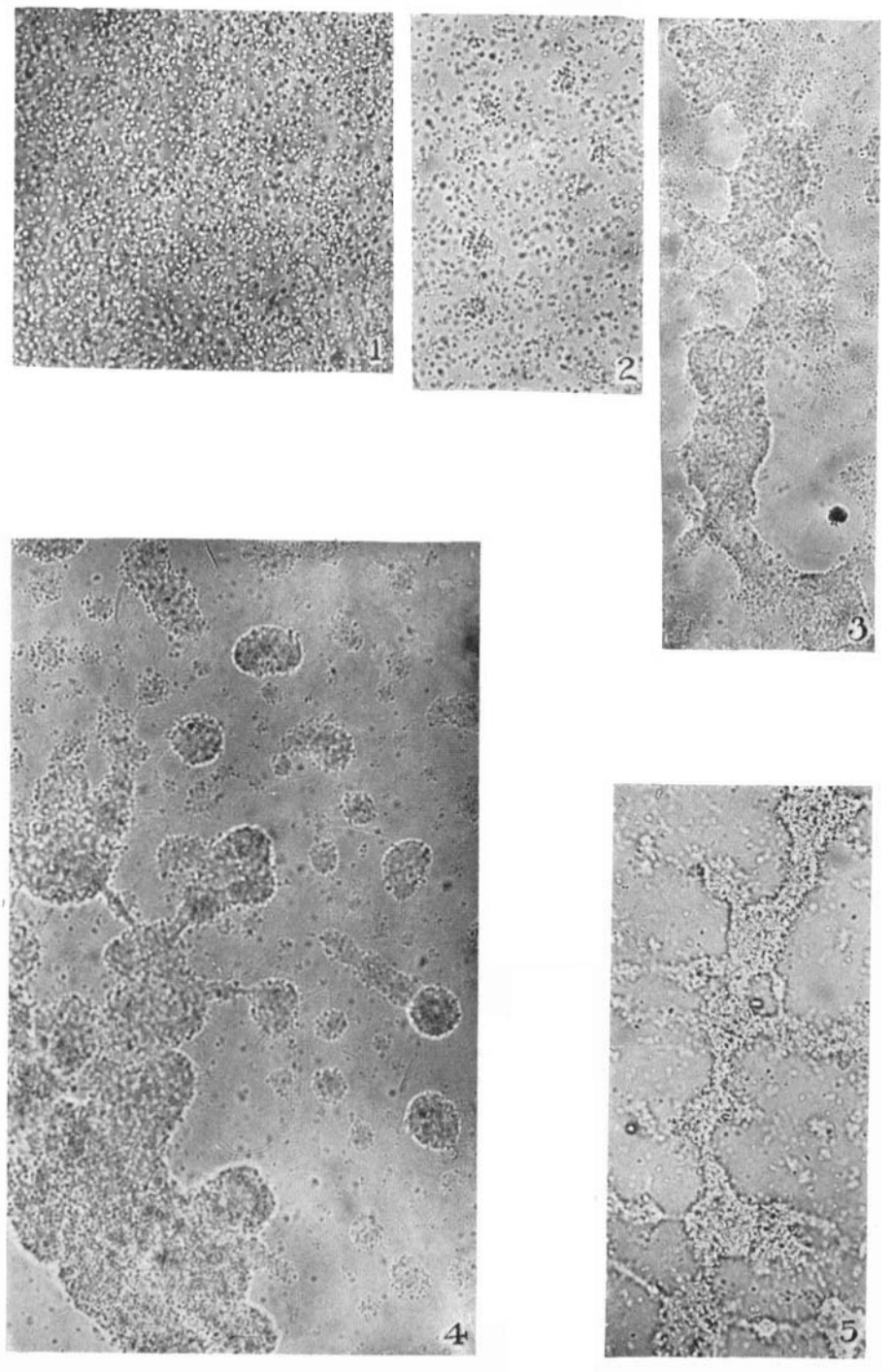
[4] The photographs were made by Mr. L. S. Brown.



(Wright and Minot: Viscous metamorphosis of blood platelets.)