Platelet aggregation

Part I Some effects of the adenosine phosphates, thrombin, and cocaine upon platelet adhesiveness

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SYNOPSIS Platelets in native blood adhere spontaneously to glass independently of temperature. If adenosine diphosphate is added to the blood the adhesiveness of the platelets is increased and this effect is largely independent of temperature. The mono- and triphosphates decrease adhesiveness at 20°C. and 37°C. but have no effect at 0°C.; cocaine inhibits adhesion at 37°C. and at 0°C.

Aggregation and viscous metamorphosis of platelets in native plasma is induced at 37°C. by adenosine diphosphate or by thrombin; these reactions do not occur at 0°C. Cocaine and all the other anti-adhesive drugs inhibit thrombin or adenosine diphosphate-induced aggregation. The mono- and tri-phosphates appear to compete with adenosine diphosphate and inhibit aggregation; they also inhibit thrombin-induced aggregation. Aggregation induced by adenosine diphosphate or thrombin is not prevented by any of the usual enzyme inhibitors or uncoupling agents at the appropriate strength. At 37°C. aggregation and viscous metamorphosis induced by adenosine diphosphate or thrombin are reversible, and the addition of more adenosine diphosphate or of thrombin again produces aggregation and viscous metamorphosis.

Platelets incubated with adenosine diphosphate but not agitated lose their power to aggregate but when more adenosine diphosphate is added with agitation, then aggregation is again produced. These observations are presumably explained by the finding that intact platelets, but not fragmented platelets, can inactivate adenosine diphosphate. From these results it is tentatively concluded that adhesion may involve intrinsic adenosine diphosphate in the platelet which may be activated by thrombin and inhibited by the added mono- or triphosphate. The anti-adhesive drugs act in a different manner. These phenomena have a remarkable similarity to those concerning mitochondrial swelling.

It is not known why a platelet sticks, but Hellem (1960) reported that a factor R isolated from red cells caused platelets to stick to glass; Øllgaard (1961) also studied a non-protein extract from platelets and red cells that caused platelet aggregation. Gaarder, Jonsen, Laland, Hellem, and Owren (1961) showed that their factor is adenosine diphosphate, that it is highly specific, and that it induces platelet aggregation in citrated platelet-rich plasma. O'Brien (1961) showed that the adhesion of native platelets to glass and to damaged cells *in vitro* and *in vivo* was inhibited by many anti-malarial, anti-histaminic and local anaesthetic and some other drugs which will be called the 'anti-adhesive' drugs. These findings stimulated the present study of the

effects of adenosine diphosphate and thrombin on platelet adhesiveness to glass and on platelet aggregation and viscous metamorphosis. The effect of the anti-adhesive drugs and adenosine monophosphate and the triphosphate and enzyme inhibitors were also studied in an attempt to understand the processes involved in adhesion, aggregation, and viscous metamorphosis.

METHODS

ADHESION OF PLATELETS TO GLASS Native blood collected in a plastic tube was used at once or after cooling for five minutes in ice-water; the blood was mixed with barbitone buffered saline as a control or with the material under study and was passed immediately through 'filter units' consisting of a standard quantity of glass beads in a

plastic tube (O'Brien, 1961) at room temperature or at 0°C. By counting the platelets in the blood before and after passing them through the filter the percentage of the platelets lost can be calculated; those had presumably stuck to the glass during their passage through the filter.

PLATELET AGGREGATION AND VISCOUS METAMORPHOSIS
Platelet-rich plasma was prepared by slow centrifugation of native, citrated (9 parts of blood to 1 of 3.8% sodium citrate) or heparinized (4 units of heparin/ml. blood) blood. This platelet-rich plasma with or without additions was examined within two hours of collection on a slide under a coverslip so supported that it could be rocked. A 1/6 microscope objective and phase illumination was used. With constant rocking, platelet aggregation and viscous metamorphosis could be observed and roughly timed. Platelet aggregation was also studied in tubes of 7 mm. internal diameter in which usually 0.2 ml. of platelet-rich plasma was mixed with 0.1 ml. of the substance under investigation. The tube was continuously agitated in a 37°C. water-bath, and frequently inspected through a powerful lens with strong transverse illumination.

Viscous metamorphosis is used here to mean the change when a clump of discrete platelets that have stuck together is observed under the microscope to change into a hyaline amorphous mass with a clearly defined, smooth, refractile edge and complete loss of platelet identity.

The reagents used were diluted with 0.9% saline buffered with barbitone buffer to pH 7.35. The adenosine di- and triphosphate and uridine diphosphate were over 99% pure and adenosine monophosphate was 95% pure, and were obtained from the Sigma Chemicals Co. Human thrombin was obtained from the Lister Institute, Ro 3/0837 from Dr. J. Marks of Roche Products, Ltd., and N. butyl 3-5-di-iodo-4-hydroxybenzoate from Dr. R. L. Greif.

RESULTS

From Table I, which reports the mean figure from three to six experiments in each situation, it will be seen that native platelets (saline control) stuck to the glass as well at 0°C. as at room temperature in either the long (8.8 cm.) or short (5 cm.) units. Adenosine triphosphate at a final concentration of 0.2 µg./ml. decreased platelet adhesion at room temperature but had no effect if the experiment was carried out at 0°C. Adhesion was also decreased by adenosine monophosphate, 200 µg./ml., and 0.2 µg./ml. probably still had some effect. Cocaine prevented platelet adhesion equally in the cold and at room temperature. Using the shorter units (fewer platelets stick spontaneously to the reduced glass surface area) it will be seen that adenosine diphosphate at a final concentration of 0.2 µg./ml. greatly increased platelet adhesiveness at room temperature and also when the whole procedure was carried out at 0°C. If adenosine diphosphate was added and the adhesiveness not tested till five

Filter Length (cm.)	Final Concentration of Addition (µg./ml.)	Percentage Platelets Adhering	
		At Room Temperature	At 0°C.
8.8	Saline	71	78
8.8	Adenosine triphosphate, 0.2	16	73
8.8	Adenosine triphosphate, 0.02	55	—
8.8	Adenosine monophosphate, 200	43	—
8.8	Cocaine 3,000	6	10
5.0	Saline	25	49
5.0	Adenosine diphosphate, 0.2	82	—
5.0	Adenosine diphosphate, 2.0	—	80
5.0	Adenosine diphosphate, 0.2 (5 min.) ¹	8	—
5.0	Adenosine diphosphate, 0.02	16	—
5.0	Adenosine triphosphate, 200 + adenosine diphosphate, 0.2	35	—
5.0	Adenosine monophosphate, 200 + adenosine diphosphate, 0.2	14	—
5.0	Cocaine 3,000 + adenosine diphosphate, 0.2	32	—

Native blood at room temperature or after cooling in ice-water for five minutes was mixed with the addition and immediately passed through the filter.

¹Adenosine diphosphate was mixed with the blood and the mixture left for five minutes before it was passed through the filter.

minutes later, the platelets had then become less adhesive than normal. The immediately increased adhesiveness of platelets produced by adenosine diphosphate was inhibited by mono- and triphosphates and by cocaine. It was also shown that when a compound with anti-serotonin activity (referred to here as Ro 3/0837) at a final concentration of 1 mg./ml. blood was added to native blood it reduced platelet adhesion to glass from 73% (saline control) to 22%.

ADENOSINE DIPHOSPHATE-INDUCED PLATELET AGGREGATION AND VISCOUS METAMORPHOSIS Platelets in native plasma, in citrated plasma, and in heparinized plasma aggregated equally when small quantities of adenosine diphosphate were added and the mixture was immediately agitated in a water-bath at 37°C. The speed with which platelet clumps were formed and their size were dependent on the amount of adenosine diphosphate added. The minimum aggregating concentration was found by preparing serial tenfold dilutions of adenosine diphosphate and adding 0.1 ml. of each dilution to 0.2 ml. platelet-rich citrated plasma with a platelet count of 700,000 per c.mm. These mixtures were continuously agitated for one minute and then transferred to a coverslip preparation and inspected for platelet clumps. Adenosine diphosphate at a final concentration of 0.007 µg./ml. produced significant clumping. It can be calculated that approximately 100,000 molecules adenosine diphosphate per platelet produced these changes.

The effect of time and temperature on platelet aggregation was investigated in tubes as follows:

To 0.1 ml. of adenosine diphosphate, 2 $\mu\text{g./ml.}$, was added 0.2 ml of platelet-rich citrated plasma and gross aggregation occurred in 15 seconds at 37°C. if agitation was continuous. If this experiment was carried out at 0°C. no aggregation occurred within five minutes but if the tube was then warmed and agitation continued, good aggregation was rapid. If the adenosine diphosphate and platelet-rich plasma were mixed and incubated at 37°C. without agitation for five minutes and then agitated no aggregation occurred: if more adenosine diphosphate was then added and the tube agitated, aggregation was rapid. Furthermore, it could be shown by the following experiment that the adenosine diphosphate originally added had been inactivated during the five minutes' stationary incubation.

After adding adenosine diphosphate in the strengths A, B, and C shown in Table II to platelet-rich plasma, the tubes were incubated for five minutes: agitation then confirmed that there was no aggregation in tubes B and C. Excess adenosine diphosphate in A had caused aggregation. The platelets in the reaction tubes A, B, and C were centrifuged and 0.2 ml. of each supernatant plasma was transferred into a subsample tube containing 0.2 ml. of the platelet-rich plasma to test for the presence of adenosine diphosphate surviving in the plasma. On agitation, no aggregation occurred in the subsample tubes B and C. As controls, adenosine diphosphate was incubated with platelet-poor plasma and with platelet-rich plasma at 0°C.; subsamples from these mixtures produced aggregation, indicating that the adenosine diphosphate had not been inactivated. If platelets were fragmented by freezing and thawing twice or by ultrasonic agitation, no adenosine diphosphate was inactivated. It is concluded that 1 ml. of this platelet-rich plasma which contained 400,000 platelets per c.mm. could almost inactivate 1,000 $\mu\text{g.}$ adenosine diphosphate, and has inactivated at least 100 $\mu\text{g.}$, or one platelet inactivates 4×10^8 molecules.

The following experiments showed that aggregation and viscous metamorphosis are reversible phenomena.

One drop of adenosine diphosphate, 2 $\mu\text{g./ml.}$, was added to one drop of platelet-rich citrated plasma under a coverslip which was rocked. In 30 seconds platelets had visibly clumped and in 15 minutes viscous metamorphosis was marked with few recognizable platelets remaining. The slide was then incubated in a moist chamber at 37°C. for two hours and inspected. Many of the hyaline masses had disappeared and clumps were recognizable, if distorted, platelets were seen. Agitation caused many platelets to float away. The addition of more adenosine diphosphate again rapidly caused complete aggregation and viscous metamorphosis.

INHIBITION OF ADENOSINE DIPHOSPHATE-INDUCED PLATELET AGGREGATION Uncoupling agents separate the normally interdependent processes of oxidation and phosphorylation in cells or mitochondria. A number of these uncoupling agents and enzyme inhibitors were tested for their ability to prevent adenosine diphosphate-induced aggregation (Table III). It will be seen that none inhibited aggregation when used at the concentration required to inhibit enzyme activity in other systems but at a higher concentration some prevented aggregation. Table III also reports the inhibitory effect of the anti-adhesive agents. In these experiments the weakest final strength of adenosine diphosphate to produce aggregation was 0.5 $\mu\text{g./ml.}$ and a final concentration of 1 mg. of cocaine per ml. prevented aggregation. If 500 $\mu\text{g.}$ adenosine diphosphate was added to platelet-rich plasma the concentration of cocaine had only to be increased to 10 mg./ml. to prevent aggregation. Adenosine triphosphate inhibited adenosine-diphosphate-induced aggregation of platelets in citrated plasma at 37°C. but the amount of the triphosphate required varied according to the amount of adenosine diphosphate present. An approximately equimolar solution did not prevent aggregation but a solution of adenosine triphosphate 10 times stronger than the adenosine diphosphate usually prevented aggregation over the whole concentration range studied (Table IV). On the other hand, the addition of 2 mg./ml. adenosine triphosphate to native or heparinized plasma at 20°C. caused aggregation and did not inhibit adenosine diphosphate-induced aggregation. It was also shown

TABLE II
INACTIVATION OF ADENOSINE DIPHOSPHATE BY PLATELETS

	Shaken after five minutes: Aggregation	Supernatant Added to Platelet- rich Plasma Aggregation
A 0.4 ml. platelet-rich plasma + 400 $\mu\text{g.}$ adenosine diphosphate	Trace	Gross
B 0.4 ml. platelet-rich plasma + 40 $\mu\text{g.}$ adenosine diphosphate	Nil	Nil
C 0.4 ml. platelet-rich plasma + 4 $\mu\text{g.}$ adenosine diphosphate	Nil	Nil
0.4 ml. platelet-poor plasma + 400 $\mu\text{g.}$ adenosine diphosphate		Gross
0.4 ml. platelet-poor plasma + 40 $\mu\text{g.}$ adenosine diphosphate		Gross
0.4 ml. platelet-poor plasma + 4 $\mu\text{g.}$ adenosine diphosphate		Trace

The mixtures were incubated undisturbed at 37°C. for five min. and then agitated. After centrifuging, 0.2 ml. of each supernatant was agitated with platelet-rich plasma.

TABLE III

INHIBITION OF PLATELET AGGREGATION

The following compounds prevent platelet aggregation induced by thrombin (0.4 u./ml.) or adenosine diphosphate (4M $\times 10^{-4}$ /ml.) in the final concentration shown:—

Quinine	1.65M $\times 10^{-3}$
Paludrine	2.5M $\times 10^{-3}$
Cocaine	2.5M $\times 10^{-3}$
Procaine	5M $\times 10^{-3}$
Xylocaine	8M $\times 10^{-3}$
Nupercaine ¹	5M $\times 10^{-3}$
Benadryl	M $\times 10^{-3}$
Phenergan	2M $\times 10^{-3}$
Chlorpromazine	7M $\times 10^{-3}$
Imipramine	7M $\times 10^{-3}$
Ro 3/0837	6M $\times 10^{-3}$
Reserpine	M $\times 10^{-3}$
Sodium citrate	M $\times 10^{-1}$
E.D.T.A.	1.25M $\times 10^{-3}$
Adenosine monophosphate	1.7M $\times 10^{-4}$
Adenosine triphosphate	1.3M $\times 10^{-4}$

The following compounds in the final concentrations shown do not prevent platelet aggregation induced by thrombin or adenosine diphosphate:—

Di-isopropyl phosphofluoridate	M $\times 10^{-3}$
2,4 Dinitro-phenol	5M $\times 10^{-3}$
Potassium cyanide	2.5M $\times 10^{-4}$
Sodium fluoride	2.5M $\times 10^{-4}$
Sodium iodoacetate	2.5M $\times 10^{-4}$
Sodium amytal	4.5M $\times 10^{-4}$
Mercuric chloride ²	2.5M $\times 10^{-5}$
Sodium arsenate ¹	2.5M $\times 10^{-4}$
Arsenious oxide ²	2.5M $\times 10^{-3}$
Sodium azide	2.5M $\times 10^{-4}$
Caffeine citrate ²	1.25M $\times 10^{-4}$
Nicotinamide ²	2.5M $\times 10^{-4}$
N. Butyl 3-5 di-iodo	
4-Hydroxy benzoate	2.5M $\times 10^{-4}$
Strophanthin G	1.5M $\times 10^{-3}$
Phloridzin	M $\times 10^{-3}$
Serotonin	6M $\times 10^{-4}$
Uridine di-phosphate	1.25M $\times 10^{-3}$
Sodium malonate	2.5M $\times 10^{-3}$

¹This compound was inhibitory when used at 10 times the concentration indicated.

²These compounds were inhibitory when used at 100 times the concentration indicated.

that the monophosphate quantitatively inhibited adenosine-diphosphate-induced aggregation in native and heparinized and citrated plasma. The inhibitory effect of minimal quantities of the mono- and tri-phosphates increased if they were incubated with the platelet-rich plasma before adding adenosine diphosphate.

THROMBIN-INDUCED AGGREGATION AND VISCOUS METAMORPHOSIS If thrombin was added to citrated platelet-rich plasma in a slide preparation or if native platelet-rich plasma was used alone, first clumping, then clotting, and finally viscous metamorphosis could be observed under the microscope. If such slides were incubated for two hours, discrete platelets in masses were again seen among the fibrin

TABLE IV

ADENOSINE TRIPHOSPHATE CONCENTRATION NEEDED TO INHIBIT ADENOSINE DIPHOSPHATE-INDUCED AGGREGATION

Adenosine triphosphate + Adenosine diphosphate (μ g.)	Aggregation
Platelet-rich plasma + saline + 2	Gross
Platelet-rich plasma + 2 + 2	Gross
Platelet-rich plasma + 20 + 2	Nil
Platelet-rich plasma + 20 + 20	Gross
Platelet-rich plasma + 200 + 20	Nil
Platelet-rich plasma + 200 + 200	Gross
Platelet-rich plasma + 2,000 + 200	Nil

To 0.2 ml. of platelet-rich citrated plasma was added the amount of adenosine triphosphate indicated contained in 0.1 ml. saline and then adenosine diphosphate also in 0.1 ml. and the tube was agitated for 30 seconds and inspected.

fibrin, but they remained adherent to the fibrin even when the coverslip was rocked. The addition of more thrombin again produced viscous metamorphosis. If thrombin was added to platelet-rich plasma from a case of congenital afibrinogenemia no fibrin threads formed but with agitation platelets clumped and viscous metamorphosis occurred, few platelets remaining free. Two hours later the platelets were again seen to be in loose clumps and with agitation could be separated, yielding large numbers of free, floating platelets. All the compounds tested for their ability to prevent adenosine-diphosphate-induced aggregation were also tested using thrombin (Table III). None of the enzyme inhibitors or uncoupling agents prevented thrombin-induced aggregation, and all the anti-adhesive drugs were inhibitory. Adenosine mono- and triphosphate also inhibited thrombin-induced aggregation in native blood or plasma or in citrated plasma.

A comparison of the concentrations of thrombin and adenosine diphosphate required to induce similar degrees of platelet aggregation in platelet-rich citrated plasma is reported in Table V. It will be seen that a fourfold increase in the concentration of thrombin covers the whole range of effects from no visible aggregation to rapid and complete aggre-

TABLE V

SENSITIVITY OF AGGREGATION TO CONCENTRATION OF THROMBIN OR ADENOSINE DIPHOSPHATE

<i>Final Concentration</i>		<i>Aggregation</i>
<i>Thrombin (units/ml.)</i>	<i>Adenosine Diphosphate (μg./ml.)</i>	
0.08	0.07	No effect
0.11	0.7	Trace
0.17	7.0	Moderate
0.33	70.0	Maximal

A range of concentrations of either thrombin or adenosine diphosphate was added to aliquots of the same platelet-rich plasma and agitated. The concentration of each substance added to produce similar degrees of aggregation is reported.

gation. On the other hand, the concentration of adenosine diphosphate has to be increased a thousand fold to produce similar results.

DISCUSSION

Platelets in native blood stick to glass spontaneously and the number sticking is the same at 0°C. as at 20°C.; the addition of adenosine diphosphate markedly increases the number of platelets that stick and this result is also independent of temperature. When either the mono- or triphosphate is added to native blood fewer platelets stick; they also inhibit the effect of the added diphosphate, but are effective only at room temperature. Cocaine prevents spontaneous or adenosine diphosphate-induced adhesion at room temperature and at 0°C.

Since the tri- and monophosphates inhibit the increased adhesiveness induced by the diphosphate, these three compounds may be acting competitively; and as the mono- and triphosphates also reduce the adhesiveness of platelets in native blood, it is possible that normal adhesiveness is due to a mechanism involving intrinsic adenosine diphosphate. The competitive situation must however be complex in view of the different effects of temperature.

The adhesion of one platelet to another, aggregation, differs from platelet adhesion to glass in that aggregation in fresh plasma does not occur in the absence of thrombin or adenosine diphosphate and these substances are effective only at 20°C. and 37°C. The mono- and triphosphates appear to interact stoichiometrically with the diphosphate in citrated plasma at 37°C. while cocaine and the other anti-adhesive drugs are acting in a different manner. Since adhesion to glass and platelet aggregation are inhibited by so many different anti-adhesive drugs (Øllgaard, 1961, also finds inhibition in the presence of quinine) and since they are effective in the cold, it may be suggested that these drugs all act in a non-specific way. It has been shown that cocaine is incorporated into a monolayer of stearic acid (Shanes, 1958) and of nerve tissue lipoids (Skou, 1954), thereby increasing the molecular packing and surface pressure. Shanes was concerned primarily with the permeability of living membranes and has evolved the concept of 'stabilizers' and 'labilizers'. On the other hand, Judah (1961a), working with a different system, suggests that anti-histamines interfere with a phosphoprotein kinase. Accordingly it is possible that the anti-adhesive drugs are incorporated into the lipids at or near the surface of the platelet, the properties of which would then be altered.

It is remarkable that we do not know the nature of the forces holding a platelet stuck to a glass surface or to another platelet, nor do we know if the forces

are similar to these two situations: clearly they are not identical. The following tentative suggestion may serve as a working model. Adhesion occurs as a result of attractive forces between 'adhesive sites' on one or both of the two adhering surfaces and few adhesive sites are needed for platelet adhesion to glass: added adenosine diphosphate even in the cold increases the number or strength of the adhesive sites, thereby encouraging adhesion to glass and at room temperature it produces even more adhesive sites which permit platelets to stick to each other.

Platelet aggregation induced by thrombin resembles adenosine diphosphate-induced aggregation in that it is inhibited by the mono- and triphosphates and also by the anti-adhesive drugs; on the other hand the concentration of thrombin added to platelet-rich plasma has to be increased fourfold to change the result from no aggregation to a maximal response but the concentration of adenosine diphosphate has to be increased by a thousand fold to produce the same results. The dynamics of the two situations are clearly different, and might suggest that thrombin is acting as an enzyme, and possibly that adenosine diphosphate is a substrate in a different system with it and the mono- and triphosphates, all competing for the same site. The observation that the triphosphate at 20°C. in native or heparinized platelet-rich plasma induces aggregation is unexplained. Whether the nature of adhesive sites is the same in thrombin-induced as in adenosine diphosphate-induced aggregation is unknown. Zucker and Borrelli (1961) report that 15 minutes after adding thrombin to washed platelets the adenosine triphosphate concentration decreased by half. Perhaps thrombin converts the tri- into the diphosphate.

Calcium ions and or possibly magnesium ions (Zucker and Borrelli, 1958) seem essential for all types of adhesion since adhesion is prevented by E.D.T.A. and by strong citrate. The concentration of calcium ions required for adhesion is considerably less than that needed for coagulation, as reported by Sharp (1958). A final concentration of 0.38% citrate permits adhesion and aggregation but prevents clotting; 1.0% citrate prevents both phenomena. It has been suggested that calcium acts by ionic bonding between two negatively charged surfaces (O'Brien, 1961). It has recently been shown by microelectrophoresis that platelets in native plasma carry a negative charge and that cocaine does not alter this overall net charge (Bangham and O'Brien, 1961). If cocaine is, in fact, incorporated at the surface it could have replaced other positively charged ions, leaving the net charge unaffected. Indeed Bangham (1961) has shown that cocaine competes with calcium for adsorption at a glass

surface, and the antagonism between calcium and cocaine has also been shown to occur in relation to ion migration and the electric potential in frog muscle (Karzel, Draper, and Friebe, 1961). It seems unlikely that adenosine monophosphate and triphosphate inhibit sticking by chelating calcium, since the diphosphate, which can also chelate calcium, has a directly opposite effect on adhesion.

Five minutes after adding adenosine diphosphate to platelets they will not stick to glass or to each other, and in time viscous metamorphosis is reversible as reported by Øllgaard (1961). These findings presumably are related to the observation that intact platelets at 37°C. can inactivate large quantities of adenosine diphosphate but fragmented platelets or intact platelets at 0°C. cannot. Indeed Øllgaard (1961) has reported that platelets and white cells can inactivate his aggregating factor, which was presumably adenosine diphosphate. If it is involved in some metabolic activity leading to stickiness it is remarkable that in the reported experiments none of the usual enzyme poisons or uncoupling agents at the appropriate strength prevented its effects. The situation is probably complex, particularly since Øllgaard (1961) and Hellem (1962) report that mono-iodo-acetate can under some circumstances prevent adenosine-diphosphate-induced aggregation or adhesion. Fig. 1 summarizes some of the observed interactions and puts forward some speculative suggestions.

These results also show that Ro 3/0837 has considerable anti-adhesive activity in many tests *in vitro*. Its effect *in vivo* is at present being investigated.

The drugs with anti-adhesive properties have many other important properties. They make red cells in plasma swell (O'Brien, 1961) and decrease the osmotic fragility of red cells in saline (Judah, 1961b). Serotonin uptake by platelets is stimulated by adenosine triphosphate (Sano, Kakimoto, and Taniguchi, 1958) and inhibited by cocaine and chlorpromazine (Stacey, 1961) and by reserpine (Hardisty, Ingram, and Stacey, 1956). Anti-histamines, local anaesthetics, and some anti-malarials inhibit both ion transport in red cells and a Na and K activated adenosine triphosphatase in red cells (Judah, 1962). Mitochondrial swelling ('damage') is influenced by many of the same drugs. Grief (1961) reports that adenosine triphosphate reverses thyroxine-induced mitochondrial swelling and Judah (1961a) states that swelling is produced by calcium ions and is prevented by adenosine triphosphate and by E.D.T.A. Swelling is also prevented by anti-histamines, quinine, procaine, and by chlorpromazine (Judah, 1961b). Judah thinks that these drugs may interfere with a phospho-protein kinase that couples one phosphate group from

FIG. 1. Diagram of the possible mechanisms of platelet adhesion and its inhibition. The inside of the platelet is on the left. The hatched area represents the platelet membrane. Adhesive sites, according to their frequency or strength, are marked □ or ▢; inhibitors of adhesion are underlined, substances promoting adhesion are not.

adenosine triphosphate onto the protein to produce a phospho-protein and the diphosphate. At the same time Bangham, Rees, and Shotlander (1962) show that many anti-adhesive drugs protect liver cells against damage caused by carbon tetrachloride and that their protective power runs parallel to their physical effects at a lipid-water interface. These findings and the overlap of pharmacological pro-

perties indicate some basic and general effect of all these drugs on cellular or organelle membranes.

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Part II Some results from a new method of study

SYNOPSIS The light transmitted through a suspension of platelets increases if the platelets aggregate. A method derived from this observation has been used to study the kinetics of platelet aggregation. Aggregation is more rapid at 37°C., with rapid stirring, with high concentrations of adenosine diphosphate, and a high platelet concentration; a decrease in any of these conditions slows the process.

The study of platelet aggregation, that is the adhesion of one platelet to another, has been handicapped by the methods used, which include the examination of a few platelets under the microscope or the macroscopic observation of platelet suspensions agitated in a small test tube. This paper briefly reports a new method of study and amplifies the observations made in Part I.

METHOD

Human citrated platelet-rich plasma, 3 ml., was placed in a cuvette with a cross section of 1 × 1.5 cm. in a simple photoelectric colorimeter. A small stirrer with a variable speed control was so placed that the blades were just submerged in the plasma. Using a green filter, the light intensity was adjusted till the reading on the optical density scale was about 0.40. When stirred at 1,000 r.p.m. without additions the optical density of the suspension remained practically constant. When 20 µg. adenosine diphosphate in 0.1 ml. of barbitone buffered saline was introduced into the stirred plasma at room temperature the optical density decreased and was read at five-second intervals; at intervals of 0, 10, 20, 40, and 80 seconds after the addition, 0.1 ml. of the stirred plasma was withdrawn and placed in 1.0 ml. of formol saline. The number of individual platelets and the number of clumps of platelets containing two to 10 platelets were counted in these subsamples. Technical difficulties prevented the counting of larger particles. Few if any platelets were broken up. The plasma in the cuvette finally contained many easily visible coarse platelet clumps; if cocaine was added, the platelets separated and could then be counted and this count was similar to the initial count.

RESULTS

From Fig. 1 it will be seen that for about the first 50 seconds the optical density decreased apparently linearly with time, that the free platelet count decreased rapidly and exponentially, and that small aggregates containing two to 10 platelets increased for 10 to 20 seconds and then decreased again. After about 30 seconds it was established visually that the bulk of the platelets were in large clumps each containing 20 to 500 platelets. The optical density decreased because the light scattering power of the comparatively few coarse particles present at the end of the experiment is less than that of the small particles, free platelets, whose diameter approaches the wavelength of light. The analysis of why the optical density initially decreased in a linear fashion is complicated and was not pursued. The addition of adenosine diphosphate without stirring did not alter the optical density, so it was concluded that the change in optical density with stirring was a faithful mirror of platelet aggregation from single platelets to dimers, to clumps of five and to clumps of 100 or more. Since the initial rate of fall in optical density is linear and maximal from zero time, i.e., from the moment the adenosine diphosphate was added, it is suggested that all the platelets had very rapidly become maximally 'sticky' and that thereafter adhesion depended solely on the chance of one platelet colliding with another or one clump with another. It was now possible with this