Abstracts

Fourth Conference on Blood Platelets*

June 23–24, 1969
Oak Ridge, Tennessee

MONDAY MORNING, SESSION I

COMPARISON OF AEROBIC AND ANAEROBIC METABOLISM IN WASHED RAT PLATELETS

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Washed rat platelets were incubated at 37 C. under nitrogen or oxygen, without glucose or with 4 mM glucose. Glycogen, glucose, lactate, ATP, and ADP were measured after a 3-minute equilibration and again after an additional 10- or 15-minute incubation period. Oxygen consumption was measured with a recording Clark electrode. Anaerobiosis caused about a twofold increase in the rate of glycolysis. Addition of glucose caused a 75 percent decrease in oxygen consumption and a 100 percent increase in glycolysis. In both anaerobic and aerobic platelets, all of the glucose and glycogen consumed could be accounted for as lactate, indicating that some other endogenous substrates were being utilized for oxygen consumption. Anaerobic platelets without glucose were not able to meet their energy requirements, as evidenced by a decline in the ATP to ADP ratio. Estimations of theoretical ATP production indicate that in aerobic platelets, respiration accounted for about 50 percent of ATP production in platelets with glucose and 90 percent in platelets without glucose. The major conclusions are: 1) washed platelets can utilize endogenous substrates other than glycogen, and 2) respiration is a major source of ATP production in washed platelets.

EFFECT OF CARNITINE, ERGOTHIONEINE, AND AMINO ACIDS ON GLUCOSE METABOLISM IN HUMAN PLATELETS

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Carnitine and ergothioneine are quaternary amines present in mammalian tissues. Carnitine functions as a carrier of activated acyl groups across mitochondrial membranes. Biologic function for ergothioneine has yet to be elucidated.

Addition of either carnitine or ergothioneine to human washed platelets in Kreb-Ringer calcium-free phosphate buffer resulted in increased 14CO2 production from pyruvate 2-14C (24.85 ± 9.22 and 24.25 ± 7.73 vs. 18.09 ± 7.59 μM/Gm. protein/hr.), decreased overall lactate production from glucose (in 6 experiments, range 35–138 and 114–181 vs. 193–313 μM/Gm. protein/hr.), and decreased 14CO2 production from glucose 1-14C (8.65 ± 2.07 and 8.48 ± 1.93 vs. 11.04 ± 2.54 μM/Gm. protein/hr.). Oxygen consumption was unaffected. No gluconeogenesis could be demonstrated with either lactate 2,3-14C or pyruvate 2-14C, but a labeled neutral compound or compounds (not identifiable chromatographically as glucose, glyceraldehyde, or glycerol) was recovered by use of mixed bed resin columns after incubation of platelets with pyruvate 2-14C. Production of this compound was also decreased when carnitine or ergothioneine was added.

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Our results suggest that in this in vitro system, addition of carnitine or ergothioneine to platelets results in stimulation of the tricarboxylic acid (TCA) cycle at the expense of other pathways tested. The lack of effect on oxygen consumption suggests that the increased TCA cycle activity relates more significantly to fatty acid metabolism than to respiration. The known function of carnitine is consonant with this interpretation. There is no evidence in the present studies for the mode of action in vitro of ergothioneine. It is probable that its action differs from that of carnitine and may be related to its sulfhydryl (SH) groups, perhaps nonspecifically by keeping Coenzyme A. SH reduced.

**Stimulation of Platelet Glycolysis**

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A number of agents have been shown to stimulate glycolysis of platelets in vitro. Without exception, substances capable of platelet aggregation—such as ADP, epinephrine, thrombin, and the like—increase either anaerobic glycolysis or hexose monophosphate (HMP) shunt activity. We have examined the effect of polystyrene latex particles on the energy metabolism of human platelets. Aerobic and anaerobic glycolysis as well as HMP shunt activity were determined by measuring $^{14}\text{CO}_2$ production and $^{13}\text{C}$-lactate formation from glucose-$1^{-14}\text{C}$ and glucose-$6^{-14}\text{C}$. Polystyrene latex particles, 0.234 $\mu$m in diameter, promoted an 8- to 12-fold increase in glucose metabolism by the tricarboxylic acid cycle (TCA), whereas formation of lactate and CO$_2$ production from the HMP shunt increased only 1- to 2-fold. Larger size particles, 1.3 $\mu$m in diameter, exhibited markedly less stimulation of the TCA cycle but still showed increased anaerobic glycolysis and HMP activity. The stimulatory effect on the TCA cycle decreased rapidly in stored platelets, being only 3–4 times the baseline value after 24 hours and virtually ceasing after 3–4 days of storage. Platelets obtained from EDTA-blood were not stimulated by polystyrene latex particles. AMP, a powerful inhibitor of ADP-induced aggregation, did not prevent the stimulatory effect of small particles. Based on the known phagocytic capacity of platelets for small polystyrene latex particles, we attribute the observed increase in aerobic glycolysis after exposure to such particles to phagocytosis. Platelets thus appear to possess a unique metabolic response to phagocytosis, basically different from that of granulocytes. The measure of this metabolic response can also serve as a biochemical test for platelet metabolic competence under stress.

**Glycoproteins of the Platelet Outer Membrane**

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Platelet membrane glycoproteins may be involved in viral and serotonin receptor sites, membrane antigens, and in platelet aggregation and adhesion to collagen. When intact platelets are treated with pronase (1 mg./10$^{11}$ platelets) about 40 percent of the total sialic acid (NANA) is released within 20 minutes, after which there is virtually no further release. Comparable but slower release is obtained with trypsin, but a maximum of only 30 percent is obtained. Gel filtration on calibrated columns of Sephadex G-75 gave, in both cases, two well resolved peaks of NANA-containing glycopeptides in approximately equal amounts. One peak emerged at close to $V_o$, and had an estimated mol. wt. of 77,000. The second peak was well resolved from contaminating peptide impurities and gave an $S_{20,w}$ of 1.65 in the ultracentrifuge, corresponding to a mol. wt. of 13,000. These two glycopeptides isolated from intact platelets appear to correspond to similar size classes isolated from purified platelet membranes (Pepper and Jamieson, in press). The relation between these size classes
and the low mol. wt. (3500) group isolated only from the membrane fraction is under investigation. (Supported, in part, by USPHS grant GM-13057.)

**PROGRESSIVE CLEAVAGE OF SIALIC ACID FROM PLATELETS AND THEIR ELECTROPHORETIC MOBILITY**

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Madoff et al. (J. Clin. Invest. 43:870, 1964) reported that only 61 percent of the sialic acid (SA) of human platelets could be removed by *V. cholera* sialidase and that concomitantly platelet electrophoretic mobility (EM) decreased from -1.14 to -0.66 × 10⁻⁴ cm² sec⁻¹ volt⁻¹ (pH 6.4, ionic strength 0.072, 25 C.). In the present study the mean normal platelet SA was found to be 32 × 10⁶ molecules per average platelet (7 donors); the EM determined in a Zeiss cytopherometer (pH 6.4, ionic strength 0.072, 25 C.) was -1.08, SD ± 0.05, 13 donors. A total of 62–65 percent SA could be removed by three successive treatments with *V. cholera* sialidase. A fourth treatment with *C. perfringens* sialidase increased this value by 3–5 percent. After exhaustive enzyme treatment the EM was reduced to -0.60. In platelets from a single donor on a given day, the decline was linear since SA was progressively removed until the EM leveled off abruptly at -0.60. No further decrease occurred with continued enzyme exposure or repeated challenge with additional enzyme, although approximately 5 × 10⁶ more SA molecules per platelet were removed. The same result was obtained at pH 7.4 in Tris-saline, ionic strength 0.15, 37 C., except that the final mobility was -0.45. When mobility vs. SA released was plotted, the initial slopes (EM/SA released) were the same in both systems and on platelets from different donors (-0.034 × 10⁻⁴ cm² sec⁻¹ volt⁻¹ per 10⁶ molecules). These experiments suggest three structural aspects of platelet SA: 1) SA removable by sialidase and influencing surface charge; 2) SA removable by sialidase but unassociated with surface charge; and 3) SA not removable by enzymes. Three may not be SA, since TLC in ethanol:water:ammonia (80:20:1) of the acid hydrolysate revealed two Warren positive spots, neither of which moved as N-acetyl neuraminic acid, in contrast to the report of Madoff et al. previously referred to. (Supported by USPHS grants HE-11447 and HE-09011.)

**LIPID PEROXIDATION IN BLOOD PLATELETS**

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The destruction of lipids by peroxidation and the accumulation of peroxides from this process have been shown to lead to loss of cell function and to widespread cellular damage. Lipid peroxide formation was studied in fresh and preserved platelets and in platelet cohorts of different age. Lipid peroxidation was estimated by measuring malonaldehyde (MA) by the thiobarbituric acid reaction. Freshly collected platelets contained 0.87 ± 0.14 nmoles MA/2 × 10⁸ cells. The choice of anticoagulant, either ACD or EDTA, had no effect on lipid peroxidation in platelets less than 5 hours old. Storage of up to 7 days resulted in a progressive accumulation of lipid peroxides, particularly in platelets obtained from ACD blood, and was significantly higher at room temperature than at 4 C. Increased H⁺ concentration in the platelet suspension decreased lipid peroxide formation, and the addition of dl-α-tocopherol prevented it. Platelet populations of different age, obtained after antibody-induced thrombocytopenia in rats, showed a striking correlation of the MA content to their age. The youngest platelets had not only the lowest values of lipid peroxides, but were also most resistant to in vitro peroxidation by iron. Microsomal enzymes, such as NADH cytochrome-C reductase, showed 80–90 percent reduction in activity after exposure of intact platelets either to metal ions or ADP and ascorbate, whereas NADPH
cytochrome-C reductase remained unaffected by this treatment, attesting to the dependence on lipids for activity of the former but not of the latter enzyme. These studies suggest an important role of lipid peroxidation for the energy metabolism of platelets and most probably for their viability as well.

**INCORPORATION OF $^{32}P$ ORTHOPHOSPHATE INTO PLATELET PHOSPHOLIPIDS**

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We have previously shown that stimulation of washed pig or rabbit platelets with ADP or thrombin causes increased incorporation of $^{32}P$ orthophosphate into platelet phospholipids. Platelets which were prepared by a new method, and which are sensitive to low concentrations of ADP, show minimum incorporation of $^{32}P$ without aggregating agents, whereas those prepared by methods which lead to insensitive platelets incorporate much more $^{32}P$ without aggregating agents. Since platelet stimulation leads to increased $^{32}P$ incorporation into phospholipids, the increased incorporation into insensitive platelets probably indicates alteration during the preparation procedure. Using the new method of platelet isolation, the extraction of Palmer and Rossiter (Can. J. Biochem. 43:671, 1965), and thin-layer chromatography for the separation of phospholipids, we found that the principal phospholipids into which $^{32}P$ is incorporated are phosphatidic acid and the phosphatidyl inositols. Upon aggregation with ADP, washed pig or rabbit platelets show increased incorporation of $^{32}P$ into the phosphatidyl inositols. With thrombin, there is increased incorporation into phosphatidic acid as well as into the phosphatidyl inositols, and it is more extensive than with ADP. Metabolic energy appears to be involved in the incorporation of $^{32}P$ into the platelet phospholipids, because it does not occur in suspensions from which glucose is omitted or in which inhibitors of glycolysis and oxidative phosphorylation are used. As in other cells, such as leukocytes, phosphatidyl inositols appear to be involved in the cellular changes associated with stimulation.

**UPTAKE OF FREE FATTY ACIDS BY HUMAN PLATELETS**

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Exposure of a platelet suspension to high concentrations of long-chain free fatty acid (FFA) causes aggregation. The present work is an attempt to elucidate the mechanism of this effect. Washed human platelets rapidly took up albumin-bound palmitate-1-$^{14}$C in unesterified form, and the labeled FFA content of the platelets was maintained relatively constant during a 1-hour incubation. In contrast, increasing amounts of labeled fatty acid were oxidized to $^{14}$CO$_2$ and incorporated into lipid esters, predominantly phospholipids, as the incubation progressed. As the molar ratio of FFA to albumin in the medium was raised from 0.7 to 2.9, the unesterified palmitate-1-$^{14}$C content of the platelets increased 10-fold, but the incorporation of labeled fatty acid into lipid esters and $^{14}$CO$_2$ increased to a much lesser extent. At a given palmitate to albumin molar ratio, there was only a small increase in labeled FFA content of the platelets when the incubation was performed at 37 C. as compared with 0 C. When platelets were exposed to palmitate-1-$^{14}$C, washed, and then incubated in FFA-poor albumin, the radioactive FFA present in the platelets was released to the medium. Release of labeled FFA was completed within 1 minute even at 0 C. These data suggest that essentially all of the FFA uptake present in unesterified form is bound to the platelet membrane. The most marked effect of increased medium FFA concentration is an increase in platelet FFA content. Hence, FFA-induced platelet aggregation might result from changes in the platelet membrane that are secondary to binding of large amounts of FFA.
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EFFECTS OF CARNITINE ON PLATELETS

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The addition of 1-carnitine, a normal constituent of body tissues, inhibited platelet aggregation induced by long-chain fatty acids or collagen. Carnitine also inhibited the usual release of adenine nucleotides from platelets incubated with sodium stearate (final concentration, 350 µM) or collagen. Carnitine caused a significant decrease in platelet adhesiveness when added to citrated blood.

The effects of carnitine upon oxidation and incorporation of albumin-bound palmitic acid-1-14C were studied with washed platelet suspensions. FFA oxidation was not enhanced by carnitine. The inhibitory effects on FFA oxidation induced by pent-4 enolic acid were not overcome by the addition of carnitine. As the concentration of carnitine was increased in the platelet incubation system, there was no increase of total palmitate-1-14C uptake by the platelet. However, less radioactivity was found in lecithin and a corresponding increase was observed in acylcarnitine. This effect was noted at several FFA to albumin ratios. Similar results were obtained with platelet-rich plasma.

Thus, in an intact platelet system, carnitine did not stimulate the oxidation of long-chain fatty acids, as has been reported in other intact tissues. Carnitine, however, increased the formation of acylcarnitine and decreased the amount of lecithin. Such effects may alter the composition of the platelet membrane, and thereby diminish its response to agents which influence aggregation and adhesiveness. These results may have important implications for platelet function in health and disease.

PLATELET FACTOR-3 ACTIVITY AND PLATELET PHOSPHOLIPIDS AND THEIR COMPOSITION IN PATIENTS WITH CORONARY HEART DISEASE AND JUVENILE DIABETES

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When platelets adhere or aggregate, their phospholipids are made available for the coagulation mechanism in the platelet atmosphere. A relation between the nature and the behavior of the platelet phospholipids and thrombotic disease has been postulated.

Platelet factor-3 activity and availability, platelet phospholipids, and their fatty acid and aldehyde composition were examined in males with coronary heart disease (CHD) and juvenile diabetes (D), and in a control group.

Increased PF-3 activity was present in PRP of both patient groups, and in PPP from the diabetics. No differences in the increase of PF-3 activity after exposure of PRP to ADP or to kaolin, or after freezing and thawing three times, were observed as compared to controls.

As estimated per 10⁹ platelets, increased amounts of ethanolamine phosphoglycerides (PE), serine phosphoglycerides (PS), choline phosphoglycerides (PC), and total lipid-P were found in diabetics.

The phospholipid to protein ratio was highest in the CHD group.

Only moderate changes were found in the fatty acid pattern. In the CHD group, decreased amounts of palmitic acid and highly unsaturated long-chain fatty acids and increased amounts of oleic acid were present in PC, PE, and PS. Diabetics had a markedly increased stearic acid level in PS and an increased dominance of saturated fatty acids in sphingomyelin. (Supported by the Norwegian Council on Cardiovascular Diseases.)
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PLATELET PROTEIN SYNTHESIS STUDIED IN A CELL-FREE SYSTEM

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Circulating blood platelets are capable of synthesizing protein, and the major portion of the newly formed protein has been identified as thrombosthenin. We have investigated the in vitro synthesis of protein by a cell-free system isolated from human blood platelets. Although polysomes could not be demonstrated in platelets by electron microscopy, we were able to isolate such structures by cell fractionation. On continuous sucrose gradients, such ribosomes could be fractionated into two major sizes of polysomes and a large portion of single 75S ribosomes. The two classes of polysomes contained 50–60 and 20–25 ribosomes, respectively. Both of them were able to incorporate 14C-amino acids into acid-precipitable material. The polypeptides released from the polysomes were separated by column chromatography, and two major peaks of radioactivity were identified, one being in excess of 100,000 mol. wt. (eluted with the effluent front), and the other of 57,000 mol. wt. To further characterize these proteins, myosin and G-actin obtained from human muscle were added to the products of cell-free synthesis and the proteins reisolated in a series of steps until constant specific activity could be achieved. By this method, it could be demonstrated that the larger polysomes of platelets were synthesizing mainly myosin and the smaller ones principally actin. Although the amount of polysomes isolated from platelets was small when compared to that obtainable from other mammalian cells, platelets still had at least two remaining species of messenger RNA which appeared to be monocistronic for the synthesis of myosin and actin.

ISOLATION FROM HUMAN PLATELETS OF A COLCHICINE-BINDING PROTEIN WITH ACTIN-LIKE PROPERTIES

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The function of platelet microtubules is as yet unknown, but proteins forming the microtubular matrix of other cells bind guanosine triphosphate and colchicine, the latter reportedly causing depolymerization. A protein isolated from platelets by ammonium sulfate precipitation between 40 and 50 percent saturation has been further purified on DEAE and Sephadex A-50 by elution between 0.5 and 0.8 M KCl. This protein is homogeneous by gel filtration and disc polyacrylamide electrophoresis, and 100 mg. binds 0.042 μM colchicine. It lacks ATPase activity, but markedly alters the cation requirements for ATPase when preincubated with either muscle myosin or thrombosthenin M, a myosin-like protein prepared from platelets. Binding of colchicine suggests that this new protein derives from the platelet microtubules. Furthermore, because it alters the Ca++ and Mg++ requirements for the activation of ATPase of either muscle myosin or thrombosthenin M, this colchicine-binding protein appears to be the actin component of thrombosthenin, the actomyosin-like protein from platelets. Thus, the microtubules could represent the storage site of actin—which, when required, can depolymerize into actin subunits to form thrombosthenin.

FACTORS AFFECTING THE SUPERPRECIPITATION OF PIG THROMBOSTHENIN

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Thrombosthenin was extracted from washed pig platelets with 0.6 M KCl containing 0.06 M N-tris(hydroxymethyl)methyl-2-aminoethanesulphonate (TES) buffer, pH 7.0, and 3 percent n-butanol. It was purified by three cycles of precipitation with two volumes of water and dissolution in 0.6 M KCl buffered with 0.06 M TES,
pH 7.0. The change in turbidity of 1:10 dilutions of the final product in water was recorded on addition of various agents.

When EGTA, which itself reduced turbidity, and excess Mg\(^{2+}\) ions were present, addition of ATP (2 \(\mu\)M to 2 mM) induced an immediate further decrease in turbidity (clearing), which was followed by a slow increase (superprecipitation). Intermediate ATP concentrations (e.g., 0.1 mM) caused the most rapid superprecipitation. Very low Ca\(^{2+}\) ion concentrations (e.g., 10\(^{-7}\) M) maintained by Ca-EGTA buffers prevented clearing, greatly accelerated superprecipitation, and stimulated ATP-ase activity. Higher Ca\(^{2+}\) ion concentrations (> 5 \(\times\) 10\(^{-7}\)) prevented superprecipitation by ATP but themselves increased turbidity. This high sensitivity to Ca\(^{2+}\) ions, comparable to that of natural actomyosin, suggests that calcium may control the physiological activity of thrombosthenin.

Addition of 0.5 mM dithioerythritol to all solutions used in the extraction and purification of thrombosthenin yielded preparations showing less superprecipitation in the absence of Ca\(^{2+}\) ions and more superprecipitation at low Ca\(^{2+}\) ion concentrations. Further evidence of a role for thiol groups in the reactions of thrombosthenin was obtained with pCM, which completely abolished all ATP-dependent turbidity changes, and with NEM, which suppressed clearing but potentiated superprecipitation. Although ADP alone had no effect on turbidity, selected concentrations (0.1–0.5 mM) accelerated the superprecipitation induced by equimolar ATP. ADP inhibited clearing. These findings suggest that the clearing and superprecipitation phenomena involve different ATP binding sites and different thiol groups.

**The Identity of Platelet Albumin**

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There is considerable controversy about the identity of the proteins which occur both in platelets and plasma. Previous studies have shown that platelet fibrinogen resembles plasma fibrinogen in coagulability, heat lability, and immunologic properties. There are, however, some striking differences between platelet and plasma fibrinogens. Albumin is another protein that is present in both platelets and plasma. This paper presents the results of a systematic study on the relation between platelet and plasma albumins.

Albumin was isolated from homogenized human blood platelets by ammonium sulphate precipitation followed by gel filtration. Under normal conditions (pH 7.0, I 0.1), the sedimentation, viscosity, gel electrophoretic, and immunologic properties of the isolated albumin were similar to those of serum albumin. At acid pH values and in the presence of 8 M urea, however, there were distinct differences in electrophoretic and sedimentation characteristics between the two albumins, and these appeared to indicate a structural difference between the two albumins. When serum albumin was mixed with the platelet suspension and the platelet and serum albumins isolated together, the two albumins were found to be indistinguishable by the above criteria. Further studies indicate that the reported differences between platelet and serum albumins are due to mild proteolytic alteration of the platelet albumin molecule. We conclude that platelet and serum albumins in vivo are identical. (Supported in part by NIH grant HE-12520-01.)

**On the Occurrence and Measurement of Monoamine Oxidase Activity in Human Blood Platelets and Plasma**

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A monoamine oxidase (1.4.3.4) whose properties are largely unknown, and which appears to be involved in thrombocyte aggregation, has been found in human
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platelets. As a first step in studying its physicochemical characteristics, its biological function, and its separation from the (1.4.3.4)-plasma monooamine oxidase, we developed a method of measurement with a new substrate, o-aminophenylethylamine, which, under the influence of monooamine oxidase (MAO), is converted into indole. Blood, treated with EDTA, was separated into red cells and platelet-rich plasma. Since other amine oxidases may occur in plasma or platelets, specific inhibitors were added to the incubation solution. With this procedure, we obtained much higher activities than previously reported, namely, 1 to 2 \( \mu \)moles indole \( \times 10^{10} \) cells hr. In platelets collected from pregnant women, the values tend to be even higher. Very small MAO activities, if any, occur in human “platelet-free” plasma. We believe that the data previously reported by two other groups seem to be derived from the various amounts of thrombocytes left in plasma after the centrifugation of blood.

For the first time, it is possible to study human MAO in vivo. We applied this method to the measurement of platelet MAO after very small amounts of pargyline (Eutonyl, Abbott) were given to volunteers. A marked depression of the activity was noticed 60 minutes or less after the administration.

MONDAY AFTERNOON, SESSION II

PLATELET KINETICS IN SPLENOMEGALY

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Measurements of platelet survival, turnover, and splenic pool size, as well as mean megakaryocyte volume and megakaryocyte mass, were compared in normal, splenectomized, and splenomegalic rats. The amount of splenic pooling was estimated indirectly by using the \( ^{51} \)Cr platelet labeling technique, and directly by determining platelet recovery during platelet-poor blood exchange transfusions. Fifteen percent of the total body platelets were localized in the splenic vasculature of normal rats, while in methylcellulose-induced splenomegaly, the splenic platelet pool was 1-1\( \frac{1}{2} \) times greater than the systemic platelet pool. Good agreement was observed between direct and indirect estimates of splenic pool size. In animals with splenomegaly, the platelet count was reduced to nearly one-half of normal, but platelet turnover was greater than normal, indicating attempted compensation, which was confirmed by increases in mean megakaryocyte volume and total megakaryocyte mass. The splenectomized animals had normal platelet counts; platelet turnover and megakaryocyte mass were decreased by 15 percent because of the absence of splenic platelet pool requirements. On the basis of these data and comparable observations in man, it was concluded that splenic enlargement increases the proportion of platelets pooled within the splenic vasculature. The resultant drain on the platelets circulating systemically stimulates thrombopoiesis. Thrombocytopenia occurs when the degree of splenic pooling exceeds the thrombopoietic response of the marrow.

MEASUREMENT OF THROMBOPOIESIS IN RABBITS USING \( ^{75} \)SeLEMINOETHIONINE

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Incorporation of selenomethionine \( (^{75} \)SeM) has been used to study platelet production in rabbits. Radioactivity of platelets was low immediately after intravenous injection of \( ^{75} \)SeM and rose to a maximum approximately 3 days later. Platelet radioactivity was independent of concurrent plasma levels. The life-span of rabbit platelets, as estimated with this technique, was 4–5 days. In vivo reutilization of \( ^{75} \)SeM previously incorporated into plasma proteins was not detected, nor was in vitro incorporation of \( ^{75} \)SeM by platelets in platelet-rich plasma. Acute hemorrhage 24 hours before administration of \( ^{75} \)SeM increased the incorporation of \( ^{75} \)SeM into platelets. Transfusion-induced thrombocytosis reduced the incorporation of \( ^{75} \)SeM to approxi-
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mately 30 percent of that observed in control animals. Suppression of bone marrow function with nitrogen mustard resulted in decreased numbers of platelets in the circulation and a decrease in incorporation of $^{75}$SeM. Delayed appearance of $^{75}$SeM was observed in circulating platelets during recovery from marrow suppression. Injection of 75–225 ml. of plasma from thrombocytopenic donors into normal rabbits increased incorporation of $^{75}$SeM into platelets, whereas normal plasma did not. The rate of appearance of $^{75}$SeM in circulating platelets appears to provide a sensitive and specific method for the study of production of platelets by megakaryocytes. The data suggest a more rapid entry of platelets into the circulation as well as a sustained increase in incorporation of $^{75}$SeM into platelet protein after stimulation of platelet production. These results are consistent with the concept of a humoral agent (thrombopoietin) that acts on megakaryocytes to regulate platelet production.

COMPUTER ANALYSIS OF PLATELET SURVIVAL CURVES

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Current methods of estimating mean platelet life-span are based on fitting linear, exponential, linear-exponential, or other functions to the survival data obtained after isotope labeling. In many cases, a clear choice between different functions may be impossible on statistical or physiological grounds. Depending on the model chosen, widely different predictions for platelet life-span will result.

There is thus a definite need for an estimate of platelet survival, valid in the absence of knowledge of the correct model. Such an analysis has been made possible by a computer program permitting this calculation for the intercept of the tangent to the survival curve with the time axis (Dornhorst’s formula). The best polynomial regression is fitted to the experimental survival data from days 0 to 8, starting when mixing of the labeled platelets is nearly completed. Calculations from this polynomial yield platelet life-spans in normal individuals that range from 9.24 to 11.18 days, with an SE of 0.33 to 2.10 days. Shortened life-spans were found in a variety of hematological diseases. Additional derived data are 1) platelet daily production, 2) mean platelet age, 3) graphs of age and longevity distributions, and 4) statistical comparisons of life-span and production before and after treatment in the same individual.

NUCLEAR MATURATION AND CYTOPLASMIC ORGANELLES AND ANTIGENS IN GUINEA PIG AND HUMAN MEGAKARYOCYTES

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Microspectrophotometric measurements of DNA using the plug method were made on guinea pig and human megakaryocytes stained by the Feulgen reaction. In guinea pigs, megakaryocytes are 8, 16, or 32N, while in man they are 8, 16, 32, or 64N. In imprints, the nuclear area increases with the DNA content, and both segmented and nonsegmented nuclei appear. In the guinea pig, 25 percent of 8N, 75 percent of 16N, and 92 percent of 32N nuclei are segmented. In man, the percentage of segmented nuclei is 22 percent of 8N, 43 percent of 16N, 78 percent of 32N, and nearly 100 percent of 64N. Tritiated thymidine labels only the nonsegmented nuclei 2 hours after injection into the guinea pig. This means that megakaryocytes can stop their DNA syntheses at any of the different ploidy levels (8, 16, or 32N).

Electron microscopy combined with autoradiography shows that, in guinea pigs, the center of the nucleus of DNA synthesizing cells is hollow, and contains most of the Golgi apparatus. In these cells, some granules, demarcation membranes, and
microtubules are already present. Organelles are formed in increasing amounts in non-DNA synthesizing cells.

Using the fluorescent antibody method, cytoplasmic fluorescence was detected only in megakaryocytes having a segmented nucleus. Thus, platelet antigens appear only after the completion of DNA syntheses.

**Ploidy Composition of Megakaryocyte Stages**

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The ploidy composition of several morphologic stages of megakaryocytes was determined by measuring the relative amount of DNA in individual megakaryocytes by the two-wavelength microspectrometric method. Megakaryocytes in bone marrow smears or squashes stained with Giemsa were classified morphologically into three stages. The most immature compartment is composed of basophilic cells having evenly stippled chromatin (Type I). Mature megakaryocytes have an acidophilic, granulated cytoplasm and more dense and unevenly distributed chromatin (Type II). The third stage is made up of nuclei without cytoplasm (Type III). The frequencies of the three types were 26, 69, and 5 percent. The preparations were then restained with the Feulgen reaction for DNA determinations. The ploidy distribution within each stage was determined and expressed as a percentage of all cells measured in that compartment. The relative percentage of $8\ C$ cells declined from the Type I to II to III compartments; the $16\ C$ cells exhibited a moderate increase; and the $32\ C$ megakaryocytes tended to increase. The percentages of cells of each ploidy class in a compartment depend on the rate of entry and on the time spent in the compartment. The results suggest that the time spent in each compartment varies among ploidy classes. In addition, $8\ C$ cells may either mature into nonreplicating (platelet-producing) megakaryocytes or undergo endoreduplication and become $16\ C$ cells. (Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.)

**The Effect of Thrombocytopenia on the Platelet Response to Sublethal Irradiation**

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Sublethal total-body irradiation with 200, 300, or 400 R produces reversible thrombocytopenia in mice. Platelet counts begin to decrease after the 4th postirradiation day, reach minimum values on the 8th or 9th day, and return to normal by about the 16th day. To determine if this response would be modified by thrombocytopenia at the time of irradiation, animals were treated with rabbit anti-mouse platelet serum (APS). Acute thrombocytopenia was induced by a single injection of APS immediately before or after irradiation, and was followed by an initial increase in platelet counts. The subsequent irradiation-induced thrombocytopenia was less severe and/or of shorter duration than in irradiated controls. When animals were irradiated after more chronic thrombocytopenia, maintained for 4 days by daily injections of APS, different responses were seen. The thrombocytopenia produced by 400 R was not modified, but, after the lower doses, the irradiation-induced thrombocytopenia was prolonged. Reticulocyte and leukocyte counts of thrombocytopenic and irradiated mice showed no consistent differences from those of irradiated controls.

Modification of irradiation damage to the erythropoietic system by acute anemia or hypoxia has been shown to be mediated by the hormone erythropoietin. Modification of the platelet response to irradiation by acute thrombocytopenia substantiates
numerous other observations which suggest the existence of a feedback mechanism by which a platelet deficit stimulates megakaryocytopoiesis. These studies also suggest that the chronically stimulated megakaryocyte system is more sensitive than normal to the damaging effects of irradiation.

THE IMPORTANCE OF "LIBERATED" PLATELET THROMBOPLASTIN IN THROMBOCYTOPENIC STATES

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In studying a number of thrombocytopenic and thrombasthenic patients, attempts have been made to correlate the following factors: 1) the abundance of megakaryocytes in the marrow and their morphology, 2) the duration of thrombocytopenia as documented in the laboratory or surmised by the history, 3) the platelet-rich plasma thrombelastographic r value, 4) the degree of r value shortening following "sublethal" ultrasonic platelet disruption, and 5) the changes in the thrombelastographic values after splenectomy for thrombocytopenia as well as for other reasons.

Based on the data accumulated, some new conclusions can be drawn and some older concepts can be strengthened. 1) Time is required for substantial megakaryocytic hyperplasia to develop in the marrow. 2) The marrow full of morphologically young megakaryocytes does indeed indicate rapid production of platelets and their delivery from the marrow. 3) Platelets which are sequestered in the spleen are not necessarily held there intact; many are ruptured and their contents liberated. In "autoimmune" situations where the spleen can recognize the platelets as being abnormal (clumped and antibody coated), platelet rupturing is more pronounced than in large spleen "hypersplenic" situations where the platelets are innocent.

PLATELET UPTAKE BY THE RETICULOENDOTHELIAL SYSTEM

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Circulating blood platelets, besides being removed from the circulation during coagulation, can also be reduced in number by alteration of reticuloendothelial system (RES) activity.

When canine blood platelets were exposed to the RES-stimulating agents, zymosan and glucan (10–30 mg. kg. body weight), in both in vivo and ex vivo test systems, adhesion of platelets to the zymosan or glucan particles could be demonstrated by electron microscopy of whole mount platelet preparations. Many aggregates were composed of one platelet and one zymosan or glucan particle. A marked thrombocytopenia resulted in vivo. The platelet reduction, however, was not associated with a fibrinogen-fibrin transformation, nor did activation of platelets occur. The platelets appeared to be removed because of their attachment to these particles.

Fluorescent antibody studies with rhodamine antiplatelet sera revealed the presence of platelet material in liver RE cells by 1 hour after zymosan injection. Zymosan particles with their pale green autofluorescence could easily be distinguished from the liver RE cells which showed specific fluorescence indicative of the presence of platelet material. No specific fluorescence of splenic RE cells was observed.

There was no evidence that an induced state of hyperphagocytosis removes normal circulating platelets. It appears that RE cells discriminate between normal platelets and ones injured by adhesion to particulate matter like zymosan and glucan. The surface nature of zymosan and glucan particles, as well as platelet-particle aggregates, was also studied with scanning electron microscopy. (Aided by NIH grant HE-04712.)
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Adrenochrome-Induced Changes in the Ultrastructure and Number of Platelets in Vivo and in Vitro

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Adrenochrome semicarbazone injected intramuscularly into apparently healthy human volunteers resulted in a decrease in the number of circulating platelets after 30 minutes. The decrease ranged from 9 to 48 percent, with an average of 24 percent. The platelet count returned to near normal by 60 minutes. A similar concentration had no effect in vitro. The sodium salicylate carrier used to solubilize the adrenochrome complex had no effect on platelet numbers in vivo or in vitro.

Blood taken 30 minutes postinjection and prepared by a fixation-shadow-cast technique contained many disintegrating platelets and a high concentration of membrane fragments and free organelles. Thin sections of platelets revealed extensive intracellular alteration, but physically intact limiting membranes. Many free vesicles of various sizes and shapes and a few free mitochondria were shown in cross section.

Two major metabolites isolated from human urine after adrenochrome semicarbazone injection caused platelet disruption when added to citrated platelet-rich plasma in vitro. The disruption was reflected by a decrease in the number of platelets and by the electron microscopic appearance of fixed-shadow-cast preparations.

It is suggested that the sequence of events observed in this study may represent, greatly exaggerated, a process which occurs in the body constantly, as a small amount of epinephrine seems to be metabolized through an adrenochrome pathway.

Alteration of the Ultrastructure of Hemostatic Plugs by Aspirin

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Branches of mesenteric blood vessels of rats were transected with razor blades 3 hours after administration of 80 mg. of aspirin by gastric tube. Blood shed from the wounds was rinsed away with Tyrode’s solution; bleeding usually ceased in 4 to 7 minutes. Platelet plugs formed at the incision were fixed immediately after cessation of bleeding or at 2 and 6 minutes after the incision, whether or not bleeding had stopped. Degranulation of platelets in plugs from aspirin-treated animals was much more marked than in untreated animals. Strands of fibrin and clusters of erythrocytes were easily found in the interior of plugs formed after aspirin, but not in plugs of untreated rats. Leukocytes were often attached to the erythrocyte clusters in plugs from aspirin-treated animals, and platelets about the erythrocyte clusters formed prominent and striking degranulated halos. Interference with normal platelet function by aspirin may result in platelet plug formation by auxiliary mechanisms. The abnormal presence of numerous erythrocytes within plugs after aspirin suggests that these cells may play an important role in the initiation of hemostatic plug formation when the platelet release reaction induced by collagen has been inhibited; the fibrin strands inside the plugs suggest greater participation of the clotting system in hemostasis under these conditions. (Supported by USPHS grant HE-5654-09.)

The Platelet and Hypothermia

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Induction of profound hypothermia (core temperature of 5–10 C.) in dogs or in man results in a hemorrhagic state of mild to exsanguinating proportions which develops during rewarming. Hemostasis is affected at all levels: platelet, coagulation, and fibrinolysis.

During cooling, the platelet count falls precipitously and platelets assume the spiny-sphere shape. During rewarming, the platelet count rises and the morphology tends to revert toward the disc form. Biopsies of the wound prior to cooling show platelet
PLUGS WITHIN SMALL VESSELS. THESE WHITE THROMBI ARE NOT SEEN IN BIOPSIES TAKEN DURING REWARMING.

THE EFFECT OF COOLING AND REWARMING ON HUMAN PLATELETS WAS EXAMINED IN VITRO. CHILLED PLATELETS BECOME SPINY SPHERES. REWARMING DOES NOT ENTIRELY REVERSE THE MORPHOLOGICAL CHANGES. STIRRED PLATELETS AGGREGATE TO A MILD DEGREE UPON COOLING. THE RESPONSE OF CHILLED PLATELETS TO ADP IS VERY SLOW AND AGGREGATION IS POOR. REWARMED PLATELETS HAVE AN ACCELERATED BUT SUBOPTIMAL RESPONSE. THE RESPONSE OF CHILLED PLATELETS TO COLLAGEN IS ALSO SLOW, BUT THE DEGREE OF AGGREGATION IS ONLY MILDLY IMPAIRED. REWARMED PLATELETS SHOW RAPID RESPONSE AND INCREASED AGGREGATION.

THE THROMBELASTOGRAM OF REWARMED PLATELETS SHOWS PROLONGATION OF REACTION AND CLOTTING TIME AND DECREASED MAXIMAL AMPLITUDE.

SOME DIFFERENCES IN RESPONSE TO COLD WERE NOTED BETWEEN DOG AND HUMAN PLATELETS.

ELECTRON MICROSCOPIC STUDY OF VENOUS THROMBOSIS INDUCED BY AORTIC CROSS CLAMPING

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PROLONGED CLAMPING OF THE CANINE AORTA HAS BEEN SHOWN BY OTHERS TO RESULT IN INFRArenal VENOUS THROMBOSIS AND IN PULMONARY MICROEMBOLI. DISSEMINATED INTRAVASCULAR COAGULATION HAS BEEN SUGGESTED AS A MECHANISM OF THROMBOSIS, BUT EXPERIMENTS DESCRIBED HERE DEMONSTRATE PLATELET ATTRACTION TO AN ALTERED VENOUS WALL. THE AORTA AND ILIAC ARTERIES OF 6 MONGREL DOGS WERE ISOLATED FROM THE RENAL ARTERIES TO THE GROIN BEFORE CLAMPING. COLLATERAL ARTERIES WERE INTERRUPTED. IN 4 DOGS, DISSECTION WAS LIMITED TO THE AORTA. THE LARGE ABDOMINAL VEINS, VEINS OF THE HIND AND FORELEG, AND THE LUNGS WERE EXAMINED BY LIGHT AND ELECTRON MICROSCOPY 30 MINUTES AFTER CIRCULATION WAS RESTORED.

SMALL PULMONARY EMBOLI WERE CONSISTENTLY FOUND AFTER EXTENSIVE DISSECTION, BUT NOT AFTER LESS ARTERIAL INTERRUPTION. FEW THROMBI WERE SEEN IN VESSELS OF THE EXTREMITIES, BUT WERE EASILY FOUND IN ILIAC VEINS AND VENA CAVA. LEUKOCYTES WERE VERY PLentiful IN BOTH THROMBI AND EMBOLI. THEY WERE FOUND TRANSVERSING THE ENDOTHELIAL BARRIER OF ABDOMINAL VEINS, BUT WERE NOT DIRECTLY ASSOCIATED WITH PLATELETS OR FIBRIN. HOWEVER, PLATELET CLUSTERS WERE CLOSELY ASSOCIATED WITH ENDOTHELIAL DEBRIS AND WITH THE VESSEL WALL IN SMALL AREAS DENuded OF ENDOTHELium. THESE FINDINGS SUGGEST THAT PLATELET ASSOCIATION WITH ALTERED CONSTITuENTS OF THE VESSEL WALL IS AN IMPORTANT FACTOR IN THE PRODUCTION OF THESE THROMBI, AND THAT A HIGH DEGREE OF STASIS IS NECESSARY. THE RELATIVE ROLE OF INFLAMMATION AND OF TRAUMA IS UNCERTAIN AND IS CURRENTLY UNDER INVESTIGATION.

MECHANISM WHEREBY PLATELETS STRENGTHEN AND NOURISH THE ENDOTHELium

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WE HAVE TRANSFUSED PLATELETS LABELED IN VIVO WITH TRITIATED DIISOPROPYLFLUOROPHOSPHONATE INTO GUINEA PIGS RENDERED THROMBOCYTOPENIC BY TOTAL-BODY RADIATION. Electron micrographs of biopsy material, taken at regular intervals from 1 to 30 minutes after completion of the transfusion, show that the platelets are attracted to the endothelium immediately on entering the blood vessel and that dense areas form between platelets and endothelial membranes. Vesicles form at these sites and the juxtaposed membranes disappear. The endothelial cytoplasm becomes very active, with formation of a great many ribosomes and vesicles. The platelet contents are mixed rapidly with the endothelial cytoplasm by the vesicular transport mechanism. Thirty minutes after completion of the transfusion, no evidence of platelet structure, except the label, can be detected in the endothelium. WHEN PLASMA CONTAINING 10 TIMES THE AMOUNT OF RADIooACTIVITY OF THE PLATELET CONCENTRATE WAS TRANSFUSED INTO SIMILAR THROMBOCYTOPENIC guinea pigs, 87 percent of the label was observed in the plasma and red blood cells, and none was concentrated in the endothelium. Electron
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autoradiographs of platelets prepared prior to transfusion show that only 20 percent of the platelets are labeled, so each label observed in the endothelium means that 5 platelets have interacted. (Research supported in part by USPHS project grant HE-12121-01.)

TUESDAY MORNING, SESSION III

THE RELEASE REACTION AND ITS GENERAL ASPECTS

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The discovery that ADP is a potent platelet-aggregating substance greatly stimulated platelet research. This research has established the platelet as a secretory cell, and studies on the release reaction of their secretory activity have revealed the following. A variety of inducers initiate the release of a number of substances from the platelets, whereas other substances remain inside. The released substances are contained in subcellular granules which seem to empty their contents by a process of reverse phagocytosis. This reaction is dependent on Ca++ and energy, and there is evidence that a contractile protein is involved. The reaction can be blocked by inhibitors of energy metabolism, α-blockers, chlorpromazine, and anti-inflammatory drugs such as butazolidine and acetylsalicylic acid.

The extrusion process of secretory granules (secosomes) seems to follow this basic pattern in other secretory cells. Thus, dependence on Ca++ and energy has been established for the release reaction in adrenal medulla, neurohypophysis, polymorphonuclear leukocytes, mast cells, nerve terminals, endocrine and exocrine pancreas, anterior pituitary, and submaxillary gland; the reverse process, phagocytosis, is also dependent on these factors. Other similarities are the occurrence of Ca/Mg-activated ATPase, of ATP, and of a specific protein in the granules. In addition, the above-mentioned agents inhibit the release reaction in several other cell systems. Since both secretion and phagocytosis are more widely operative in the body than earlier anticipated, these similarities have great interest to physiology, pathology, and pharmacology. (See Stormorken, Scand. J. Haemat. Suppl., 1969, for a more detailed study.)

SELECTIVITY OF THE THROMBIN-INDUCED BLOOD PLATELET RELEASE REACTION

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The thrombin-induced platelet release reaction has been shown to release non-metabolic adenine nucleotides, serotonin, and β-glucuronidase from intact platelets. Investigations into the selectivity of the reaction were undertaken by combining subcellular fractionation and the effect of thrombin upon washed platelets. Platelets were homogenized and applied to sucrose density gradients after ultracentrifugation. The homogenate yielded a soluble portion, membranes, and a granular band which could be further subdivided into fractions containing lighter α-granules and mitochondria above a collection of heavier, denser α-granules. Biochemical determinations were made of over 21 platelet enzymes and substances. Washed human platelets were incubated with 1–5 NIH units/ml. of thrombin at 37 C. for varying periods of time. Sixty percent of nonradioactive labeled adenine nucleotides and 80 percent of serotonin were rapidly released. Between 14 and 50 percent of 6 acid hydrolases were also released with the exception of acid nitrophenolphosphatase, which was not actively released. The platelets retained lactate dehydrogenase, pyruvate kinase, adenosine kinase, adenosine deaminase, and adenosine phosphohibosyltransferase, constituents of the soluble portion. Enzymes of mitochondrial membranes—adenylate kinase, hexokinase, 5′nucleotidase and cytochrome C oxidase—were also retained, as were metabolically active ATP and ADP.
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All substances released—acid hydrolases, serotonin, and inert metabolic nucleotides—were present in platelet granules. The former were found in a fraction associated with lighter, less dense α-granules and the latter in heavier, more dense granular structures.

These findings indicate that the thrombin-induced platelet release reaction consists of extrusion of granular material and is not caused by changes in membrane permeability, cell lysis, or pore formation in membranes.

EFFECT OF THROMBIN ON PLATELET RELEASE OF GLYCOLYTIC INTERMEDIATES

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Changes in platelet metabolism caused by thrombin could be due in part to alterations in the compartmentation of metabolic systems. To test this, preliminary studies have been carried out to determine if thrombin causes a "release" of glycolytic intermediates from platelets. Washed rat platelets, suspended in saline buffered with tris-HCl pH 7.4, and containing Ca²⁺, Mg²⁺, and glucose were incubated at 37°C, either with or without thrombin. After 10 minutes, the suspensions were quickly cooled and separated by centrifugation into a platelet pellet and a supernatant fraction. Glycolytic intermediates and adenine nucleotides were measured in perchloric acid extracts of each fraction by enzyme fluorometric methods. In suspensions without thrombin, only pyruvate was found in the supernatant fraction, consistent with the free diffusion of pyruvate across cell membranes. In the suspensions with thrombin, the supernatant fraction contained significant amounts of glucose-6-phosphate, fructose-6-phosphate, ADP, and ATP, as well as pyruvate. This fraction also contained small amounts of dihydroxyacetone phosphate and traces of fructose-1,6-diphosphate. The other glycolytic intermediates appear not to be released to a great extent, but the small amounts present in platelets make an exact assessment of the extent of release difficult. It is concluded that thrombin leads to extensive release of some, but not all, glycolytic intermediates.

PATTERNS OF ENDOGENOUS SEROTONIN RELEASE FROM HUMAN PLATELETS

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Serotonin release from human platelets has been used as a parameter in the study of platelet release reactions. Taking advantage of the platelet's avidity for exogenous serotonin, most investigations have been carried out by introducing labeled serotonin into platelets and measuring released radioactivity. Serotonin, a known platelet-aggregating agent and releaser of platelet factor 3, produces reversible aggregation at the doses used in those experiments.

Human platelet endogenous serotonin release was measured by a spectrophoto-fluorometric assay. This method obviates the exposure of the platelet to exogenous 5HT and, by comparison with labeling experiments, provides information as to whether exogenous 5HT equilibrates with and therefore behaves in a fashion representative of endogenous 5HT.

Platelets from 8 normal donors were evaluated for release of serotonin by using bovine thrombin, adenosine diphosphate, and connective tissue as aggregating agents. With the serotonin released by doses of thrombin sufficient to produce clot retraction used as the 100 percent release value, connective tissue was found to produce an average release of 29 percent. Adenosine diphosphate at 2 μM gave no release,
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whereas 10 mM lead to release in 1 of 8 subjects. Less 5HT was released when thrombin was used at doses which produced aggregation but not retraction.

The results obtained by measurement of endogenous release differ from reports of those obtained with exogenous serotonin and suggest that the introduced labeled serotonin does not equilibrate and is therefore not representative of the total serotonin pool.

EFFECT OF ACETYLSALICYLIC ACID AND OTHER NONSTEROIDAL ANTI-INFLAMMATORY AGENTS ON THE RELEASE OF 14C-SEROTONIN FROM HUMAN AND RABBIT PLATELETS

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Release of platelet-bound 14C-serotonin induced by connective tissue particles (CT) is inhibited by nonsteroidal anti-inflammatory drugs. The degree of inhibition has been used to assess the effect of these drugs. Human citrated platelet-rich plasma (PRP) or rabbit citrated or heparinized PRP was tested after 15-minute incubation with the drugs. With a maximally effective concentration of acetylsalicylic acid, the amount of platelet-bound 14C-serotonin released in all samples of rabbit PRP and some samples of human PRP was reduced by a constant amount (about 20 percent of the bound radioactivity). In other samples of human PRP, release was inhibited to a considerably greater degree. The efficacy of drugs in preventing 14C-serotonin release in human PRP followed the same order as inhibition of CT-induced aggregation reported by O'Brien (Lancet 1: 894, 1968). The concentration (mM) which caused about 50 percent inhibition of release from human platelets were: indomethacin, 0.004; acetylsalicylic acid, 0.020; mefenamic acid, 0.125; flufenamic acid, 0.125; phenylbutazone, 0.55; sulfipyrazone, 1.1; and sodium salicylate, 9.0. These drugs also increased the amount of 14C-serotonin bound to the platelets in control samples not shaken with CT.

CELL MEMBRANE DAMAGE, PLATELET STICKINESS, AND SOME EFFECTS OF ASPIRIN AND COCAINE

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Aspirin and other anti-inflammatory drugs are reported to inhibit the release of ADP from platelets. This protective or stabilizing effect might be related to the anti-inflammatory effect of these drugs. Accordingly, platelets and red cells were exposed to various insults, and the nature of the resulting "damage" was studied. The effect of pretreatment of the cells with aspirin and with cocaine was also investigated.

Within 2 seconds after addition of ADP to platelet-rich plasma, the platelets developed heparin-neutralizing activity (HNA) and platelet factor-4 activity. The addition of adenosine or cocaine, either before or after the ADP, prevented the platelets from becoming sticky and also prevented the appearance of HNA. Aspirin added before ADP decreased the HNA but had no effect on stickiness. Serotonin- and thrombin-induced stickiness also occur in association with HNA, and normal serum contains a soluble form of HNA. Adrenaline-induced aggregation is atypical and no HNA is exposed unless and until aggregation occurs. Thus, HNA and stickiness are closely related phenomena.

Red cells and platelets were damaged by lysolecithin, saponin, and deoxycholate, which also shortened the stypven time, indicating the presence of phospholipid activity (PA). No HNA appears, and aspirin does not protect the cells. These compounds probably penetrate the lipid in the membrane, which has been weakened by a related exposure of phospholipid. Hypotonie saline and freezing and thawing expose both HNA and PA. Pretreatment with aspirin decreases the amount of HNA and decreases "damage" assessed in other ways. Cocaine increases the damage as
judged by PA release and hemolysis. Aspirin thus stabilizes the membrane and has some special relation with the heparin-neutralizing sites. Perhaps this effect is concerned with the anti-inflammatory action of this group of drugs.

A Comparison of the Effects of Guanidinosuccinic Acid and Aspirin on Platelet Factor-3 Activation and Platelet Aggregation

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An activity in uremic blood inhibits activation of platelet factor 3 (PF-3) by ADP in citrated plasma. Previously reported studies of the uremic inhibitor (Blood 30: 331, 1967) and direct measurement of guanidinosuccinic acid (GSA) in uremic serum are in accord with the hypothesis that they are identical. GSA and aspirin (ASA) at equimolar concentrations inhibit PF-3 activation by ADP in citrated platelet-rich plasma. The inhibition induced by GSA can be overcome by $2 \times 10^{-5}$ M Ca$^{++}$, whereas that of ASA cannot. Both GSA and ASA inhibit the second wave of platelet aggregation at 37 C. when induced by ADP or 1-epinephrine, and reduce the extent of aggregation induced by low concentrations of connective tissue. Increments of ADP reestablish patterns of secondary aggregation with GSA but not with ASA. Addition of $2.7 \times 10^{-3}$ M EDTA to citrated plasma mimics the inhibitory activity of GSA, further suggesting that the latter acts to bind Ca$^{++}$. However, GSA inhibits PF-3 activation in heparinized plasma, indicating that it is active at physiological concentrations of Ca$^{++}$. In electron microscopic studies, platelets incubated with GSA are well preserved morphologically, but have a diminished wave of contraction of granules and marginal microtubules when ADP is added. Thus GSA appears to act like ASA in limiting the release of endogenous ADP and thereby decreasing PF-3 activation and platelet aggregation.

The Effect of Epinephrine-Induced Elevation of Plasma-Free Fatty Acids on Platelet Aggregation in Man


In vitro studies and animal experiments have shown that epinephrine (EPI) induces platelet aggregation. It has also been reported that EPI injection produces elevation of plasma-free fatty acids (FFA). Hoak (Circulation Res. 20: 11, 1967) has suggested that fatty acids can potentiate platelet aggregation and activate the early stages of coagulation.

Our studies show that the plasma FFA levels are often abnormally elevated in diabetic patients with acute myocardial infarction, a condition frequently associated with thromboembolic complications. An intramuscular injection of 0.1 mg. EPI in well-controlled fasting diabetics and in normal fasting individuals significantly raises plasma FFA and glucose levels 3½ hour after the injection. FFA and glucose levels in the diabetics remain elevated even 2 hours after the EPI injection, whereas FFA and glucose levels in the nondiabetic individuals return to normal within 2 hours. Orally administered propranolol (10 mg. qid., for 1 day) effectively blocked the effect of EPI on lipolysis in normal human volunteers, but it did not block the effect of EPI on glycolysis. Furthermore, on intramuscular injection of 0.1 mg. EPI in normal human volunteers produced, in addition to a significant rise in FFA levels, a considerable increase in platelet aggregation (induced by collagen, ADP, or thrombin), as measured by the Aggregometer and by Chandler’s loop. Both the rise of FFA and the increase in platelet aggregation produced by the exogenous administration of
EPI was significantly inhibited by propranolol pretreatment, whereas the glycolytic effect of EPI was not altered.

Our data suggest that the EPI-induced increase in platelet aggregation may be the result of an increased lipid mobilization upon release of FFA into the blood stream, and action which is inhibited by beta-adrenergic receptor-blocking agents.

**Divalent Cations, Platelet Membrane Fibrinogen, and Platelet Aggregation**

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To examine the role of cations and fibrinogen in ADP-induced platelet aggregation, we isolated platelets with as little alteration as possible. It was important that Mg\(^{2+}\), K\(^+\), and glucose be in the washing and suspending fluids. The addition of a protein (albumin or gelatin) enhanced the stability of platelets in the final suspension. To obtain washed platelets sensitive to low concentrations of ADP, it was necessary to maintain platelet Mg\(^{2+}\). Studies with anti-fibrinogen-coated polystyrene showed that platelet membrane fibrinogen was preserved by the preparation method used. Addition of a plasma cofactor was unnecessary for ADP-induced aggregation. Aggregation of platelets by ADP led to the release of fibrinogen. Platelets suspended in solutions without added Ca\(^{2+}\) or Mg\(^{2+}\) showed the initial shape change upon addition of ADP, but did not aggregate until Ca\(^{2+}\) was added. Mg\(^{2+}\) was necessary for platelet deaggregation, and inhibited aggregation in the presence of Ca\(^{2+}\). The pattern of aggregation and deaggregation was therefore dependent upon the Ca\(^{2+}\) to Mg\(^{2+}\) ratio in the suspending fluid. To determine whether calcium added to the suspension was required as ionized or bound calcium, we examined the relationship between ionized calcium and platelet aggregation. Platelets suspended in a solution containing Ca\(^{2+}\) (but no Mg\(^{2+}\) or protein) aggregated upon the addition of ADP in the presence of concentrations of EDTA adequate to bind the ionized calcium (determined by a calcium electrode). However, the concentration of EDTA required to inhibit aggregation was greater than that required to bind the ionized calcium, and thus bound calcium may be the form that is involved in ADP-induced aggregation.

**The Relationship of Immunologically Induced Rabbit Platelet Aggregation to ADP and to Platelet Membrane Changes Independent of ADP**

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We have investigated the relationship of rabbit platelet aggregation by antigen-antibody reactions to ADP-induced aggregation and to changes in platelet stickiness unrelated to ADP. Rabbit platelet-rich plasma (PRP) containing heparin (FC 40 \(\mu\)g/mL) was challenged with egg albumin (EA) (FC 0.55 \(\mu\)g EA N/mL) and rabbit anti-EA gamma globulin (FC 0.11 \(\mu\)g AB N/mL). Aggregation was measured with a Chronolog aggregometer; adhesion was measured by passage of PRP through celite glass bead columns.

Addition of antigen and antibody to heparin PRP produced aggregation after a latent period of 1 to 2 minutes. Addition of AMP to PRP in a final concentration of 5 mM prior to challenge diminished the extent of aggregation. Since this concentration of AMP inhibits aggregation produced by concentrations of ADP greater than those released from platelets during the course of this reaction, and since the onset of aggregation occurs sooner than the bulk of ADP release, it was concluded that aggregation produced by antigen-antibody reactions was partially independent of ADP-mediated mechanisms.

Changes in surface stickiness will produce aggregation in a stirred system. The
notion that the AMP-indifferent aggregation was due to surface stickiness was tested by passing PRP through celite columns at intervals after the addition of antigen and antibody. The results indicated increased adherence beginning about 1 minute after challenge, which was only partially inhibited by AMP (5 mM FC).

It was concluded that platelet aggregation after challenge with antigen and antibody is in part due to membrane changes not mediated by ADP and probably preceding release of intracellular platelet components.

SPECIES DIFFERENCES IN PLATELET AGGREGATION

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Platelet aggregation induced by collagen, ADP, or thrombin, in citrated platelet-rich plasma (PRP) obtained from 7 species (human, monkey, dog, rabbit, rat, chicken, and pigeon), was studied with the Aggregometer® or by the modified Chandler's loop technique. Though platelets from all species responded to the exogenous stimuli, there were considerable differences in the degree of response among the species. All mammalian samples aggregated faster than those of chicken and pigeons. Prothrombin times of mammalian plasmas were also considerably shorter than those of the birds. Among the mammals, human and monkey platelets showed similar aggregation characteristics. There was considerable variation in aggregation parameters obtained on recalcification of citrated PRP from human, monkey, and rabbit, whereas PRP from the rest of the 4 species showed comparatively consistent aggregation in the system. Chicken and pigeon PRP did not produce any significant aggregation with exogenous ADP. Platelet counts of chicken and pigeon were considerably lower (less than one-tenth) than that of mammalian samples. Our results suggest that among the various species tested important qualitative and quantitative differences exist in platelet aggregation, and that platelet concentration, platelet size, and possible presence of aggregation inhibitors in plasma might be important factors that contribute to these differences.

PLATELET ADHESIVENESS AND THROMBOSIS INDUCED BY ALKALINE PHOSPHATASE IN THE RABBIT

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A sixfold increase in total serum alkaline phosphatase (ALPh), accompanying a decrease of platelets to 54 percent of controls, was observed in averaged blood samples of 20 rabbits 1 minute after intravenous injection of 10 mg./kg. purified ALPh. Small or large platelet aggregates were noted in counting chambers. A glass bead test of platelet adhesiveness, with the 1 minute postinjection platelet-rich plasma in the presence of 0.1 μg./ml. ADP, gave values at least twice that in controls. In thromboelastograms (TEG), a significant shortening of r and k values paralleled the increase in platelet adhesiveness and the shortening of the earlobe bleeding-time test. There was also a very significant 4+ thrombosis of temporarily occluded segments of experimentally exposed mesenteric veins. Since various coagulation tests and factor assays did not show changes of sufficient magnitude, it may be concluded that the ALPh-induced thrombophilia is primarily related to the increased platelet aggregation. In similar studies, 15 minutes postinjection, platelet counts had risen again, no aggregates were seen, and the thrombosis test was negative. Platelet values reached normal in about 2 hours, when the serum ALPh returned to control levels. In 15 rabbits, dicumarolized for 3 days to prolong the PT to > 60 seconds, the effect of ALPh injection on platelet aggregation was still found, but now the vein
thrombosis test was negative. Changing patterns in postinjection isoenzyme electrophoresis showed that the significant alkaline phosphatase effects correlate with certain slow-moving components, cathodic to β-globulin. These patterns are conspicuous in data with the original intestinal enzyme preparation. (Supported by NIH research grant HE-01510.)

OPTICAL DENSITY CHANGES DURING PLATELET AGGREGATION IN THE CHANDLER TUBE

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Alterations of turbidity of platelet-rich plasma (PRP) in the Chandler tube were evaluated in normal humans, rabbits, and selected experimental models. Optical density (OD) changes during aggregation were monitored continuously. The mean thrombus formation time and duration of aggregation were 15.2 and 4.4 minutes (human PRP) and 19.1 and 5.0 minutes (rabbit PRP). The decrease of OD during aggregation exceeded 30 percent of four out of five of the human PRP specimens. A small decrease of OD (<12 percent) was observed in 10 percent of specimens. Similarly, 8–26 percent of rabbit PRP specimens in different groups had small changes of OD with aggregation. Ellagic acid and adrenaline injected in vivo and stearic acid, collagen, and Russell’s viper venom added in vitro shortened thrombus formation time and duration of aggregation, and minimized the decrease of OD during aggregation. Small changes of OD were associated with widespread tiny aggregates of platelets in the Chandler tube. Electron microscopy showed that fibrin strands were prominent in such samples and were within platelet aggregates and in proximity to platelet membranes. An OD change during aggregation in less than 30 percent occurred in three-fourths of the patients with advanced malignant disease. Thus, alterations in aggregation of PRP in the Chandler system are found in human disease states associated with an increased incidence of thromboembolism or disseminated intravascular coagulation. Preliminary evidence suggests that the abnormalities of aggregation are related in part to pertinence alterations in the aggregation system.

PLATELET-LEUKOCYTE AGGREGATION PATTERNS AND THEIR CORRELATION TO THROMBOTIC STATES IN MAN

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Highly specific “rosette” patterns of clumped platelets surrounded by a ring of leukocytes have been found in specially prepared blood smears obtained from patients undergoing thrombotic episodes. Experience with over 200 patients and control subjects shows that individuals with coronary occlusion and cerebral arterial occlusion have the greatest number of platelet-leukocyte aggregates.

Blood samples were taken from subjects by peripheral venous tap and treated with heparin-EDTA. Divalent cation was replaced by strontium chloride in concentrations ranging from 0.005 M to 0.50 M. A thin smear was then made on a slide stained with Wright’s solution.

Since leukocytes are known to appear in thrombi in an orderly sequential manner (in vivo) rather than as simple thrombotic inclusions, the in vitro platelet-leukocyte aggregates noted above may reflect platelet-leukocyte interaction at the site of thrombosis. Certainly, many individuals with intra-arterial thromboses have some alterations in their peripheral venous blood which may be detected by formation of the platelet-leukocyte aggregates described.

Studies are now in progress to determine the surface (membrane) phenomena responsible for this aggregation. (Supported by the Michigan Heart Association.)
Pseudokalemia

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Spurious elevation of the serum potassium (K) concentration (i.e., “pseudokalemia”) was initially described in patients with thrombocythemia. The true circulating plasma K was shown to be normal, and the “hyperkalemia” (e.g., serum K 8 mEq./liter.) was ascribed to the release of K from platelets during in vitro clotting. Available evidence suggests that the pseudokalemia is related to both an increase in total platelet mass and to an abnormally great release of K from thrombocytemic platelets, and not primarily to increased K per unit platelet mass (weight or H2O content). Platelet counts notoriously underestimated the extent of the true platelet mass in thrombocytemia.

Pseudokalemia has also been ascribed to release of K from leukocytes in an occasional case of leukemia. In contrast to platelet-related pseudokalemia, coagulation may not be necessary for pseudokalemia in leukemic patients, and simple incubation of anticoagulated blood may suffice for significant K release. It is important to differentiate leukemic pseudokalemia from true hyperkalemia, which may also occur in leukemia as a result of renal impairment and perhaps therapy.

“Pseudoelevations” of other substances (e.g., acid phosphatase) in serum, as compared with cell-free plasma, have also been described and related to release from platelets and/or leukocytes. It can be reasonably predicted that with the future increase in automated laboratory tests on serum, other chemical determinations will be shown to be potentially influenced by abnormalities in the cellular compartment. Thus, in certain pathologic states, knowledge of the status of the cellular compartment of blood may be important in interpreting tests carried out on the serum compartment. The phenomenon of pseudokalemia emphasizes the necessity of maintaining sufficient “overview” so that laboratory tests are not only precise (i.e., chemically reproducible) but also accurate (i.e., biologically meaningful).

TUESDAY AFTERNOON, SESSION IV

New Familial Defect in Platelet Function Due to Impaired Release of ADP

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Recent studies indicate that abnormalities of platelet function in some patients with primary, but sporadic, disorders of hemostasis may be attributed to impaired release of platelet adenosine diphosphate (ADP). We have recently studied 4 male and 2 female members, representing 3 generations, of a large family in whom the defect appears to be transmitted as an autosomal dominant. Platelet counts varied from 130,000 to 200,000 per cu. mm., and clot retraction was normal. Bleeding time was variably prolonged, and platelet adhesion to glass was decreased in 3 of 4 patients tested. Serum prothrombin was abnormal and was corrected by the addition of lipid prior to clotting. Platelet factor-3 activity, assayed by incubating platelet-rich plasma with kaolin, was decreased and was associated with decreased release of ADP. The following tests of platelet aggregation were abnormal: aggregation by a standard concentration of connective tissue was either absent or minimal due to impaired ADP release, although aggregation occurred with a 10 times more concentrated suspension. Primary aggregation by epinephrine (5 and 50 μM, 37 C.) occurred in 4 subjects and was absent in 2. The second wave of aggregation was absent in all 6 patients. Primary aggregation with ADP (2 μM, 37 C.) occurred normally but was followed by rapid disaggregation. Although no morphologic abnormalities were detected by light or electron microscopy, the patients’ platelets were smaller than normal, as determined from size distribution studies using the Coulter Counter. Platelet ATP and
ADP, expressed as mM per $10^9$ platelets, was decreased. The findings of impaired function, decreased size, and reduced nucleotides are similar to the recent findings (S. Karpatin, J. Clin. Invest., in press) reported for "old" platelets and suggest the presence of an older population of platelets in our patients, possibly due to a more selective destruction of younger platelets.

**Wiskott-Aldrich Syndrome: Qualitative Platelet Defects and Short Platelet Survival**

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The Wiskott-Aldrich syndrome is characterized by hemorrhagic diathesis, eczema, and recurrent infections.

The platelet function in 3 boys (4, 5, and 7 years old) with this congenital disease was studied. They had severe thrombocytopenia and prolonged bleeding time.

The morphology of the blood platelets was abnormal with reductions in size and variations in shape. Electron microscopy revealed decreased numbers of $a$-granules, mitochondria, and very dense bodies.

Biochemical studies showed that the storage pool of nucleotides in platelets was almost completely lacking, and that insignificant amounts of ADP were released by collagen. Since the nucleotides are located in very dense bodies and $a$-granules, there is good correlation between the morphological and biochemical findings. The metabolic activity of the platelets measured as uptake of $^{32}$P in nucleotides was high.

Platelet electrophoretic mobility was normal, but after addition of collagen, the normal decrease in platelet mobility was absent. Platelet aggregation induced by collagen and ADP was deficient. Platelet adhesiveness in whole blood in vitro was reduced. Platelet factor-3 availability was normal in one and reduced in another of the patients.

The survival time of normal crosstransfused platelets was normal, whereas autologous platelets in 2 of the patients survived less than 2 days. Bone marrow had a normal number of megakaryocytes. Electron microscopy showed that platelets were phagocytized in macrophages and reticulum cells. The main cause of the thrombocytopenia is, therefore, increased platelet destruction by phagocytosis of platelets in RES. It is suggested that these pathological platelets are handled as foreign particles in RES.

**Platelet Glycolysis in the Wiskott-Aldrich Syndrome**

Byung K. Kim, Atsushi Kuramoto, Manfred Steiner, and Mario Baldini, Brown University and Memorial Hospital Division of Hematologic Research, Pawtucket, R.I.

Our previous investigations have shown that thrombocytopenia in the Wiskott-Aldrich syndrome is associated with rapid platelet destruction due to an "intra-corpuscular" defect. Deficiencies in platelet function were also demonstrated: 1) lack of aggregation with ADP, collagen, and epinephrine, 2) depressed serotonin uptake, and 3) impaired protein synthesis. In the present study, platelet glycolysis was investigated in 3 brothers with the syndrome, a normal sibling, and their parents. In the platelets of the affected children, glucose consumption and lactate production were 50 to 60 percent of normal. Accumulation of G-6-P, FDP, and triose phosphate during incubation was depressed. ATP content was reduced to 45–70 percent of normal and glycogen to 20–30 percent of normal; glycogen synthesis was negligible. Oxidative glycolysis was also investigated. Production of $^{14}$CO$_2$ from differentially labeled glucose was reduced with both G-1-$^{14}$C and G-6-$^{14}$C, indicating that aerobic, as well as anaerobic, glycolysis was depressed. With latex particles present in the suspending medium, there was no increase in $^{14}$CO$_2$ production from G-6-$^{14}$C, while it
was increased 8 to 12 times with normal platelets. By this test, a definite but partial defect could also be demonstrated in the platelets of the mother. No abnormalities were found in the platelets of the normal sibling or of the father. Further studies included measurement of various platelet glycolytic enzymes. The enzyme activity significantly reduced was that of hexokinase (38 to 55 percent of normal). We conclude that in the Wiskott-Aldrich syndrome there is a metabolic abnormality in platelets which is characterized by depressed anaerobic and aerobic glycolysis, with reduced energy metabolism caused by a severe defect in the first phase of the glycolytic process.

A Three-Dimensional Study of Platelet Surface Responses to Drugs

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Surface detail of the platelet membrane was revealed by the scanning electron microscope and contrasted with transmission electron microscopy of whole mount platelet preparations (Thromb. Diath. Haemorrhag. Suppl. 26:87, 1967). Whole blood platelets (human) in a sodium citrate environment were exposed in siliconized equipment ex vivo to either thrombin (0.01–0.2 Iowa units/ml.), serotonin (0.01–0.2 µg./ml.), Adrenosem or adrenochrome semicarbazone (0.01–2 µg./ml.), sodium salicylate (0.1–5 mg./ml.), acetylsalicylic acid (0.3–3 mg./ml.), Rheomacrodex (5–20 mg./ml.), or uric acid (0.12–0.25 mg./ml.). Platelets were fixed with glutaraldehyde. Membrane features were intensified by further treatment with either 5 percent FeCl₃, propylene oxide, or saturated difluorodinitrobenzene. The preparations, or either a glass or formvar surface, were simultaneously rotated and coated at a low angle with metal. A Joelco scanning electron microscope was operated at 25 kV to obtain 3-D information about platelet membranes. Platelets fixed immediately with glutaraldehyde were round or oval with some surface folding, but pseudopods were rare or minute. Exposure to Adrenosem, adrenochrome semicarbazone, acetylsalicylic acid, citrate, or citrate buffered with tris had a similar morphology, with wrinkled surfaces and numerous pseudopods. Platelets with histamine and Rheomacrodex resembled glutaraldehyde platelets with few pseudopods. Platelet spreading and aggregation with reorganization of organelles was prominent in the presence of thrombin, epinephrine, norepinephrine, and serotonin. There was also evidence of platelet disruption. Single platelets and aggregates formed after uric acid addition showed blebs on their surfaces, as well as spreading and granule dispersion. (Aided by the Michigan Heart Association and the S. E. Massingill Co.)

The Measurement of Membrane or Ecto-ATPase Activity in Intact Human Platelets

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A membrane or “ecto-ATPase” has been demonstrated in intact human platelets by a number of investigators. Histochemical procedures and the measurement of inorganic phosphate released from ATP have been the techniques used. We have confirmed the presence of a platelet ecto-ATPase by using a simple enzymatic assay. Blood was drawn from volunteers, and platelet-rich plasma was prepared by differential centrifugation. The platelets were washed twice in a Ringer’s solution containing citrate, with pH adjusted to 6.7. Care was taken to avoid excessive trauma to platelets during washing, since this results in the release of ATPase activity to the ambient medium. The platelets were finally suspended in Ringer’s solution or 5 percent glucose.
ABSTRACTS: BLOOD PLATELETS

in water containing citrate. ATP, $3.6 \times 10^{-4} M$; calcium and magnesium, each present at $5 \times 10^{-6} M$; and tris buffer, pH 7.5, were incubated with reduced diphosphopyridine nucleotide (DPNH), phosphoenolpyruvate (PEP), lactic dehydrogenase (LDH), and pyruvate kinase (PK). The concentration of DPNH was followed with a spectrophotometer, and ADP contaminating the ATP substrate was removed by the enzyme mixture. When the spectrophotometric reading became stable the ATPase assay was started by the introduction of a measured amount of platelet suspension. At 20°C., $2.0 \times 10^{-7} M$ of ADP was released per $10^5$ platelets per hour. The $K_m$ of ecto-ATPase by this technique was $3.6-5.0 \times 10^{-4} M$. This technique requires small numbers of platelets and eliminates the need for acid extraction.

MAGNESIUM AND STORAGE OF BLOOD PLATELETS

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The storage of blood platelets is characterized by changes in the aggregative and adhesive properties of platelets as well as by structural changes that can be demonstrated by electron microscopy. The addition of 2.5 mEq. of magnesium per liter of platelet-rich plasma collected in ACD solution and stored at 5°C. with a final pH of 7.0–7.1 was found to retard these changes. There was a reduction of the spontaneous aggregation of platelets normally seen after storage for 24 to 48 hours. In addition, the response to ADP, collagen, and agar, when studied by a modified Wright technique at 37°C., was maintained at relatively high levels. The platelets stored in the presence of magnesium at pH 7.0 resemble fresh platelets when studied with the electron microscope. They remain discoid in shape and exhibit a full complement of platelet granules after 48 hours storage. Platelets acidified to pH 6.5 and stored with added magnesium show ultrastructural degenerative changes, with the combination of magnesium and acidity also reducing their response to aggregative agents.

IN VIVO RESYNTHESIS OF PLATELET GLYCOGEN AFTER DEPLETION DURING STORAGE AT 22°C.

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Human platelet concentrates (PC) can be stored with satisfactory maintenance of viability for transfusion purposes if 22°C. is chosen as storage temperature rather than 4°C. During the first 24 hours of such storage, platelet glycogen, measured chemically, falls to less than 10 percent of the control values; the glycogen organelle disappears from electron micrographic (EM) preparations as well. This occurs in spite of an essentially unchanged plasma glucose level, a linear production of lactate, and maintenance of ATP stores. Stored platelets resuspended in Krebs-Ringer buffer demonstrate rates of glycolysis and glucose oxidation greater than those of fresh platelets, but net resynthesis of glycogen does not occur. In three studies, patients with aplastic anemia and severe thrombocytopenia were infused with multiple units of stored PC so that platelet counts over 80,000 per mm$^3$ were achieved. Platelet glycogen was rapidly resynthesized in vivo, reaching normal levels within 3 hours after transfusion. Glycogen particles reappeared in EM preparations of circulating platelets obtained 24 hours after transfusion. We conclude that biochemical defects which develop during platelet storage can be corrected in vivo if storage conditions permit maintenance of viability. Current biochemical and functional studies of stored platelets cannot be relied upon to predict either subsequent in vivo viability or in vivo functional capacity.
Observations on Human Blood Platelets under Hypertonic Stress, Supercooling and Slow Freezing

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During slow (equilibrium) freezing of a cell suspension, the hypertonic solution produced by the formation of extracellular ice leads to shrinking of the cells. Packed platelet volume (crit) was found to be a linear function of the reciprocal of osmolality up to approximately $4 \times$ isotonic. At this osmolality, crit was 55 percent of control. Further increase in osmolality caused no further shrinking, and the following signs of injury appeared: loss of platelet K and $^{14}$C-serotonin to plasma, and reduction of clot retraction (CR) and of $^{14}$C-serotonin uptake (SU). Osmolality higher than $4 \times$ isotonic was tolerable only for periods shorter than 1 minute at room temperature.

Supercooling not only failed to affect SU or CR when they were tested after rewarming of the platelets, but at $-5$ C. platelets could tolerate up to $10 \times$ isotonic environment for at least 8 minutes. Packed platelet volume at $-5$ C. was 10–20 percent larger than at 37 C. at all osmolalities up to $6 \times$ isotonic. At $10 \times$ isotonic, the crit of supercooled platelets was the same as at $6 \times$ isotonic, while that of 37 C. platelets had considerably increased and showed signs of injury.

Platelet-rich plasma could be frozen at $-5.5$ C. and thawed without reduction of SU or CR, but after freezing at $-6$ C. both were reduced. The solute concentration in frozen plasma at $-5.5$ C. corresponds to $10 \times$ isotonic. These observations support the hypothesis that freezing injury is related to the reduction in cell size below a minimum tolerable volume.