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FIXATION OF PLATELET AGGREGATE SIZE DISTRIBUTION IN HUMAN BLOOD

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ABSTRACT

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An experimental study has been carried out on the use of aldehydes for fixation of human platelet aggregate size distributions. The objective of the work was to develop a methodology of stopping aggregation and disaggregation processes for subsequent analysis. The results are intended to facilitate study of rates of aggregation and disaggregation as influenced by various stimuli.

Platelet aggregation was induced in citrate-anticoagulated platelet-rich plasma (PRP) by addition of adenine dinucleotide (ADP) in final concentration ranging from 0.5 to 20 μM. The aggregated PRP specimens were diluted (158.5 to 1) in a counting medium (isoton) for size distribution analysis. An electronic particle counter was used to study the aggregate size distributions in the range 13-101 μm in equivalent spherical diameter. Parameters used to monitor the size distributions were cumulative volume and cumulative population of the aggregates, mean aggregate size, and volume available for aggregation from free (unaggregated) platelets.

In preliminary studies evidence was obtained that glutaraldehyde was a more promising fixative than formaldehyde. Glutaraldehyde in

appropriate concentrations caused no important problems in resuspension or in aggregate size change for times of fixation of several minutes. Dilution of aggregated PRP specimens in isoton for counting induced rapid disaggregation. However, it was found that this disaggregation could be avoided by use of glutaraldehyde in the isoton counting diluent.

Glutaraldehyde addition to both the aggregated PRP specimen and to the isoton counting diluent to final concentration of 0.048 wt.% was selected as the recommended procedure. Detailed studies were made of aggregate size distributions fixed at various times in the aggregation process. The results indicate that the fixative stops the reactions and stabilizes the distribution for times of 3 to 5 minutes. Thus, the procedure should be useful in studies on rates of platelet aggregation.

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ABBREVIATIONS AND SYMBOLS

Å	Angstrom unit
ADP	adenosine diphosphate
ATP	adenosine triphosphate
°C	degrees Centigrade
cc	cubic centimeter
cm	centimeter
CP	cumulative population
CV	cumulative volume*
CPn	cumulative population to channel n
CVn	cumulative volume to channel n
EDTA	ethylenediaminetetraacetate
gm	gram
Ig	immunoglobulin
Kg	kilogram
ml	milliliter
µl	microliter
M	molar concentration
M.A.S.	mean aggregate size
mm	millimeter
µM	micromolar
µm	micrometer (micron)
m Osmol	milliosmole

PF1	platelet factor 1
PF2	platelet factor 2
PF3	platelet factor 3
PF4	platelet factor 4
PFP	platelet-free plasma
PPP	platelet-poor plasma
PRP	platelet-rich plasma
c-PRP	citrated platelet-rich plasma
PC	platelet count
TV	total volume**
%	percent (either 1/100 or concentration in gm/100 ml)
s.d.	standard deviation
s.e.m.	standard error of the mean
Vavail	available volume

*The term cumulative volume as used in this work is applied to results from channels of the Coulter Counter which pertain to platelet aggregates. Thus, the figures do not include free platelets.

**The term total volume pertains to a count of the unaggregated specimen prior to aggregation using 70 μm aperture of the Coulter Counter. Thus, the total volume corresponds to the total platelet volume available for aggregation.

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I. INTRODUCTION

There are many disorders, involving platelets, that can cause either insufficient or excess platelet activity. One of the normal platelet functions is aggregation, where platelets attach to each other. In applications involving contact of blood with foreign materials excessive platelet aggregation can lead to formation of microemboli.

The present study was undertaken with the overall aim of enhancing our understanding of factors that influence rates of platelet aggregation and disaggregation. The specific objective was to develop a method for fixing platelet aggregates to stop the aggregation process or prevent breakup. Then this method can be used to determine aggregate size distributions in the fixed suspensions.

The basic research tool used in this study was the electronic Coulter Counter (model T) for particle size analysis. The three primary parameters measured were the cumulative volume (volume concentration) of the aggregates, the cumulative population (number concentration) of the aggregates, and the mean aggregate size (mean platelet size with free platelets).

II. BACKGROUND

A. Platelets

Whole blood consists of liquid plasma as well as formed blood cells. In normal adult males, the hematocrit, the percent volume of the formed elements in whole blood, is approximately 45 percent. In normal adult females, the hematocrit is normally about 40 percent. The blood cells are of three main types, the red blood cell (erythrocyte), the white blood cell (leukocyte), and the platelet. The higher density of red blood cells makes it possible to separate them centrifugally from the rest of the blood elements in a platelet-rich plasma. Unfortunately, white blood cells have a comparable density to that of platelets, so leukocytes may remain in the plasma with the platelets after centrifugation. Since leukocyte diameters range up to 13 microns, studies on platelet aggregation by use of electronic particle counters usually are restricted to larger aggregates. In studies on platelet aggregation in whole blood, one has to deal with red blood cell interference as well as white blood cell interference.

Platelets are highly variable in size, ranging from less than 5 to more than 20 cubic microns in volume. In general, older platelets are lighter and smaller, whereas the younger ones are larger and heavier. The two key roles of the platelet are the hemostatic and thromboplastic functions. The hemostatic function is accomplished by physical occlusion of openings in blood vessels by masses of platelets to stop bleeding from injured blood vessels. The thromboplastic function is carried

out by the participation of chemical constituents of platelets (phospholipoprotein parts) in the blood coagulation mechanism. The term platelet aggregation refers to platelet attachment to each other, whereas the term adhesion refers to platelet attachment to a non-platelet surface.[44]

Platelets have most cellular components that other cells have except DNA (deoxyribonucleic acid). Platelets are 77 percent water and 12 percent proteins by weight. Proteins are 52 percent of the dry weight. Other components include carbohydrates, lipids, purines, pyrimidines, nucleotides, minerals and vitamins. In the platelet granule-membrane fraction, one finds fibrinogen, which is 13 percent of the total platelet protein, and the contractile proteins, which are 15 percent of the platelet proteins. The main contractile proteins are ATPase and thrombostein, which is somewhat similar to muscle actomyosin. Platelets also possess 30 to 50 percent of the total blood factor XIII intracellularly. Other platelet membrane proteins include IgG, IgM, plasminoger, factor V (platelet factor 1), factors VIII and XI.[44]

Two of the major platelet proteins are platelet factors 2 and 4. Platelet factor 2 acts as a fibrinogen activating factor along with thrombin, when fibrinogen is converted into fibrin monomer, and is synergistic with ADP-induced aggregation. Platelet factor 4, an anti-heparin factor, is a glycoprotein released from platelets following platelet aggregation induced by ADP, thrombin, or epinephrine. Other characteristics of platelet factor 4 are: it precipitates fibrinogen, neutralizes fibrinogen products, nonenzymatically clots soluble fibrin monomer complexes, shortens thrombin clotting time in presence of

heparin, and like platelet factor 2, it enhances ADP-induced platelet aggregation in vitro. Platelet factor 4 release is mostly from the larger platelets, and it is also worthwhile to note in passing that acid phosphatase is another protein released by platelets, but only during irreversible platelet degrannulation, which is characteristic of irreversible platelet aggregation.

The morphology of platelets will now be discussed. Platelets circulate as flattened discs, and such a shape can be preserved in vitro by collecting blood in citrate or heparin anticoagulants and separating the platelet-rich plasma (PRP) at 37° C. Light microscopy helped in recognizing outstanding features of platelet function such as their shape, their attachment to sites of vascular injury and to each other, their participation in coagulation, and involvement in clot retraction. However, the most basic questions concerning the mechanisms of platelet hemostatic function remain unanswered. In the 1950's and 1960's, there was an increased interest in hemostasis and thrombosis which promoted a rapid accumulation of new information on fundamental aspects of platelet activity. Electron microscopy has been of great assistance in this endeavor. Improved methods of fixation and staining as well as cytochemistry, immunochemistry, and autoradiography applicable to electron microscopy have all contributed to the study of structural physiology of platelets along with its morphology.

The platelet is thought of in terms of three morphological zones, namely, the peripheral zone, the sol-gel zone, and the organella zone.[44] The peripheral zone is involved in converting the platelet from the non-sticky to the adhesive state by releasing endogenous chemical constituents

essential for propagating platelet aggregation or platelet adhesion itself. The peripheral zone includes the exterior coat, the unit membrane, and the submembrane area. The exterior coat is in immediate contact with the surrounding plasma, and covers unit membranes of the platelet surface as well as the linings of the tortuous canalicular system penetrating the platelet substance. Exterior coat material is 150-200 Å in thickness and remains on platelets before, during, and after aggregation. The exterior coat contains acid mucopolysaccharides, glycoproteins as well as magnesium dependent ATPase. The unit membrane itself is a typical trilaminar membrane and is essential to the integrity of internal milieu of platelet. The platelet membrane has two outer electron-dense layers 20 Å thick and a less dense inner layer. The outer two layers are protein, whereas the inner layer is a lipid bimolecular layer. On the membrane surface, one finds proteins, of course and mucopolysaccharides (possibly chondroitin sulfate) which may account for the negative surface charge. This negative surface charge is removed by neuraminidase, so is thought to be due to the sialic acid, and N-acetyl neuraminic acid, a part of the chondroitin sulfate. Isolated platelet membranes have been shown to have acetyl-cholinesterase activity. The platelet membrane has a small amount of carbohydrates with sugar components such as glucose, galactose, mannose, hexosamine, sialic acid and fucose. The membrane is mostly proteins and lipids. Changes in the platelet membrane are characterized by change in surface contour as well as increased permeability resulting in platelet swelling. Surface-active agents such as antihistamines, local anesthetics, chelating agents, high and low salt concentrations,

and lipid solvents injure membrane and cause platelet damage. The submembrane area serves as a transition between the peripheral zone and the sol-gel matrix. The fine filaments in the submembrane area are peripheral to the circumferential band of microtubules (from the sol-gel zone), and probably help to support the platelet discoid shape. These fine filaments also take part in retraction of surface projections during the contraction and fusion of a platelet plug (viscous metamorphosis) as a result of platelet aggregation.[44]

The sol-gel zone comprises the hyaloplasm, which is the platelet interior and appears structureless except for a few granules. Under electron microscopy, the interior is composed of fibrous elements, which are closely associated with the fine filaments in the submembrane area of the peripheral zone as well as the cell wall. The annular bundle of 250 Å microtubules that lies under the cell wall along its greatest circumference is the most prominent fibrous system of the hyaloplasm. Microfilaments, 50 Å in diameter constitute a second system of fibers in the hyaloplasm. For any microtubule system, there are 12-15 microfilaments in the annular bundle. The normal platelet functions such as contraction or clot retraction are dependent upon the fibrous elements in the platelet sol-gel zone.[44]

As one would anticipate, the organelle zone consists of the important parts, namely the organelles, of the platelet. Three of the main organelles include the granules, dense bodies, and mitochondria. Other organelles include flattened saccules, glycogen particles in the matrix, endoplasmic reticulum, giant granuoles, centrioles, and rarely nuclear remnants. The granules represent an important source of substances

secreted by platelets during viscous metamorphosis, i.e. contraction and fusion of the platelet plug during platelet aggregation. Each granule, enclosed by a unit membrane, contains much of the substances released during the platelet release reaction, in particular ADP, ATP, ATPase, fibrinogen, and serotonin. Dense bodies are primary secretory organelles that contain serotonin, ADP, catecholamines, and platelet factor 4. These dense bodies are electron opaque due to nucleation of heavy metals within them and most opaque organelles in human platelets originate from granules. Granules transforming into a dense body are directly related to serotonin uptake. During this internal transformation following exposure to aggregating agents, some dense bodies move toward the platelet surface where they release serotonin, while granules are shifted to the platelet centers. The number of dense bodies decreases rapidly during viscous metamorphosis.[44] Mitochondria are simple and few in platelets, and serve as calcium repositories, as in smooth muscle cells, and a metabolic pool of ATP. Platelet mitochondria become more opaque during viscous metamorphosis.

Finally, there are three membranous systems in the platelet. Firstly, one that communicates with the plasma, and is part of the platelet surface and has been discussed. Secondly, the Golgi Apparatus, but its physiological role is limited. Thirdly, the dense tubular system, which is closely associated with the circumferential band of microtubules and may serve as a template for its organization.[5] The third membrane system is associated with the submembrane area and sol-gel zone.

Serotonin, a smooth muscle vasoconstrictor, is plentiful in platelets, and virtually nonexistent in the plasma. Serotonin concentration

in platelets is roughly 60 micrograms per gram. Serotonin is acquired by platelets from secretive cells by active transport, and in the platelets, it is bound to very electron dense granules. Platelets also have serotonin metabolizing enzymes, so platelets are capable of metabolizing serotonin. In coagulation or platelet aggregation, about one quarter of the total platelet serotonin is released into the serum, so it is the serum serotonin concentration that serves as the parameter for the platelet release reaction.

In the platelet release reaction, adenine nucleotides (especially ADP), catecholamines, orthophosphate, potassium, albumin, globulins, platelet factors 2 and 4, fibrinogen, beta lipoproteins, lipids associated with procoagulant activity, amino acids, sulfated mucopolysaccharides, beta glucuronidase, acid phosphatase, and adenylate kinase are all released as well as serotonin. Clearly, the platelet release reaction requires energy and the extrusion of many platelet constituents. Young, larger, and heavier platelets release less adenine nucleotides than older, smaller, and lighter platelets following osmotic shock.[44] However, it is the opposite way around following exposure to ADP, thrombin, or epinephrine. In order for the platelet release reaction to occur, particularly ADP, serotonin, platelet factor 4, or platelet degranulation, a temperature above 30° C (86° F), which is above room temperature is necessary.[44] Optimal temperature for the platelet release reaction is body temperature, 37° C.

The platelet release reaction serves as the significant event to start the irreversible (second) phase of platelet aggregation. The platelet release reaction is not all-or-none and builds in intensity as

the second wave proceeds. The rate and extent to which the release reaction proceeds reflects the nature and concentration of the stimulating agent as well as the sensitivity of the platelet sample. If release fails or is blocked, then the second wave of aggregation does not occur.

Release does not necessarily result from platelet damage, because constituents associated with injury are not extruded from platelets with products of the release reaction. Release is actually the manifestation of platelet secretory function. Platelet secretion is energy dependent and is accomplished by its contractile mechanism and occurs only after internal transformation has developed. It is the internal changes which govern the degree of platelet response and outcome of the aggregation process. It is this mechanism that provides protection against overreaction as well as the dynamic means for extruding the platelet secretory products. As the second wave of aggregation proceeds, the dense bodies in the clumped cells decrease in number and ultimately disappear. It is the discharge of dense bodies and their products that serve as the physical correlate of the platelet release reaction. The dense bodies are the only structural elements that disappear from the platelets during early stages of the second wave of aggregation.

B. Platelet Aggregation

Most in vitro studies involving PRP prepared from citrated blood could not use EDTA as an anticoagulant since aggregation needs calcium ions, and EDTA is a calcium chelating agent. Since citrated PRP is unphysiologic, interpretation of in vitro studies should be made with care,

since artifacts are likely to be introduced. The in vitro artifact is not as serious in whole blood as in PRP. The big difference between in vivo and in vitro samples is that blood is in motion in vivo.

Physical changes which develop in platelets during hemostatic reactions are directly related to physiologic and biochemical events occurring in the process. If one directly adds an aggregating agent, such as thrombin to citrated platelet-rich plasma (C-PRP), then morphologic changes are induced. With thrombin, platelet response is proportional to concentration, and the sample can be fixed at selected intervals as the aggregation proceeds allowing study of its morphologic changes. The process can also be followed by an aggregometer, which records changes in platelet aggregation by measuring changes in optical density or light transmission through the sample. PRP is cloudy since platelets deflect the incident light, so as platelets aggregate to form larger masses, the platelet count is effectively reduced, light transmission increases and optical density decreases. As aggregates disperse, light transmission decreases and optical density increases.[7,8] If the thrombin concentration is less than 0.1 units/ml c-PRP, then shape change is the only alteration. The platelet takes on a spherical shape with multiple pseudopods instead of its characteristic discoid shape. Under electron microscopy, one observes some internal reorganization, where organelles move toward the platelet center and the circumferential (annular) bundle of microtubules shift internally. However, the centrally clumped organelles are loosely arranged and there is no fusion.[30] Ten minutes after addition of such a low thrombin stimulus, all of the above mentioned changes reverse themselves. Therefore, platelet shape and organization can be altered with no accompanying

aggregation occurring.

As the concentration of the aggregating agent increases further, i.e. thrombin concentration above 0.2 units/ml c-PRP, a double or "second" wave of aggregation appears. The first phase of clumping reverses partially, and then a second wave of aggregation occurs which is irreversible and seems to involve most platelets since light transmission increases to nearly that of platelet-poor plasma (PPP). Usually the first wave of aggregation resembles morphologically aggregation observed in samples which experience complete reversal. The second wave exhibits individual platelet aggregates which are more tightly held together and surrounded by a close-fitting band of microtubules and microfilaments. The centrally clumped particles or organelles inside the platelets fuse with each other and the encircling band of microtubules is broken down into its component subfilaments. The periphery of the platelets clump and become more electron transparent as degranulation becomes prominent, and the central area more electron dense. Other morphological features of the altered platelet are that mitochondria remain discrete and are usually more dense than in unaltered cells, and glycogen disappears. Platelet swelling has also been reported during the second phase of aggregation (Salzman, et. al.).[29] With ADP-induced aggregation, sucrose has been shown to prevent platelet swelling.[29]

Increasing the thrombin concentration slightly above the critical amount which produces a second wave of aggregation, i.e. above 0.2 units/ml c-PRP, will result in a single irreversible wave of clumping. This level is well below that needed to clot the c-PRP sample. Other chemical agents that can initiate platelet aggregation and the platelet release

reaction include arachidonic acid, poly-L-lysine, collagen, kaolin, ADP, catechalamines, ristocetin, and serotonin. These produce physical changes similar to those caused by thrombin. Collagen produces only a single massive wave of aggregation, and ADP produces the various patterns thrombin does.[44] With ADP, shape change is virtually simultaneous with the development of aggregation, hence shape change can occur without aggregation, but only if the platelet sample is studied at a pH below 6. The catecholamines cause platelets to aggregate without loss of discoid shape nor swelling, but for the most part show an aggregation profile similar to that of ADP and thrombin. Serotonin only induces reversible aggregation. Other aggregating agents include gamma globulin, polymerized fibrin, trypsin, and proteolytic enzymes in snake venoms. Aspirin, chlorpromazine, and imipramine and other such drugs prevent the second wave of aggregation and serotonin release induced by ADP, epinephrine, or collagen.[9] Increasing the ADP and collagen concentrations may induce aggregation under inhibiting conditions. ADP-induced aggregation is prevented by blocking both glycolysis and oxidative phosphorylation but not either alone. Other aggregation inhibitors act by increasing cyclic-AMP levels in the platelets, whereas mercurials and other chemicals inhibit aggregation by reacting with sulfhydryl groups. Other aggregation inhibitors include adenosine, vasodilators, and prostaglandin E1.[44] Other variables involved in ADP-induced aggregation, aggregation in general, or release, comprise initial anticoagulant, temperature, composition of suspending fluid, fibrinogen concentration, and condition of platelets themselves.[29]

The crushing force of contraction inside platelet aggregates ultimately causes individual platelets to lose their integrity and fuse

with each other. This change is late and represents the end stage of platelet viscous metamorphosis (fusion and contraction of platelets) or clot retraction. Products do not necessarily leak through the cell wall as a result of increased permeability, but likely through the open canalicular system and the gaps between the aggregated platelets.[5,44] Contraction of individual platelets facilitates the discharge of material into the surrounding plasma. The same applies to secretion. The pattern of contraction is individual cells and aggregates dominates transformation.

In summary, there are three basic platelet functions, namely adhesion (aggregation), contraction, and secretion (release reaction). Adhesion and contraction are independent of each other, but secretion is dependent on contraction, which can occur up to a point without secretion. The morphologic point of no return is the fusion of centrally clumped organelles and release of dense bodies, i.e. once fusion (consolidation) has taken place, internal contraction is irreversible. Thrombin and ADP cause consolidation of the platelet plug (aggregate), and thrombin also clots plasma fibrinogen. It is the fibrin that reinforces the platelet plug, particularly at the periphery. Fibrin combines to form as the platelets disintegrate. Fibrin strands surround platelet masses, but are rarely seen within a platelet mass. Red and white blood cells are also seldom seen within a platelet mass.[44]

Within one or two seconds after a blood vessel injury, a few platelets adhere to the edges of the lesion, where the collagen is exposed. It is the amino groups on the collagen molecule that induce the reaction, and calcium ions are not necessary, since adhesion can

take place in the presence of EDTA, a calcium chelating agent. Platelets in contact with collagen swell and undergo degranulation releasing ADP and other platelet constituents and subsequent platelets contact only the layer of platelets below, which in turn contacted the collagen, i.e. the first layer. Over the next few minutes, platelets from blood flowing through the wound adhere to platelets already anchored. The resulting platelet mass fills the gap in the vessel wall and stops the bleeding in five minutes. The growth of the platelet plug depends on the cohesive force between platelets mediated by ADP. In case of a blood vessel injury, where there is no wall break, endothelial cells are damaged exposing the basement membrane.[44] Platelets can then adhere to injured endothelial cells and the exposed basement membrane, and can proceed to fill up the gap by a platelet plug. In the basement membrane, it is the noncollagenous microfibrils that act as the initiator. In any case, the wound tract becomes filled with clotted blood, i.e. a fibrin meshwork with entrapped red blood cells after 15-20 hours. At this point there is much fibrin within the platelet plug and the platelets themselves have essentially degenerated.

There are several clinical tests used to monitor platelet function. These include bleeding time, platelet aggregation, platelet adhesiveness to glass, clot retraction, phospholipoprotein activity, and microelectrophoretic mobility. Microelectrophoretic mobility is decreased by epinephrine and ADP, and this phenomenon is maximal in 10 minutes.

C. Fixation and Related Investigations

Fixation involves preserving or holding the state of the system at a particular time. This is helpful when one wants a true picture of the

situation at a specific time kept for later investigation. Clearly, fixation procedures are useful in many areas. However, they are especially useful in areas of medicine where one deals with a biological system in which the state is constantly changing.

There are many fixative agents used in a variety of situations in medicine. These include hydroxyadipaldehyde, acetaldehyde, glutaraldehyde, formaldehyde (formalin), paraformaldehyde, mercuric chloride, pieric acid, and osmium tetroxide. Some of the common mixtures that act as fixative agents are Bouin's fluid, Zenker's fixative, and Carnoy's fixative. Bouin's fluid is a mixture of pieric acid, formalin (37 wt.% in water), acetic acid, and water. Zenker's fixative is a mixture of formaldehyde, potassium dichromate, mercuric chloride, and water. Carnoy's fixative consists of alcohol and glacial acetic acid. The most common simple fixative agents are formalin and glutaraldehyde, in various concentrations.[18,30] For electron microscopy, buffered glutaraldehyde is often used in combination with osmium tetroxide.

All fixatives have both desirable and undesirable effects, i.e. the possibility of an artifact being introduced by a fixative is always present. Mercuric chloride and pieric acid promote precipitation or clumping of proteins, whereas formalin and glutaraldehyde have been shown to promote coagulation, but not coarse precipitation of proteins. For the most part, glutaraldehyde seems to be the best fixative agent, especially for platelets. This conclusion is supported by studies conducted in the present work as well as Silver and Gardner, Seamen et. al., and Nichols and Bosman.[6,23,30,42]

The chemistry of the fixation process is complex and somewhat obscure. However, formaldehyde and glutaraldehyde are known to react with amine groups of proteins that constitute the tissues. With glutaraldehyde, the fixative property is reinforced by the fact that it is a dialdehyde unlike formaldehyde. Therefore, glutaraldehyde is capable of forming stabilizing bonds between protein molecules.[18]

There has been much work involving tissue fixation for purposes of light and electron microscopy. In the general preparation of tissue, the usual steps involve fixation, dehydration, clearing the tissue of the dehydrate, infiltration and impregnation with the paraffin or araldite, embedding into a block (methacrylate), sectioning on a microtome, and then finally staining.[13,14,43] At this point, the specimen is ready for analysis under the microscope. In the fixation step, 10 wt.% formalin and 2-6 wt.% glutaraldehyde are most commonly used although there are quite a number of fixatives. In the dehydration step, one uses increasingly concentrated alcohols, usually ethanols. It is this step that helps in hardening the tissue and at the same time preventing shrinkage. Propylene oxide is usually used to clear the dehydrant from the tissue. The tissue is now prepared in a block form with paraffin and hardened with araldite, then sectioned on the microtome, as stated above. There are many stains used prior to microscopic analysis such as lead citrate, uranyl acetate, Periodic Acid Schiff stain, amyloid stain and many others.[13] Another stain of interest is osmium tetroxide since that is both a fixative and stain for electron microscopy.[14] More extensive detail of tissue fixation can be found in any textbook covering electron microscopy, particularly in histology, pathology, or anatomy.

Fixation has also been applied to individual cell types as well as tissue. Maunsbach conducted a study on fixation of rat kidney proximal tubule cells using different strengths of glutaraldehyde solutions. The most critical factor that determined the quality of preservation of the ultrastructure of these cells, was the osmolality of the fixative solution.[21] Anderson prepared peripheral leukocytes for electron microscopy in the middle sixties.[2] Sutera, Mehrjardi, et. al. used a fixation technique to study erythrocytes under the electron microscope after being subjected to shear stress. The red blood cells are washed with distilled water instead of alcohols and propylene oxide, as was the case with tissue fixation.[40,41]

Fixation has been useful when specimens for parasitic analysis have to be collected at a patient's home. A two-vial preservation technique is used in which one part of the specimen is fixed in three parts of 5-10 wt.% buffered formalin. Another part of the specimen is fixed in three parts of polyvinyl alcohol fixative.[15,22]

There has been relatively little work done involving fixation of platelets and platelet aggregates, especially in conjunction with Coulter EPC Measurements. In 1967 an anatomical study was carried out with the electron miscroscope by Behnke on rat platelets. Platelets were fixed with 2-4 wt.% glutaraldehyde in a variety of buffers.[5] In 1970 Silver and Gardner conducted a comparative analysis of various aldehyde fixatives, in conjunction with osmium tetroxide, namely acetaldehyde, paraformaldehyde, glutaraldehyde, and hydroxyadipaldehyde. All of these aldehydes had their strong and weak points, e.g. acetaldehyde is best in preserving membranes, acetaldehyde and paraformaldehyde were

good at preserving cytoplasmic microfibrillae, and hydroxyadipaldehyde in general gave very poor results. Glutaraldehyde (5 wt.%)/osmium tetroxide fixation preserves platelet shape and ultra-structure by far the best. Furthermore, glutaraldehyde fixation caused the least swelling and is the only one that preserved the platelet coat and its elliptical (disc) shape. However, glutaraldehyde has the disadvantage that cytoplasmic microfibrillae are not well preserved. Hence, Silver and Gardner concluded that, "until better fixatives are available, glutaraldehyde/osmium tetroxide should be mandatory in any study planned to define changes in platelet ultrastructure."[30] In 1972, Sixma et. al., studied 1 wt.% glutaraldehyde fixation of human blood platelets in phosphate buffers of varying osmolality. In the hyperosmolar phosphate buffer of 0.1 molar (more than 300 mosmol), the platelets tended to shrink. On the other hand, at low concentrations, 0.01 molar phosphate buffer (hypoosmolar), the platelets swelled. Optimal results were obtained with a fixation fluid that contained 1 wt.% glutaraldehyde in 0.07 molar phosphate (275 mosmol, which is isoosmolar). It is worth noting that in any of these cases the osmolality changed only slightly during the 60 minutes of the fixation process.[31] In 1976, Hung, Sutera, et. al., used 2.5 wt.% glutaraldehyde in 0.1 molar phosphate buffered saline in observing studies on shear-induced aggregation and lysis of platelets.[17]

Born carried out the first quantitative studies of platelet aggregation by optical methods.[7] The formation of platelet aggregates in plasma affects its optical density. The relationship between the optical density and the numbers and sizes of aggregates has been

explored by Born and Hume.[9] They used formaldehyde (1 wt.% final concentration) to arrest aggregation after a certain time interval subsequent to ADP addition.

Wu and Hoak[45,46] developed another means of quantifying platelet aggregation involving taking platelet counts of blood samples drawn into a buffered EDTA solution, in comparison to counts in samples drawn into buffered EDTA-formalin solution. The platelet aggregate ratio is the ratio of the platelet count in the buffered EDTA-formalin solution to that in the buffered EDTA solution. Wu and Hoak have tabulated this ratio in normal donors as well as patients with transient ischemic attack, myocardial infarction, acute and chronic peripheral arterial insufficiency.[45,46] Maca, Hoak, and Fry used the aformentioned buffered EDTA-formalin solution as a fixative, which prevented the dissociation of circulating platelet aggregates into singly dispersed platelets.[20] It was later found that age, sex, fasting versus the postprandial state, and the degree of stasis prior to drawing the sample had no bearing on the platelet aggregate ratio in normal subjects.[27] The buffered EDTA-formalin solution fixes the aggregates which are subsequently precipitated during centrifugation. The platelet aggregate ratio is used as a measure of the number of platelet aggregates. Note that this method makes no direct measurement of platelet aggregate sizes or volumes, but only deals with platelet counts. Another disadvantage of this method is that the formalin fixation is employed prior to centrifugation. It is likely that the radius and specific gravity of the particle, and the specific gravity of the fluid medium are altered.[28]

Formalin has also been used to fix platelets for assays of ristocetin-induced platelet aggregation as well as von Willebrand's factor (vWF) activity.[26] Allain and Cooper et. al., used platelets fixed with paraformaldehyde (a triple monomer of formaldehyde), as a reagent for assay of vWF and platelet aggregating factor.[1,11] Washed human platelets were fixed for 48 hours with 4 wt.% paraformaldehyde, washed twice in phosphate buffer, pH 6.4, and stored at 4° C. Washed platelets did not aggregate with ADP, collagen, epinephrine, and thrombin, but did so with bovine or porcine plasma, poly-L-lysine, and ristocetin with normal human plasma, but not with von Willebrand's disease plasma. These fixed washed platelets remained unchanged after one month of storage at 4° C.

Recently, glutaraldehyde has been used in fixation studies concerning platelet aggregation. Seamen used glutaraldehyde to fix platelet aggregates in platelet-rich plasma as well as blood microaggregates in whole blood.[6,42] A study by Benner, Tambly, Swank, and Seamen defined another aggregation parameter, namely the platelet count ratio, which is comparable to the platelet aggregate ratio in the method by Wu and Hoak.[6,45,46] This parameter is the ratio of the platelet count, a specified time after addition of an aggregating agent, to the original platelet count of the platelet-rich plasma. In both cases the systems are stabilized by fixation with glutaraldehyde. They also used a Payton aggregation module for light transmission analyses, as well as the platelet counts by use of the Electrozone/Celloscope PDP 8/M Minicomputer Analysis Package equipped with a 19 μm aperture.

They used an isoton-glutaraldehyde mixture (final glutaraldehyde concentration: 0.5 wt.%).

Tamblyn, Nordt, Swank, Zukorski, and Seamen[42] used 0.06 wt.% glutaraldehyde in 0.15 molar NaCl solution to stabilize microaggregate counts in whole blood. They used electronic particle size distribution and screen filtration pressure measurements in studies on blood filters.

The closest work to the present study was carried out at the University of Rochester by Nichols and Bosman in 1978 and 1979. They worked with formaldehyde and glutaraldehyde as fixative agents for platelet aggregates in platelet-rich plasma. Nichols and Bosman deduced that formaldehyde was an unsatisfactory fixative agent since it caused individual platelet swelling much more than glutaraldehyde. This finding is consistent with the work of Silver and Gardner.[30] Nichols and Bosman primarily concerned themselves with platelet aggregate counts, i.e. cumulative population. However, they did some studies on the cumulative volume and mean aggregate size. They used a range of ADP concentrations to study both reversible and irreversible aggregation. They also carried out some light transmission and absorbance studies to correlate with population measurements on the Coulter Counter (Model TAII, 70 μm and 280 μm apertures).[38] They found that the aggregates started to break up even immediately upon dilution in isoton containing no fixative. It takes 10-15 seconds to carry out this particle size analysis on the 280 μm aperture. Therefore the counts under these conditions can not be considered to be completely accurate. Hence, they used 1 wt.% glutaraldehyde in the isoton diluent. With irreversible

aggregation (high ADP levels), they found that the largest aggregates exceed the size of the largest particle detectable with the 280 µm aperture. They used no fixative (glutaraldehyde) in the platelet-rich plasma prior to dilution in the isoton-glutaraldehyde diluent.

III. MATERIALS AND METHODS

A. Electronic Coulter Counter

The electronic particle size analyzer in this study (model T, Coulter Electronics, Hialeah, Fla.) counts particles in 15 preset channels simultaneously. Each channel counts particles of twice the mean volume of those counted in the previous channel. The lower channel numbers are indicative of increasing size (channel 0 is largest and channel 14 is smallest).

The data are usually reported as the number (population) and the volume of particles counted in each channel. The primary datum given by the instrument is the population of particles counted in each channel. The volume is the product of the population and the geometric mean volume in cubic microns of those particles detected in each channel. Although the instrument detects the volume of particles, the size of particles counted in each channel is reported as the diameter of a sphere having a volume equal to the arithmetic mean size of particles detected within the channel.[36,38]

On the Coulter Counter, there are several apertures in order to cover a wider size range. For each of these apertures, there are 15 preset channels (0-14) as previously described. The 5 apertures include the 70 μm, 100 μm, 200 μm, 280 μm, as well as the 400 μm apertures. These apertures are described in further detail in the chart in Appendix A (Table A).

The two apertures used in this study were the 70 μm and 280 μm apertures. In the free platelet size range, the 70 μm aperture is used from channels 12 through 8, which covers particles ranging from 1.59-4.0 microns in equivalent spherical diameter. When analyzing platelet aggregates 13-101 microns in diameter, the 280 μm aperture is used from channels 9 through 0. The 400 μm aperture is used for aggregates larger than 101 microns, i.e. larger than that which would be detected in channel 0 of the 280 μm aperture. As a consistency check on the electronic measurements, comparison was made of the cumulative volume of the platelet aggregates to that of the free platelets (available volume for aggregation) prior to aggregation.

The particle size is measured by detecting changes in current proportional to the volume of particles suspended in an electrolyte solution passing through an orifice. The electrolyte solution is isoton, a physiologic buffer with pH 7.35, that is used as the counting diluent by Coulter Electronics, Hialeah, Fla. Isoton is a balanced electrolyte solution that has been referred to as Eagle's solution.[35,38] However, aggregates in platelet-rich plasma (PRP) were found to break up upon dilution in isoton. Hence, the need for an isoton-glutaraldehyde mixture. In the Coulter accuvettes, 30 ml of isoton are used to dilute 0.2 ml of the aggregated sample, when dealing with platelet aggregates on the 280 μm aperture. One uses 20 ml of isoton to dilute 0.5 μl of the sample, when dealing with free platelets on the 70 μm aperture. When dealing with platelet aggregates, 1.5 ml of glutaraldehyde, in various concentrations, was added to the 30 ml of isoton for the final desired glutaraldehyde concentration.

B. Blood Collection and Preparation

All the blood donors were characterized as hematologically normal and were not on any medication for a week preceding donations. Venipuncture was performed by either Janet Horak at St. Lukes Hospital or Marcella Estrella at Rice University. Fresh human blood was drawn into plastic syringes and from there transferred to glass test tubes containing sodium citrate (Fisher Scientific, Fairlawn, N.J., final concentration: 3.2 gm/100 ml). The sodium citrate solution was 10% of the final citrated blood volume. The purpose of sodium citrate was to prevent the blood from clotting prior to addition of the aggregating agent, adenosine diphosphate (ADP). The ellapsed time from donation to arrival at Dr. Solis' Laboratory never exceeded 30 minutes. All experiments were carried out at room temperature in Dr. Solis' Laboratory at St. Lukes Hospital.

PRP was prepared by centrifugation three times, 10 minutes apiece, at 144.4 g, 215.7 g, and 1114.3 g, which corresponded to 900, 1100, and 2500 revolutions per minute, respectively. The first two centrifugations yielded PRP, and the final centrifugation (1114.3 g) was used to prepare platelet-poor plasma (PPP).

Platelet aggregation was induced by 0.1 ml of ADP (disodium salt; Sigma Chemical; St. Louis, Mo.; final concentration: 2×10^{-7} to 2×10^{-5} M) dissolved in veronal buffer and isoton (pH 7.35) added to plastic test tubes containing 0.9 ml aliquots of PRP at room temperature. The test tube was then shaken on the vortex (Fisher Scientific Products

Deluxe Mixer, 2 setting) for 55 seconds. The aggregated sample was then ready for particle size analysis on the Coulter Counter. The original concentrated ADP (2×10^{-3} M) was made up with veronal buffer, and diluted with isoton to the desired ADP level.

C. Glutaraldehyde Preparation and Use in Coulter Accuvettes

Glutaraldehyde was prepared in several different concentrations by dilution with isoton from concentrated glutaraldehyde (25 wt.%, Sigma Laboratories, St. Louis, Missouri). All glutaraldehyde solutions were stored at -5° C.

Glutaraldehyde solutions were prepared as follows:

i) 1% glutaraldehyde:

1 ml of 25% glutaraldehyde + 24 ml of isoton →

25 ml of 1% glutaraldehyde

ii) 2% glutaraldehyde:

2 ml of 25% glutaraldehyde + 23 ml of isoton →

25 ml of 2% glutaraldehyde

iii) 3% glutaraldehyde:

3 ml of 25% glutaraldehyde + 22 ml of isoton →

25 ml of 3% glutaraldehyde

iv) 6% glutaraldehyde:

6 ml of 25% glutaraldehyde + 19 ml of isoton →

25 ml of 6% glutaraldehyde

v) 10.5% glutaraldehyde:

10.5 ml of 25% glutaraldehyde + 14.5 ml of isoton →

25 ml of 10.5% glutaraldehyde

vi) 13% glutaraldehyde:

13 ml of 25% glutaraldehyde + 12 ml of isoton →

25 ml of 13% glutaraldehyde

vii) 21% glutaraldehyde:

21 ml of 25% glutaraldehyde + 4 ml of isoton →

25 ml of 21% glutaraldehyde

viii) 25% glutaraldehyde:

no addition

These solutions were stored in accuvettes or plastic test tubes in the freezer. However, once these solutions were used or added to the isoton in the Coulter Counter accuvettes that particular day, they were used in that given experiment planned that day.

The final glutaraldehyde concentration and osmolality in the Coulter accuvettes are given below for 30 ml of isoton added to 1.5 ml of various glutaraldehyde solutions.

	final glutaraldehyde concentration (wt.%)	final osmolality ($\frac{mOsmol}{Kg}$)
All isoton	0	339
i)	0.048	343
ii)	0.095	353
iii)	0.143	361
iv)	0.286	370
v)	0.500	*
vi)	0.619	408
vii)	1.000	*
viii)	1.190	*

*Osmolality was not measured for these particular solutions (v, vii, viii).

D. Fixation Procedure Prior to Dilution

In fixing aggregates, 50 µl of glutaraldehyde solution was added to 1 ml of the aggregated sample immediately after the vortex mixing subsequent to ADP addition. The fixed sample was then diluted in one of the isoton-glutaraldehyde solutions (diluents) just described in C. above and analyzed with the Coulter Counter. Both the time interval after sample fixation and time interval after dilution in the isoton-glutaraldehyde solution were varied in certain experiments. The glutaraldehyde concentration in the aggregated sample was also varied. Glutaraldehyde concentrations higher than 0.23 wt.% in the undiluted sample caused gel formation within a few minutes, and thus could not be used on the Coulter Counter. This gelling presumably occurred due to the cross-linking of plasma proteins. Once the sample was diluted in isoton, the plasma protein concentration was dramatically reduced, and no gelling was evident, even when the glutaraldehyde concentration in the isoton diluent was as high as 1.190 wt.%.

In some preliminary experiments formalin solutions were tried as fixative agents, in concentrations of 0.048 and 0.190 wt.%. These formalin solutions were prepared by diluting 37 wt.% formalin (Fisher Scientific) with isoton to 1 and 4 wt.% formalin, respectively, in the additive solutions. These additive solutions in turn lead to final formalin concentrations of 0.048 and 0.190 wt.% (50 µl of additive + 1 ml of aggregated sample). In these preliminary experiments, Coulter observations were made immediately, 1 hour, and 24 hours after adding the fixative solution to the aggregated sample. No fixative agent was added to the isoton counting diluent in the preliminary experiments.

Wu and Hoak solutions were also prepared according to the procedure in their papers,[45,46] i.e. both the buffered EDTA and the buffered EDTA/formalin solutions. The formalin concentration in the buffered EDTA/formalin was 1 wt.%. These 2 solutions and isoton were added in 4 parts of volume to 1 volume part of the sample (a 5-fold dilution), as was done in the Wu and Hoak experiments.[45,46] Consequently, the final formalin concentration was 0.8% in the observed sample. Since this was a 5-fold dilution, whereas all the other experiments used a 1.05-fold dilution, we compensated on the Coulter accuvette by adding 1 ml of the sample to the 30 ml of isoton, instead of the normal 0.2 ml of sample. These samples were observed immediately, 1 hour, and 2 hours after dilution with either isoton, or the buffered solutions. No fixative agent was added to the isoton counting diluent in the Coulter accuvette.

IV. PRELIMINARY INVESTIGATIONS

A series of preliminary investigations were carried out in which various fixative agents were added to the aggregated sample with no fixative agents in the isoton diluent counting solution. As will be shown in the next section, the procedure finally found to be most promising was different (fixative agent was found to be desirable in both the aggregated sample and the isoton diluent counting solution). However, there were a number of findings from the preliminary studies which served to guide subsequent work. These findings are summarized below.

A. Studies on 24 Hour Fixation and Resuspension

Preliminary experiments had as an objective fixation of platelet aggregates for as long as 24 hours. Prior to particle size analysis, it is necessary to mix the sample, since the platelet aggregates settle to the bottom of the test tube. Sedimentation is not a problem in Coulter Counter studies done within a few hours of aggregation. In these cases, inverting the test tube twice yielded reproducible suspensions. However, after the sample was left for 24 hours, 2 inversions of the test tube was not sufficient to resuspend the platelet button. Therefore, studies were carried out on resuspension by several methods: 2 inversions, 8 inversions, 2 and 5 seconds on vortex (Scientific Product Deluxe Mixer; 2 setting) and use of a continuous rotating turntable.

In these experiments, the final ADP concentration was 2.0 μM, and the final glutaraldehyde concentration was 0.048 wt.% in the test

samples. In control samples, isoton was added, whereas in test samples, glutaraldehyde was added to fix the platelet aggregates. In the controls platelet aggregates disaggregated when placed in the isoton counting solution diluent.

The results given in Table IV-1 provide several guidelines for subsequent work. (1) Results for 24 hour fixation are much less encouraging than that of 1 hour, because of apparent swelling and break-up of the platelet aggregates. (2) Continuous rotation on a turntable does not appear to be promising. Evidently, disaggregation is increased. (3) For fixation for periods of one hour the other two methods of resuspension are not significantly different. Other results indicate that 2 inversions of the tube are sufficient for one hour intervals.

Table IV-1: Effects of Different Resuspension Methods

Rotating Turntable		(mean ± 1 s.e.m., n=4)	
time	cumulative volume	cumulative population	mean aggregate size
0	109± 4	100±13	115±15
1 hour	61± 8	82±32	95±21
24 hours	19± 3	53±12	40±10
8 inversions		(mean ± 1 s.e.m., n=5)	
time	cumulative volume	cumulative population	mean aggregate size
0	108± 3	99±14	117±15
1 hour	109± 7	89±13	132±19
24 hours	141±16	204±15	68± 6
2 second vortex		(mean ± 1 s.e.m., n=5)	
time	cumulative volume	cumulative population	mean aggregate size
0	107± 2	99± 5	109± 6
1 hour	108± 3	83± 5	131± 9
24 hours	169±13	172±15	99± 8

All figures in Table IV-1 are the ratio of results for the test sample (glutaraldehyde added to sample) to the results for the control sample (no glutaraldehyde added, counted immediately after aggregation), expressed as a percentage. The cumulative volume, population, and aggregate size are for the range 13-101 μm in equivalent spherical diameter, i.e. channels 9 through 0 on the 280 μm aperture of the Coulter Counter.

B. Various Fixative Agents

Preliminary studies were carried out using 5 different glutaraldehyde concentrations as well as 2 formaldehyde (formalin) concentrations. Final glutaraldehyde concentrations used were 0.619, 0.286, 0.095, 0.048 and 0.024 wt.%, and final formalin concentrations used were 0.190 and 0.048 wt.%. In all cases PRP samples were aggregated with 2 μM ADP.

No results could be obtained at 0.619 and 0.286 wt.% glutaraldehyde levels, because the samples gelled within 4 minutes. The results of Table IV-2 illustrate that 0.024 wt.% glutaraldehyde and 0.048 wt.% formalin did not fix the platelet aggregates for even 1 hour. The cumulative population increased drastically, which indicates breakup. The 0.095 wt.% glutaraldehyde resulted in apparent swelling at 1 hour as evidenced by increases in cumulative volume and mean aggregate sizes. The 0.190% formalin also yielded large increases in cumulative volume and mean aggregate size in one hour.

The glutaraldehyde level of 0.048 wt.% produced the best result at 1 hour observations. None of these were encouraging for 24 hours.

Table IV-2: Effects of Different Fixative Agents

0.048 wt.% glutaraldehyde (mean, n=2)

time	cumulative volume	cumulative population	mean aggregate size
0	113	116	97
1 hour	106	94	114
24 hours	151	261	58

0.095 wt.% glutaraldehyde (mean, n=2)

time	cumulative volume	cumulative population	mean aggregate size
0	122	107	115
1 hour	148	89	171
24 hours	155	74	213

0.024 wt.% glutaraldehyde (mean, n=3)

time	cumulative volume	cumulative population	mean aggregate size
0	100	114	89
1 hour	105	251	43
24 hours	94	295	33

0.190 wt.% formalin (mean, n=2)

time	cumulative volume	cumulative population	mean aggregate size
0	126	131	98
1 hour	151	107	145
24 hours	173	95	183

(Continued)

Table IV-2: Effects of Different Fixative Agents

	0.048 wt.% formalin	(mean, n=2)	
time	cumulative volume	cumulative population	mean aggregate size
0	108	144	76
1 hour	118	909	13
24 hours	180	1216	15

All figures in Table IV-2 are the ratio of results for the test sample (fixative added to sample) to the results for the control sample (no fixative added, counted immediately after aggregation), expressed as a percentage. The cumulative volume, population, and aggregate size are for the range 13-101 μm in equivalent spherical diameter, i.e. channels 9 through 0 on the 280 μm aperture of the Coulter Counter.

C. Studies on the Wu-Hoak EDTA/Formalin Fixative

In these experiments, buffered EDTA and EDTA/formalin solutions were made by the procedure of Wu and Hoak.[45,46] Dilutions were carried out in the same proportion of Wu and Hoak, i.e. 4 ml of both the EDTA and EDTA/formalin solutions to 1 ml of PRP sample. PRP samples diluted in isoton (1:4) were also analyzed.

The control samples had nothing added to them so different procedures were used to yield the same platelet concentration on the Coulter Counter. One ml of the diluted samples was added to the 30 ml of isoton, whereas 0.2 ml of the control aggregated PRP was added to the 30 ml of isoton. As in the other preliminary studies, the final ADP concentration in the samples prior to dilution was 2.0 μM.

The results in Table IV-3 indicate that the aggregates break up immediately upon dilution with isoton or the buffered EDTA solution. One and 2 hours after such dilutions, virtually all original aggregates disaggregated to particles smaller than 13 μm in equivalent spherical diameter. The buffered EDTA/formalin fixative solution results in considerable swelling at both 1 and 2 hours as shown by the increase in cumulative volume. The cumulative population even shows some of the aggregates coming together.

Table IV-3: Effect of the Wu-Hoak Fixative

Aggregated PRP (1 ml) + 4 ml of Isoton (mean ± 1 s.e.m., n=8)

time	cumulative volume	cumulative population	mean aggregate size
0	97.4±3.4	601.1±56.6	17.6±2.4
1 hour	5.5±1.3	12.1± 1.3	40.6±0.8
2 hours	7.6±1.4	26.0±10.5	41.5±7.0

Aggregated PRP (1 ml) + 4 ml of EDTA Solution (mean ± 1 s.e.m., n=8)

time	cumulative volume	cumulative population	mean aggregate size
0	99.6± 4.4	613.5±54.8	17.0± 1.2
1 hour	4.9± 0.6	17.1± 1.5	29.8± 3.2
2 hours	7.1± 1.0	18.6± 1.2	37.5± 4.7

Aggregated PRP (1 ml) + 4 ml of EDTA/Formalin Fixative Solution (mean ± 1 s.e.m., n=10)

time	cumulative volume	cumulative population	mean aggregate size
0	109.1± 4.2	106.3± 3.9	102.7± 3.0
1 hour	123.6± 4.7	81.7± 2.8	152.6± 6.5
2 hours	130.3± 5.3	81.9± 3.8	161.7± 7.8

All figures in Table IV-3 are the ratio of results for the test sample (diluted 1:4 after aggregation) to the results for the control sample (nothing added, counted immediately after aggregation), expressed as a percentage. The cumulative volume, population, and aggregate size are for the range 13-101 μm in equivalent spherical diameter, i.e. channels 9 through 0 on the 280 μm aperture of the Coulter Counter.

There is one important difference between the present work and that of Wu and Hoak. They drew venous blood (0.5 ml) directly into 2 separate syringes, one containing 2 ml of buffered EDTA/formalin solution and the other 2 ml of buffered EDTA solution, prior to any centrifugation. In the present work PRP was prepared from citrated blood by centrifugation, and then aggregated by ADP before the 5-fold dilution.

D. Influence of Glutaraldehyde on Free Platelets

Results of the preliminary studies discussed above seem to indicate that glutaraldehyde is a promising fixative agent for platelet aggregates. Other investigators, e.g. Silver and Gardner,[30] Seamen,[6,42] and Nichols and Bosman[23] have produced evidence that glutaraldehyde is a suitable fixative agent.

Therefore, an additional preliminary study was made to determine if glutaraldehyde causes platelet aggregation or any other artifact in total volume of platelets (TV), total population of platelets (platelet count (PC)), or mean platelet size. The background counts for both volume and population were small (less than 5% compared to the raw TV and PC of PRP samples in both isoton and in the isoton-glutaraldehyde mixtures. In any case, the background counts should be subtracted off from the sample counts, i.e. raw TV and PC (derived by taking channels 12 through 8 on the 70 μm aperture)for both the sample and background mixture. Background counts for the isoton-glutaraldehyde mixtures were comparable to those of pure isoton.

The measurements, obtained with the 70 μm aperture and expressed as volume in μm^3 (TV) and particles (platelets) per μl of sample (PC) were made immediately and 5 minutes after addition of glutaraldehyde to the unaggregated platelets (Table IV-4). See Appendix A for further detail on treating raw data from the 70 μm aperture.

Table IV-4: Effect of Glutaraldehyde on Free Platelets

(mean ± 1 s.e.m., n=4)

Glutaraldehyde Concentration	Time (min.)	TV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	PC($\frac{\text{platelets}}{\mu l \text{ of sample}}$) X 10^{-5}	Mean platelet size (μm^3)
pure isoton 0 wt.%	0	4.93±0.07	4.046±0.087	12.19±0.11
	5	5.07±0.15	4.212±0.134	12.05±0.14
0.048 wt.%	0	5.16±0.12	4.449±0.104	11.61±0.10
	5	5.19±0.21	4.550±0.204	11.42±0.07
0.095 wt.%	0	5.20±0.15	4.417±0.140	11.80±0.14
	5	5.17±0.16	4.430±0.140	11.68±0.08
0.286 wt.%	0	4.85±0.20	4.152±0.231	11.71±0.19
	5	4.90±0.25	4.357±0.272	11.28±0.25
0.619 wt.%	0	4.88±0.22	4.197±0.223	11.66±0.14
	5	4.85±0.18	4.283±0.121	11.30±0.12

Table IV-4 shows that glutaraldehyde does not introduce any artifacts in the free platelets, nor that size range, except that glutaraldehyde causes slight shrinkage in the mean platelet size. Although the mean platelet sizes in the isoton-glutaraldehyde mixtures are significantly lower than those in the pure isoton ($p < 0.05$, Student t-test for paired data), the maximum shrinkage of the mean platelet size due to glutaraldehyde is 6.4% after 5 minutes of suspension in the isoton-glutaraldehyde mixture. The maximum initial shrinkage of mean platelet size due to glutaraldehyde is 4.7%, and the average platelet shrinkage is even less. Regarding the 5 minutes time interval, only the highest glutaraldehyde levels, i.e. 0.286 and 0.619 wt.%, showed a significant decrease in the mean platelet size. In both cases, the immediate and 5 minute mean platelet sizes differed by less than 4%.

The total volume and platelet count did not differ significantly over the 5 minute time interval ($p > 0.05$). Finally, the total volumes and platelet counts did not differ significantly for the different isoton-glutaraldehyde mixtures, nor did these differ significantly from the total volume and platelet count of the specimen in pure isoton ($p > 0.05$).

E. Influence of Glutaraldehyde on Isoton

Background Counts for the 280 μm Aperture

This study was to ascertain that isoton-glutaraldehyde mixtures, used to carry out particle size analysis on platelet aggregates, does not contribute a significant part to the cumulative volume and population of particles 13-101 μm in equivalent spherical diameter. Pure isoton (Coulter Electronics, Hialeah, Fla.) has been used extensively as the counting diluent in Coulter Counter particle size analysis of particles 13-101 μm in equivalent spherical diameter, when dealing with blood or PRP samples.[32,33,34,36,37,38] The background counts for the isoton-glutaraldehyde mixtures and isoton alone are given in Table IV-5.

CV = cumulative volume

CP = cumulative population

CV9 = cumulative volume to channel 9

CP9 = cumulative population to channel 9

For the background counts:

$$CV = CV9 \times \frac{6066\ \mu m^3}{2000\ \mu l \text{ of sample}} \text{ and } CP = CP9 \times \frac{1 \text{ particle}}{2000\ \mu l \text{ of sample}}$$

No dilutions with PRP, only diluents are analyzed. For further details, see Appendix A.

Table IV-5: Effect of Glutaraldehyde on Isoton Background Counts

(280 μm Aperture)

(mean ± 1 s.e.m., n=8)

Glutaraldehyde Concentration	CV9	CP9
pure isoton - 0 wt.%	136±40	32±10
0.048 wt.%	77±26	29± 4
0.095 wt.%	101±20	51±10
0.286 wt.%	113±23	42±13
0.619 Wt.%	102±19	42± 8

The results from Table IV-5 indicate that glutaraldehyde does not cause interference in the cumulative volume nor the cumulative population. Comparison of the isoton-glutaraldehyde mixtures with isoton alone reveals no significant differences ($p > 0.05$). This can be further illustrated by considering typical values for cumulative volume and population up to channel 9, on the 280 μm aperture, for PRP or blood samples. Our samples have cumulative volumes to channel 9 above 3000 and cumulative populations to channel 9 from 2000 up to 8000, and in all cases, the background counts were small compared to these values. However, there can be circumstances involving relatively large aggregates (e.g. studies to be discussed later involving a final ADP concentration of 20 μM) in which the cumulative populations are low (less than 400). In such cases, the background interference can cause a compromise in accuracy.

At the other extreme, consider cases of very low cumulative volumes and populations. In such circumstances, background interference becomes more significant and mean aggregate size becomes inaccurate in light of

the high variation observed in Table IV-5. This arises in control samples (not fixed), and in samples where aggregates break up (e.g. studies involving a final concentration of 0.5 μM ADP).

V. PRINCIPAL RESULTS

After the preliminary investigations, a study was carried out to determine the effect of adding glutaraldehyde to the isoton diluent on the measurement of platelet aggregates in PRP. Adding 0.2 ml of aggregated PRP to 30 ml of isoton alone causes aggregates to disaggregate shortly after dilution. Particle size analysis using the 280 μm aperture takes 6.3 seconds. Mixing the sample with the isoton diluent in the Coulter accuvette prior to particle size analysis requires an additional 3 seconds. Thus, 10 seconds ellapse between isoton dilution and the end of the counting process. During this interval considerable disaggregation occurs. Therefore, studies were carried out in which glutaraldehyde was added to the isoton counting diluent as well as to the aggregated sample prior to dilution.

The studies are divided into three categories as outlined below. In section "A," studies will be discussed in which glutaraldehyde in various concentrations was added to the isoton solution used for dilution in the accuvettes prior to counting. In section "B," studies will be discussed in which glutaraldehyde fixative was used in both the aggregated sample and in the isoton counting solution diluent. From these studies, a final glutaraldehyde concentration of 0.048 wt.% was selected for use both in the aggregated sample and in the isoton diluent. The final series of studies, described in section "C," involved use of the selected procedure on PRP samples aggregated with various final

concentrations of ADP. Throughout the present work, statistical significance (p-values) of observed differences in data was determined by the Student's t-test for paired data (See Appendix B for further explanation).

A. Studies on Use of Glutaraldehyde Fixative in Isoton Counting Solution Only

Immediately after aggregation, 0.2 ml of aggregated PRP was added to 31.5 ml of counting diluent (pure isoton or isoton-glutaraldehyde mixture) in Coulter accuvettes. Particle size analysis was carried out in each Coulter accuvette immediately, 1, 2, and 3 minutes after dilution as well as 30-90 minutes after dilution. In the first series of these experiments, PRP was aggregated with 2 μM ADP, then isoton alone, zap isoton (Coulter Electronics, Hialeah, Fla.) added to isoton, and isoton-glutaraldehyde mixtures (glutaraldehyde concentrations: 0.048 and 0.619 wt.%) were used as diluents. In the remainder of these experiments, PRP was aggregated with 0.5 μM ADP and isoton alone, and isoton-glutaraldehyde mixtures (glutaraldehyde concentration: 0.048-1.190 wt.%) were used as counting diluents. Platelet aggregates induced by 0.5 μM ADP are much less firmly held together than those from 2 μM ADP, so breakup of platelet aggregates after isoton dilution presumably is more critical. For each observation, the 3 parameters measured were mean aggregate size (M.A.S.), cumulative volume (CV), and cumulative population (CP). Procedure for treatment of raw data is given in Appendices A and C.

1. 2 μM ADP-Glutaraldehyde Concentration up to 0.619 wt.%

In this study, 4 treatments of the isoton counting diluent were used.

Treatment	Glutaraldehyde Concentration
a) 31.5 ml of sioton	0 wt.%
b) 31.5 ml of isoton + 3 drops of zap isoton	0 wt.%
c) 30 ml of isoton + 1.5 ml of 1 wt.% glutaraldehyde	0.048 wt.%
d) 30 ml of isoton + 1.5 ml of 13 wt.% glutaraldehyde	0.619 wt.%

A summary of the results is given in Table V-1 and Figure V-1. The time column in the Table gives the elapsed time after dilution before the count was taken. The total platelet volume available (from counts of unaggregated platelets in the PRP with the 70 μm aperture) for aggregation was $2.290 \pm 0.089 \times 10^6 \frac{\mu m^3}{\mu l}$.

The results of Table V-1 indicate that platelet aggregates disaggregate rapidly following dilution in isoton alone. Treatment "b" (zap isoton added) appears to bring some stability to platelet aggregates in the counting diluent for 3 minutes. However, there is some breakup initially, and disaggregation was appreciable by the late observation. The immediate observations are the same for both treatments "a" and "b," but disaggregation is much more rapid in treatment "a," without the zap isoton. This finding is consistent with prior work by Solis and colleague[36] in whole blood studies, where zap isoton was used to lyse red blood cells. Zap isoton does not stabilize the aggregate size distribution as much as glutaraldehyde. Immediate observations were the same whether zap isoton was added to isoton alone or not, and these were close to the immediate observation of treatment

Table V-1: Effect of Glutaraldehyde in Isoton Diluent on Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter (2 μM ADP). (mean ± 1 s.e.m., n=4)

Glutaraldehyde Concentration (wt.%)	Time (min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
0	0	5.69±0.32	1.926±0.019	56.5±3.6
	1	0.89±0.16	1.194±0.140	231.4±19.8
	2,3,(30-90)	-	--	--
0 (zap isoton)	0	5.74±0.37	2.043±0.246	58.3±4.2
	1	4.11±0.08	1.709±0.094	68.6±3.7
	2	4.19±0.11	1.827±0.087	71.8±2.5
	3	4.16±0.17	1.761±0.110	69.7±2.2
	Late(30-90)	1.95±0.11	0.616±0.054	51.7±2.3
0.048	0	6.34±0.27	2.179±0.088	57.3±4.6
	1	6.36±0.30	2.014±0.105	52.2±0.9
	2	6.44±0.22	1.944±0.078	49.9±2.4
	3	6.31±0.23	2.079±0.025	54.7±2.4
	Late(30-90)	7.33±0.14	2.228±0.112	50.1±1.4
0.619	0	10.09±0.61	2.413±0.163	39.5±1.6
	1	9.55±0.44	2.076±0.121	35.9±1.9
	2	8.69±0.41	2.070±0.079	39.4±1.0
	3	7.90±0.37	2.172±0.097	45.5±2.6
	Late(30-90)	8.02±0.18	2.500±0.085	51.5±1.4

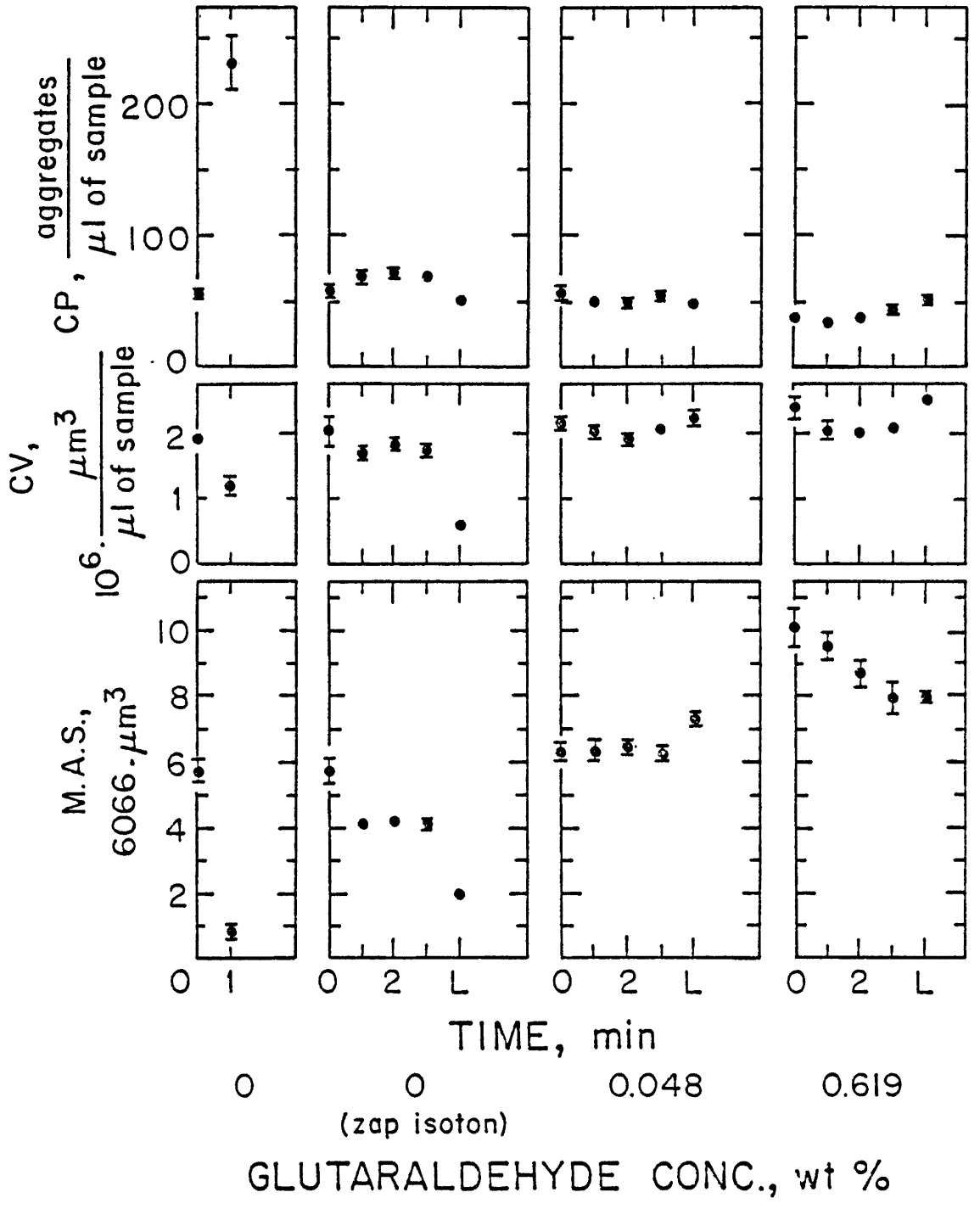

Figure V-1: Effect of glutaraldehyde and zap isoton added to isoton counting diluent on stability of platelet aggregates 13-101 μm in equivalent spherical diameter induced by ADP (2.0 μM final concentration). Measurements were made immediately, 1,2,3, and 30-90 (L) minutes after dilution of aggregated platelet-rich plasma for particle size analysis. The total platelet volume available for aggregation is (2.290±0.089) X 10^6 $\mu m^3/\mu l$ of sample. (mean ± 1 s.e.m., n=4)

"c," except mean aggregate size was lower without the glutaraldehyde by 10%, than that shown in treatment "c."

Treatments "c" and "d" show comparable cumulative volume profiles except there is evidence of swelling caused by the higher glutaraldehyde concentration (0.619 wt.%) at the late observation. This cumulative volume exceeds that available for aggregation from the free platelets (measured on 70 μm aperture).

It is not likely that treatment "d" reflects the true aggregate size distribution. The cumulative population in treatment "d" is significantly lower than in the other treatments for 3 minutes after dilution of PRP in the isoton diluent ($p < 0.05$). The mean aggregate size progressively decreases and the cumulative population increases up to the late observation. However, the mean aggregate size is not significantly different at the late reading from that 3 minutes after dilution ($p > 0.05$). Therefore, this higher glutaraldehyde concentration (0.619 wt.%) in the diluent appears to cause weak cross-linking of the aggregates initially as well as swelling by the late observation.

Immediate results are actually reported 10 seconds after isoton dilution. Extrapolation of the initial mean aggregate size for treatment "a" back 10 seconds gives results consistent with treatment "c." For treatment "a," mean aggregate size decreases by 80-90% in 1 minute, so in 10 seconds, a 10-15% decrease is expected. The initial mean aggregate size for treatment "a" is 88% of that shown for treatment "c," and 56% of that shown for treatment "d." Hence, treatment "c" accounts for the projected course for treatment "a," in terms of mean aggregate size. Thus, there is evidence that a counting diluent,

with 0.048 wt.% glutaraldehyde, best reflects the true aggregate size distribution.

2. 0.5 μM ADP-Glutaraldehyde Concentration (0.286-1.190 wt.%)

This study used 6 counting diluents; isoton alone and 5 isoton-glutaraldehyde mixtures (glutaraldehyde concentration: 0.286-1.190 wt.%). Zap isoton was no longer used since it did not completely stabilize the relatively large and firm aggregates (2 μM ADP) discussed above. A summary of results is given in Tables V-2-a and V-2-b. The time column gives the elapsed time after dilution before the count was taken. The total platelet volume available (from counts with the 70 μm aperture) for aggregation was $(2.907\pm0.112) \times 10^6 \frac{\mu m^3}{\mu l}$ for Table V-2-a, and $(2.698\pm0.140) \times 10^6 \frac{\mu m^3}{\mu l}$ for Table V-2-b.

Results from Tables V-2-a and V-2-b show that there are no significant differences in any of the aggregate size distribution parameters; neither for the 5 isoton-glutaraldehyde counting diluents nor for any of the timed observations within each isoton-glutaraldehyde counting diluent ($p > 0.05$). Comparison of cumulative volumes of the aggregates with available volume for aggregation from free platelets prior to aggregation reveals slight swelling for all isoton-glutaraldehyde diluents. Hence, isoton-glutaraldehyde counting diluents stabilize aggregate size distributions in PRP aggregated with 0.5 μM ADP equally well for any glutaraldehyde concentration from 0.286 to 1.190 wt.%. Of course, isoton alone used as the diluent caused rapid disaggregation.

Note, the 5 runs of Table V-2-b are a subset of the 9 runs of Table V-2-a. The 5 runs were averaged and recorded separately since late observations were made only for this subset of the total data.

These results are presented also in Figure V-2.

3. 0.5 μM ADP-Glutaraldehyde Concentration (0.048-0.286 wt.%)

This experimental series used 4 counting diluents; isoton alone and 3 isoton-glutaraldehyde mixtures (glutaraldehyde concentration: 0.048-0.286 wt.%). The highest glutaraldehyde concentration (0.286 wt.%), in this series, produced results that were not significantly different statistically from that of diluents with glutaraldehyde levels 0.286-1.190 wt.%, as shown in the preceding section. A summary of these results (0.048-0.286 wt.%) is given in Table V-3 and in Figure V-3. The time figures column give the elapsed time after dilution before the count was taken. The total platelet volume available (from counts with 70 μm aperture) for aggregation was (2.805±0.220) $\times 10^6 \frac{\mu m^3}{\mu l}$.

Results in Table V-3 indicate that all isoton-glutaraldehyde diluents produce similar results for all the aggregate size distribution parameters. There were no significant differences in any of the aggregate size distribution parameters between the 0.143 and 0.286 wt.% glutaraldehyde diluents for any timed observation up to the late observations ($p > 0.05$). For both 0.143 and 0.286 wt.% glutaraldehyde diluents, no significant differences across any of the timed observations up to the late observations ($p > 0.05$). Hence, the same results can be expected for any isoton-glutaraldehyde counting diluent (glutaraldehyde concentration: 0.143-1.190 wt.%).

The diluent (with 0.048 wt.% glutaraldehyde) produced the same cumulative populations as those with more glutaraldehyde, for all timed observations. However, this diluent produced slightly lower cumulative

Table V-2-a: Effect of Glutaraldehyde in Isoton Diluent on Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter (0.5 μM ADP). (mean ± 1 s.e.m., n=9)

Glutaraldehyde Concentration (wt.%)	Time (min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
0	0	1.12±0.11	2.498±0.115	396±43
	1	0.39±0.01	1.114±0.085	433±37
	2	--	--	--
	3	--	--	--
0.286	0	1.86±0.22	3.063±0.146	311±43
	1	1.95±0.27	3.098±0.114	307±42
	2	1.95±0.29	3.085±0.109	306±42
	3	1.94±0.28	3.124±0.087	309±41
0.500	0	2.05±0.25	3.164±0.099	289±38
	1	2.01±0.24	3.094±0.115	289±38
	2	2.03±0.25	3.136±0.106	289±38
	3	2.07±0.24	3.178±0.130	284±36
0.619	0	1.93±0.28	3.178±0.124	333±56
	1	1.93±0.28	3.139±0.098	332±56
	2	1.92±0.29	3.088±0.103	326±54
	3	1.92±0.28	3.166±0.120	335±56
1.000	0	1.74±0.19	3.165±0.134	328±36
	1	1.73±0.19	3.051±0.127	321±36
	2	1.72±0.18	3.051±0.127	322±36
	3	1.73±0.18	3.100±0.135	323±34
1.190	0	1.83±0.22	3.149±0.118	324±43
	1	1.82±0.21	3.112±0.121	321±42
	2	1.80±0.20	3.138±0.123	322±41
	3	1.81±0.20	3.171±0.136	323±41

Table V-2-b: Same as V-2-a with late observations included

(mean ± 1 s.e.m., n=5)

Glutaraldehyde Concentration (wt.%)	(min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
0	0	1.09±0.12	2.363±0.180	377±55
	1	0.39±0.02	1.111±0.145	409±66
	2,3,(30-90)	--	--	--
0.286	0	2.14±0.33	2.865±0.229	253±56
	1	2.28±0.42	2.929±0.151	252±57
	2	2.32±0.45	2.975±0.176	252±57
	3	2.30±0.43	3.061±0.150	259±57
	Late(30-90)	2.43±0.44	3.249±0.230	257±56
0.500	0	2.19±0.35	3.042±0.157	256±47
	1	2.16±0.35	2.979±0.166	256±48
	2	2.16±0.35	3.015±0.172	257±47
	3	2.19±0.37	2.980±0.179	254±49
	Late(30-90)	2.30±0.36	3.217±0.243	258±49
0.619	0	2.23±0.40	3.025±0.189	274±71
	1	2.23±0.41	3.018±0.150	273±70
	2	2.25±0.42	2.999±0.173	271±71
	3	2.22±0.42	3.040±0.179	280±75
	Late(30-90)	2.34±0.42	3.134±0.206	270±70
1.000	0	2.03±0.30	3.023±0.208	290±56
	1	2.02±0.29	2.918±0.197	285±55
	2	1.99±0.27	2.920±0.199	282±54
	3	2.04±0.27	3.026±0.226	285±54
	Late(30-90)	2.05±0.31	3.084±0.198	290±54

Continued

Table V-2-b: Same as V-2-a with late observations included

(mean ± 1 s.e.m., n=5)

Glutaraldehyde Concentration (wt.%)	(min.)	M.A.S. (μM^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
1.190	0	2.15±0.30	3.068±0.202	266±58
	1	2.09±0.30	2.987±0.186	267±59
	2	2.08±0.27	3.006±0.191	268±57
	3	2.11±0.27	3.122±0.240	273±60
	Late(30-90)	2.23±0.30	3.188±0.176	265±55

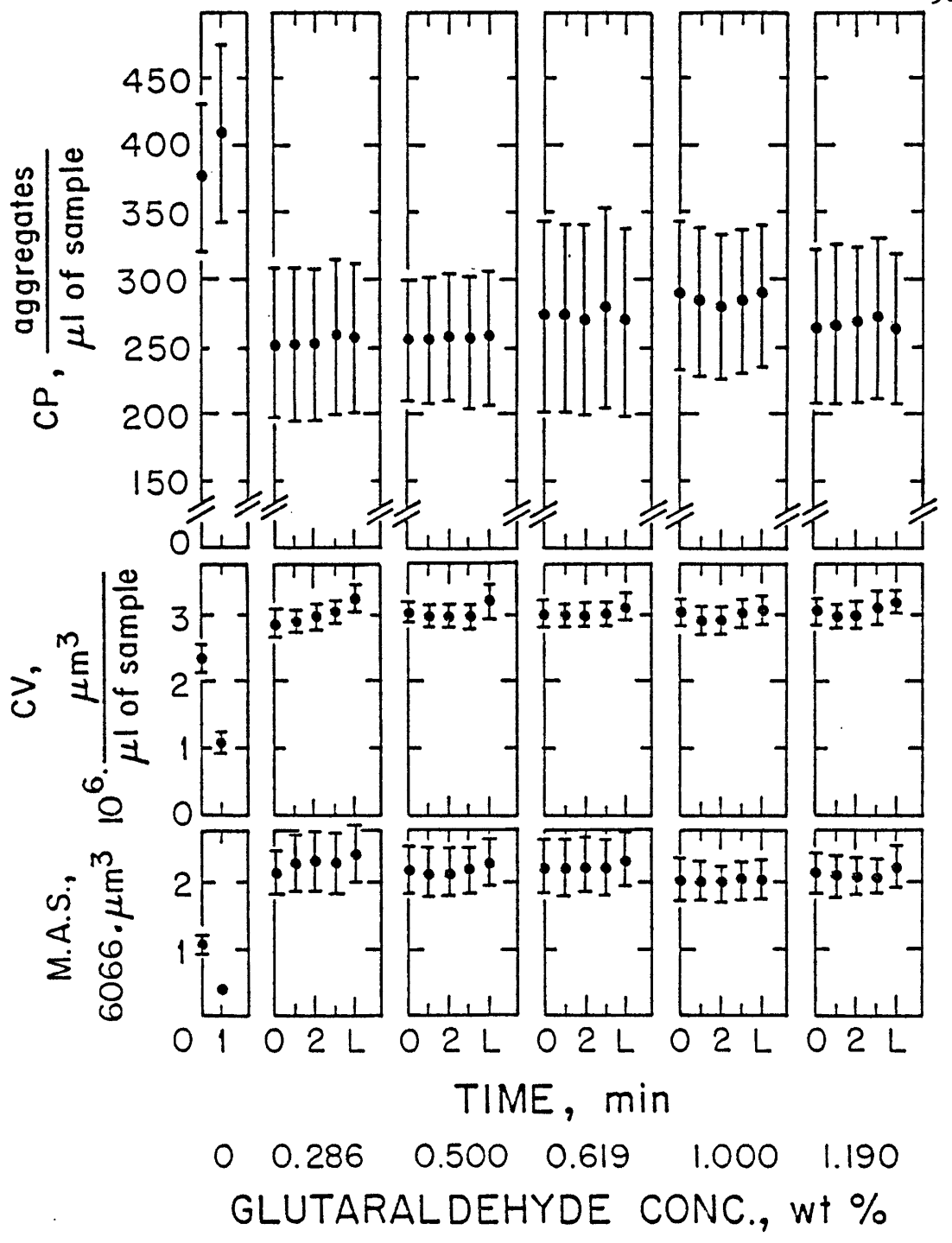

Figure V-2: Effect of glutaraldehyde added to isoton counting diluent on stability of platelet aggregates 13-101 µm in equivalent spherical diameter induced by ADP (0.5 µM final concentration). Measurements were made immediately, 1,2,3, and 30 to 90 (L) minutes after dilution of aggregated platelet-rich plasma for particle size analysis. The total platelet volume available for aggregation is $(2.698 \pm 0.140) \times 10^6$ $\mu m^3/\mu l$ of sample. (mean ± 1 s.e.m., n=5)

Table V-3: Same as Tables V-2-a and V-2-b except different glutaraldehyde levels.

(mean ± 1 s.e.m., n=15)

Glutaraldehyde Concentration (wt.%)	Time (min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
0	0	0.70±0.06	2.089±0.223	500±42
	1	0.36±0.01	0.694±0.121	316±58
	2,3,(30-90)	--	--	--
0.048	0	0.85±0.08	2.443±0.209	532±50
	1	0.86±0.08	2.423±0.193	523±50
	2	0.89±0.09	2.518±0.208	520±50
	3	0.92±0.09	2.569±0.217	522±50
	Late(30-90)	1.10±0.13	3.100±0.301	539±59
0.143	0	0.96±0.10	2.585±0.220	506±51
	1	1.00±0.11	2.670±0.248	504±52
	2	1.01±0.11	2.679±0.234	500±51
	3	1.02±0.11	2.711±0.247	504±51
	Late(30-90)	1.04±0.12	2.857±0.295	518±55
0.286	0	1.02±0.11	2.744±0.244	506±48
	1	1.03±0.11	2.731±0.245	504±49
	2	1.03±0.11	2.749±0.243	503±49
	3	1.02±0.11	2.749±0.245	502±47
	Late(30-90)	1.09±0.12	3.032±0.294	516±48

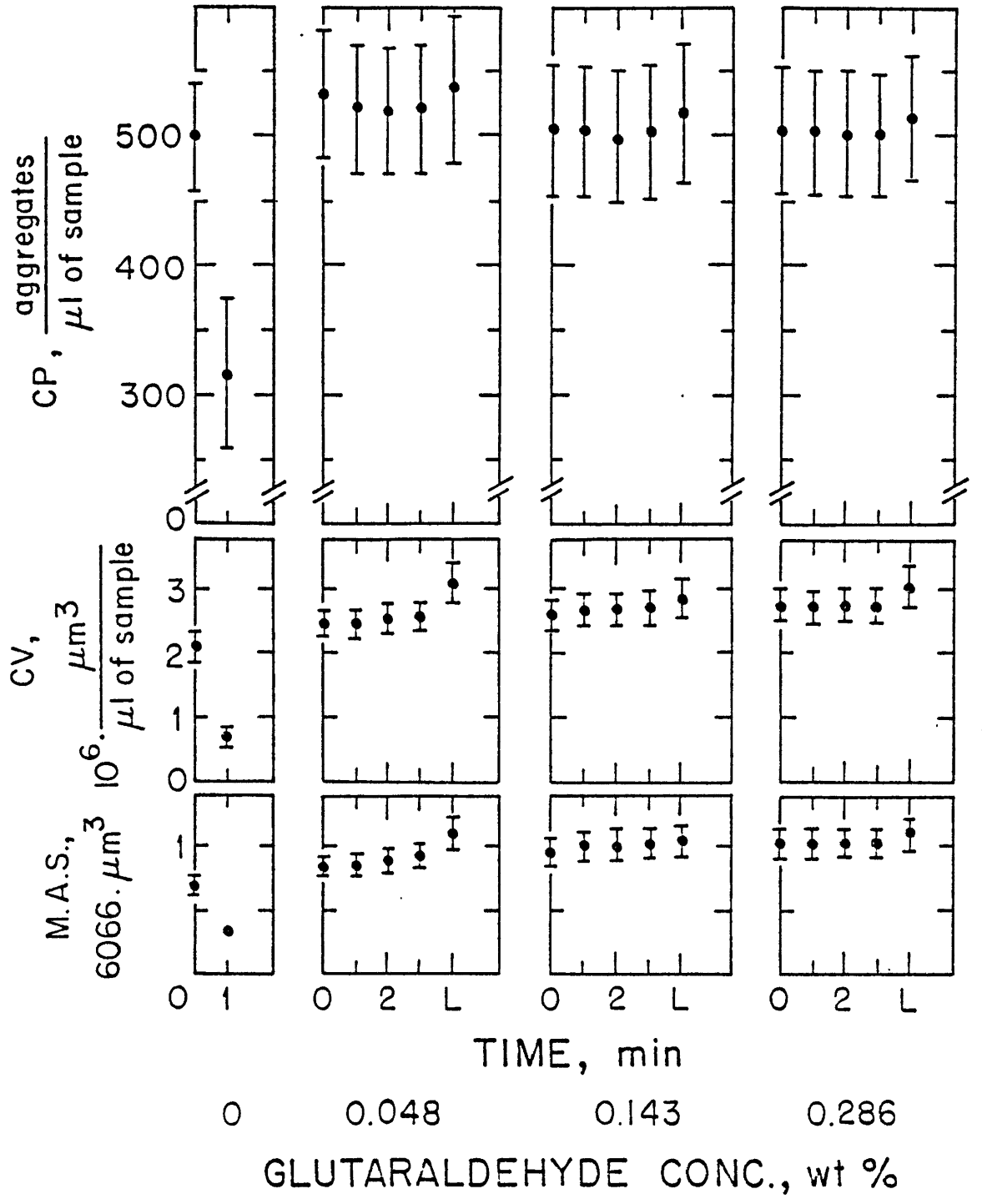

Figure V-3: Effect of glutaraldehyde added to isoton counting diluent on stability of platelet aggregates 13-101 µm in equivalent spherical diameter induced by ADP (0.5 µM final concentration). Measurements were made immediately, 1,2,3, and 30 to 90 (L) minutes after dilution of aggregated platelet-rich plasma for particle size analysis. The total platelet volume available for aggregation is (2.805±0.220) X 10^6 μm^3/µl of sample. (mean ± 1 s.e.m., n=15)

volumes and mean aggregate sizes for the immediate, 1,2, and 3 minute observations, but slightly higher or the same cumulative volumes and mean aggregate sizes at the late observation, when compared to diluents with more glutaraldehyde.

If it is supposed that either of the higher glutaraldehyde concentrations (0.143 and 0.286 wt.%) reflects the true aggregate size distribution, then the diluent (0.048 wt.%) is allowing some initial disaggregation (of the order of 10%). Conversely, if it is supposed that the 0.048 wt.% glutaraldehyde counting diluent reflects the true aggregate size distribution, then those with more glutaraldehyde are causing slight swelling or volume increase. It should be noted that all the glutaraldehyde solutions (of Table V-3) tend to cause an apparent volume increase in the late reading. However, comparison with the volume of free platelets available for aggregation indicates that there was only a small volume increase of the aggregates. Thus, there is evidence that 0.048 wt.% glutaraldehyde diluent gives an adequate estimate of the initial aggregate size distribution for 3 minutes after dilution, with only slight error in 30 to 90 minutes after dilution. As previously noted, use of isoton alone allowed disaggregation within 1 minute.

Recall that "immediate" observations are really made 10 seconds after isoton dilution. Extrapolation of the initial mean aggregate size for pure isoton diluent back 10 seconds appears to support the validity of the results produced by the 0.048 wt.% glutaraldehyde counting diluent. For isoton alone, mean aggregate size decreased by 45-60% in 1 minute, so in 10 seconds, an 8-15% decrease is expected. The initial mean aggregate size for pure isoton is about 85% of that for the 0.048 wt.%

glutaraldehyde counting diluent and about 70% of that for the counting diluents with more glutaraldehyde. Hence, the 0.048 wt.% glutaraldehyde diluent accounts for the projected course of the mean aggregate size for the pure isoton diluent.

B. Studies on Glutaraldehyde Fixative in both the Aggregated Sample and in the Counting Diluent

In this study, glutaraldehyde was added to both the counting diluent and the aggregated PRP sample prior to dilution. Since 0.048 and 0.143 wt.% glutaraldehyde in the diluent produced slightly different results, the diluent was varied here for further confirmation. Prior to dilution, 50 µl of isoton or glutaraldehyde solution was added to 1 ml of aggregated PRP.

1. 0.048 wt.% Glutaraldehyde in Aggregated PRP Prior to Dilution

Observations were made immediately, 1,2, and 3 minutes after dilution, as well as "late" (30-90 minutes after dilution), for each Coulter accuvette. It was necessary to vary the ADP concentration due to circumstances that could not be avoided. For the first 3 samples, 1.0 µM ADP was used to bring about aggregation, since 0.5 µM ADP could not elicit any significant aggregation, due to a 3 hour delay caused by a blown fuse in the Coulter Counter. For the next 3 samples (4-6), 0.5 µM ADP was used for aggregation. For the last 3 samples (7-9), the donor exhibited unusually reactive platelets, as shown by the unusually large aggregates of this donor's first sample (7) with 0.5 µM ADP. For the next sample (8), 0.2 µM ADP was used, and for the last sample (9), 0.5 µM ADP was used again. Hence, parameter averages may be less informative due to high variance in light of the circumstances just discussed.

Six different treatments were used in this series as follows:

*50 µl of 1% glutaraldehyde + 1 ml of aggregated PRP.

**50 µl of isoton + 1 ml of aggregated PRP.

Treatment	Glutaraldehyde Concentrations Diluent	PRP Sample
a) 31.5 ml of isoton; 50 µl of 1% glutaraldehyde + 1 ml of aggregated PRP	0 wt.%	0.048 wt.%
b) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde; *	0.048 wt.%	0.048 wt.%
c) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde; *	0.143 wt.%	0.048 wt.%
d) 31.5 ml of isoton; 50 µl of isoton + 1 ml of aggregated PRP	0 wt.%	0 wt.%
e) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde; **	0.048 wt.%	0 wt.%
f) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde; **	0.143 wt.%	0 wt.%

Averaged results are given in Table V-4. The time column gives the elapsed time after dilution before the count was taken. The total platelet volume available (from counts with the 70 µm aperture) for aggregation was $(2.757 \pm 0.120) \times 10^6$ $\mu m^3/\mu l$.

Table V-4 indicates that treatments "b," "c," "e," and "f" fix platelet aggregates for 3 minutes, where swelling occurs by the late observations. Treatment "d" did not stabilize the aggregates at all, since there was no glutaraldehyde added to either the counting diluent nor the aggregated PRP, prior to dilution. Treatment "a" did not stabilize the aggregates either, but disaggregation here was not nearly as rapid as in treatment "d," since glutaraldehyde was added to the PRP sample. Hence, glutaraldehyde must be added to the isoton counting diluent.

The question arises as to which of the other 4 treatments ("b," "c," "e," or "f") give the best reflection of the true aggregate size distribution. Treatment "c" shows the highest mean aggregate sizes, whereas treatments "f," "e," and "b" produce similar results, except

Table V-4: Effect of Glutaraldehyde in Isoton Diluent and PRP on Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter (variable ADP levels). (mean ± 1 s.e.m., n=9)

Treatment	Time (min.)	(M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
a	0	1.08±0.30	2.206±0.187	446±58
	1	0.83±0.22	2.030±0.193	504±57
	2	0.67±0.15	1.861±0.167	523±46
	3	0.52±0.09	1.585±0.185	529±38
	Late(30-90)	0.45±0.06	1.367±0.244	487±51
b	0	1.05±0.24	2.456±0.155	481±61
	1	1.05±0.24	2.434±0.163	476±60
	2	1.09±0.24	2.524±0.179	473±60
	3	1.10±0.24	2.615±0.202	479±57
	Late(30-90)	1.30±0.31	3.043±0.217	492±65
c	0	1.42±0.45	2.565±0.168	434±67
	1	1.53±0.51	2.635±0.196	428±67
	2	1.56±0.55	2.643±0.220	424±66
	3	1.55±0.51	2.748±0.172	429±66
	Late(30-90)	1.62±0.55	2.858±0.202	438±67
d	0	0.91±0.22	2.187±0.169	429±45
	1	0.42±0.04	1.086±0.181	374±61
	2,3,(30-90)	--	--	--
e	0	1.18±0.30	2.307±0.223	419±58
	1	1.19±0.30	2.370±0.231	419±59
	2	1.22±0.30	2.427±0.183	428±61
	3	1.26±0.32	2.500±0.206	430±61
	Late(30-90)	1.57±0.44	2.991±0.215	443±72

Continued

Table V-4: Effect of Glutaraldehyde in isoton Diluent and PRP on Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter (variable ADP levels). (mean ± 1 s.e.m., n=9)

Treatment	Time (min.)	(M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
f	0	1.21±0.36	2.354±0.282	418±59
	1	1.29±0.38	2.520±0.188	447±62
	2	1.32±0.39	2.673±0.221	456±62
	3	1.33±0.42	2.609±0.213	454±62
	Late(30-90)	1.23±0.29	2.634±0.181	459±63

treatment "e" shows the largest increase in mean aggregate size at the late observation. Cumulative populations reveal no significant differences for treatments "b," "c," "e," and "f," nor for the timed observations. Large variances make it more meaningful to analyze individual samples on a scattergram (Figure V-4). Data are exhibited in detail in Appendix C. From study of Figure V-4, it can be seen that treatment "b" is the most promising, i.e. adding glutaraldehyde to both isoton counting diluent and aggregated PRP in a final concentration of 0.048 wt.%. All three parameters seem to be stabilized effectively despite the wide variation in initial aggregate size distributions in the various samples.

2. 0.5 μM ADP-0.143 wt.% Glutaraldehyde in Aggregated PRP Prior to Dilution

This limited series was designed to study addition of 50 μl of 3 wt.% glutaraldehyde to 1 ml of aggregated PRP (0.5 μM ADP, 0.143 wt.% glutaraldehyde) prior to dilution for fixation of platelet aggregates. The sample itself was the focus for fixation, not the counting diluent. Observations were made immediately, 3,5, and 10 minutes after glutaraldehyde addition (immediately after completion of aggregation). In each case, the count was made immediately upon dilution. For each timed observation, a fresh Coulter accuvette (counting) diluent was used. From the total 1.05 ml of fixed aggregated PRP, 0.2 ml was used for each of the 4 timed observations. The diluents used were pure isoton and isoton-glutaraldehyde mixtures (0.048 and 0.143 wt.% glutaraldehyde).

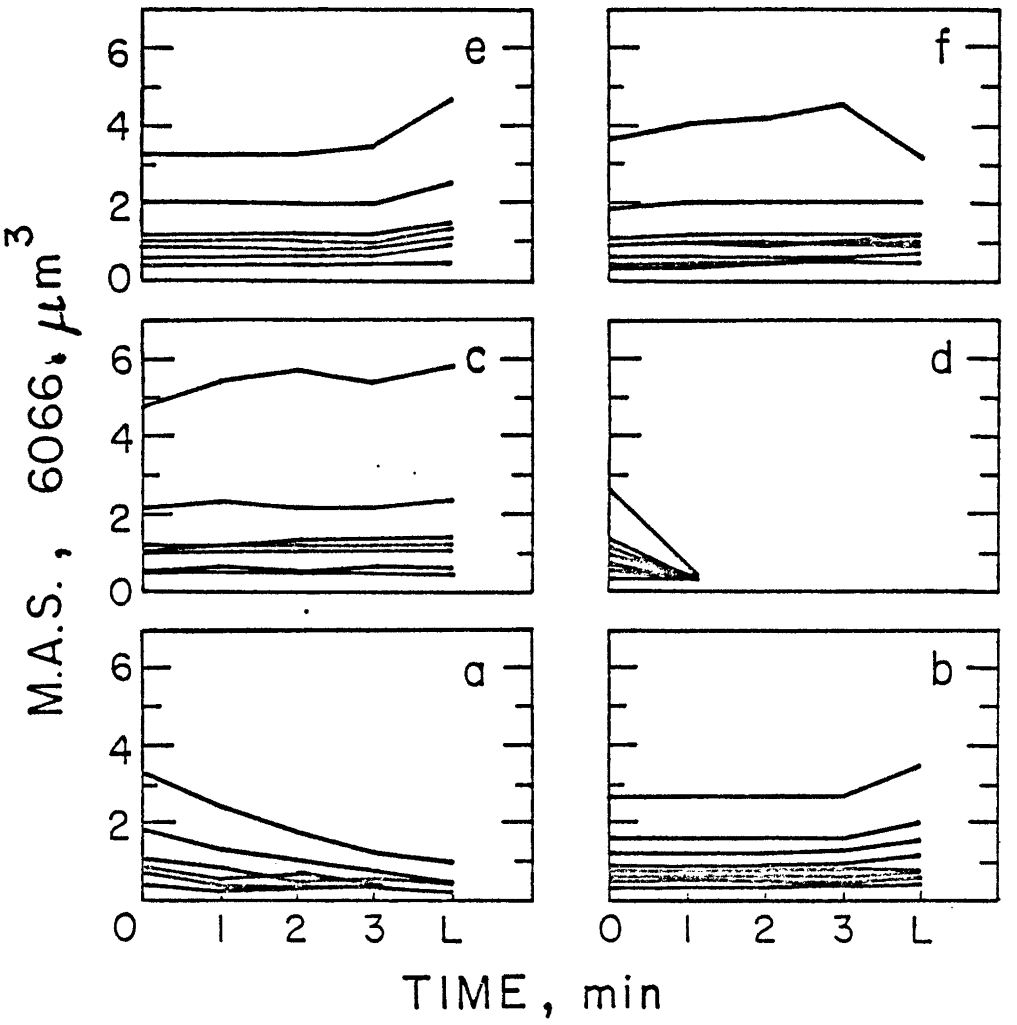

Figure V-4: Effect of various glutaraldehyde treatments on stability of platelet aggregates 13-101 μm in equivalent spherical diameter induced by ADP (final concentration: 0.2 to 1.0 μM). For treatments "a," "b," and "c," glutaraldehyde was added to aggregated PRP (final glutaraldehyde concentration: 0.048 wt.%), prior to dilution in the isoton counting diluent. In treatments "d," "e," and "f," no glutaraldehyde was added to aggregated PRP prior to dilution in isoton counting diluent. The final glutaraldehyde concentration in the isoton counting diluent was; 0 wt.% in treatments "a" and "d," 0.048 wt.% in treatments "b" and "e," and 0.143 wt.% in treatments "c" and "f." Measurements were made immediately at 1,2,3, and 30 to 90 (L) minutes after dilution of platelet-rich plasma for particle size analysis. Mean aggregate size (M.A.S.) is the ratio of the cumulative volume to the cumulative population of the aggregates. Ordinate represents CV9/CP9 which is M.A.S. ÷ 6066 (See Appendix A). Similar trends would be observed for cumulative volume (CV) and cumulative population (CP), which have less variance than M.A.S. (the ratio of CV to CP). See Appendices B and C for further explanation. (n=9)

The 3 treatments went as follows:

*50 µl of 3% glutaraldehyde + 1 ml of aggregated PRP.

Treatment	Glutaraldehyde Concentrations Diluent	PRP Sample
a) 31.5 ml of isoton; 50 µl of 3% glutaraldehyde + 1 ml of aggregated PRP	0 wt.%	0.143 wt.%
b) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde; *	0.048 wt.%	0.143 wt.%
c) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde; *	0.143 wt.%	0.143 wt.%

A summary of these results is given in Table V-5. The time column gives the elapsed time after addition of glutaraldehyde to aggregated sample prior to dilution, when the count was taken. The total platelet volume available (from counts with the 70 µm aperture) for aggregation was $(2.780 \pm 0.009) \times 10^6$ $\mu m^3/\mu l$.

The results of Table V-5 indicate that regardless of the counting diluent used, fixing platelet aggregates in PRP with a glutaraldehyde concentration of 0.143 wt.% causes aggregates to cross-link, as evidenced by the increased mean aggregate size and depressed cumulative population within 3 minutes after glutaraldehyde addition to PRP. Comparison of cumulative volumes for aggregates (13-101 µm) with available volume for aggregation from free platelets implies swelling immediately. Cumulative volumes are slightly decreased by 5 minutes, but somewhat reduced by 10 minutes.

C. Studies at Various ADP Concentrations Using a Set Glutaraldehyde Procedure

In this series, the counting solution diluent was isoton with 0.048 wt.% glutaraldehyde. For each control PRP sample, particle size analysis was carried out immediately, 3 and 5 minutes after completion of

Table V-5: Effect of 0.143 wt.% Glutaraldehyde in PRP Prior to Dilution, on the Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter. (mean ± 1 s.e.m., n=4)

Treatment	Time (min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregates}}{\mu l \text{ of sample}}$)
a	0	2.12±0.31	2.917±0.117	239±29
	3	2.88±0.45	2.957±0.154	184±30
	5	2.82±0.47	2.626±0.160	164±22
	10	2.85±0.46	2.266±0.094	146±30
b	0	1.93±0.16	2.918±0.091	255±21
	3	2.63±0.34	2.944±0.142	196±28
	5	2.60±0.35	2.666±0.066	179±25
	10	2.61±0.35	2.176±0.104	146±21
c	0	2.01±0.25	3.067±0.117	263±30
	3	2.69±0.42	3.109±0.078	209±40
	5	2.62±0.40	2.620±0.062	180±33
	10	2.61±0.38	2.283±0.051	161±37

aggregation on the vortex (55 seconds). For each test (fixed) PRP sample, particle size analysis was carried out immediately, 3, and 5 minutes after glutaraldehyde addition (immediately after aggregation). For each particle size analysis, fresh diluent was used, i.e. count taken immediately after dilution in Coulter accuvette.

The following 6 treatments were used in this final series:

Treatment

a) 0.9 ml of PRP + 0.1 ml of ADP (5 μM) → 1.0 ml of aggregated PRP
nothing added to aggregated PRP prior to dilution
a') 0.9 ml of PRP + 0.1 ml of ADP (5 μM) → 1.0 ml of aggregated PRP
50 μl of 1 wt.% glutaraldehyde added prior to dilution
b) 0.9 ml of PRP + 0.1 ml of ADP (20 μM) → 1.0 ml of aggregated PRP
nothing added to aggregated PRP prior to dilution
b') 0.9 ml of PRP + 0.1 ml of ADP (20 μM) → 1.0 ml of aggregated PRP
50 μl of 1 wt.% glutaraldehyde added prior to dilution
c) 0.9 ml of PRP + 0.1 ml of ADP (200 μM) → 1.0 ml of aggregated PRP
nothing added to aggregated PRP prior to dilution
c') 0.9 ml of PRP + 0.1 ml of ADP (200 μM) → 1.0 ml of aggregated PRP
50 μl of 1 wt.% glutaraldehyde added prior to dilution

	Glutaraldehyde Concentration in Sample	ADP Concentration in Sample
a)	0 wt.%	0.5 μM
a')	0.048 wt.%	0.5 μM
b)	0 wt.%	2.0 μM
b')	0.048 wt.%	2.0 μM
c)	0 wt.%	20 μM
c')	0.048 wt.%	20 μM

The purpose of observing 20 μM ADP was to determine if glutaraldehyde stops the aggregation process as well as disaggregation. A summary of results is given in Table V-6. The time column gives the elapsed time after completion of aggregation on vortex before count was taken (immediately after dilution) for control samples, and elapsed time after glutaraldehyde addition (immediately after completion of aggregation on vortex) before count was taken (immediately after dilution). The total

Table V-6: Effect of 0.048 wt.% Glutaraldehyde both in Diluent and PRP Prior to Dilution on the Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter.

Treatment	Time (min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregate}}{\mu l \text{ of sample}}$)
a	0	1.68±0.26	2.981±0.075	403±65
	3	0.45±0.02	0.835±0.207	292±74
	5	0.43±0.03	0.587±0.165	201±59
a'	0	1.63±0.31	3.059±0.121	434±67
	3	1.57±0.34	3.006±0.196	449±66
	5	1.47±0.31	2.842±0.120	453±66
		Vavail ($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6} = 3.149±0.164		(mean ± 1 s.e.m., n=12)
b	0	7.87±0.36	3.172±0.092	68± 3
	3	8.72±1.30	3.136±0.127	81±16
	5	7.61±1.58	2.539±0.114	92±19
b'	0	7.78±0.40	3.292±0.101	72± 5
	3	6.82±0.62	3.030±0.126	84±12
	5	6.46±0.73	2.778±0.135	87±15
		Vavail ($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6} = 3.188±0.146		(mean ± 1 s.e.m., n=12)

Continued

Table V-6: Effect of 0.048 wt.% Glutaraldehyde both in Diluent and PRP Prior to Dilution on the Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter.

Treatment	Time (min.)	M.A.S. (μm^3) ÷ 6066	CV($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6}	CP($\frac{\text{aggregate}}{\mu l \text{ of sample}}$)
c	0	10.94±0.45	3.138±0.138	48±2
	3	20.06±1.25	3.568±0.207	30±2
	5	18.42±1.23	2.198±0.193	20±2
c'	0	12.14±0.75	3.170±0.165	44±3
	3	12.46±0.52	2.878±0.114	39±2
	5	10.97±0.51	2.481±0.151	38±2

Vavail ($\frac{\mu m^3}{\mu l \text{ of sample}}$) X 10^{-6} = 3.212±0.158 (mean ± 1 s.e.m., n=11)

platelet volume available (from counts with the 70 μm aperture) for aggregation is denoted by Vavail under the cumulative volume column for each ADP concentration.

For all 3 ADP concentrations, there is no significant difference between the immediate observations in the control samples and those in the test samples, i.e. where glutaraldehyde was added to the aggregated PRP immediately after aggregation, prior to dilution ($p > 0.05$). Comparison between the cumulative volumes of platelet aggregates in the test sample with total platelet volume available for aggregation, demonstrates that there was never any swelling within 5 minutes after fixation with glutaraldehyde.

In PRP aggregated with 0.5 μM ADP, platelet aggregates in the controls broke up within 3 minutes. There was no significant difference between the immediate and 3 minute observations in the test samples ($p > 0.05$). The 5 minute results in the test samples show a slight decrease in mean aggregate size, and cumulative volume from the immediate and 3 minute readings. The cumulative populations are the same for all timed observations (up to 5 minutes) in the test samples ($p > 0.05$). Hence, (0.048 wt.%) glutaraldehyde successfully fixed the aggregates in PRP aggregated with 0.5 μM ADP for 5 minutes.

In PRP aggregated with 2.0 μM ADP, cumulative volume and mean aggregate size in test samples steadily decreased over 5 minutes ($p < 0.05$), even though the mean aggregate sizes at 3 and 5 minutes were not significantly different from each other ($p > 0.05$). The decrease in mean aggregate size is significant within 3 minutes after aggregation ($p < 0.05$). In the controls, differences for all parameters (CV, CP, and M.A.S.) over

5 minutes were not statistically significant, because of the large variance in data by 3 and 5 minutes after aggregation. In the test samples, cumulative population showed no significant differences over 5 minutes ($p > 0.05$). The high variance in controls is due to 2.0 μM ADP serving as a medium ground between reversible and irreversible platelet aggregation, whereas 0.5 μM ADP is clearly reversible and 20 μM ADP is clearly irreversible. The cumulative populations show no significant differences over 5 minutes either in the test nor control samples ($p > 0.05$). The much lower variance in test samples could indicate (0.048 wt.%) glutaraldehyde fixed the platelet aggregates in PRP aggregated with 2.0 μM ADP for 5 minutes. Further detailed information will be presented subsequently in aggregate size distributions.

In PRP aggregated with 20 μM ADP, platelets in the control samples continue to aggregate irreversibly over 3 minutes after the PRP is removed from the vortex, as evidenced by mean aggregate size and cumulative population. In all control samples, the aggregates become too large for all of them to be counted in the 280 μm aperture. Therefore, results in the controls can have no quantitative validity. There was no significant differences between the immediate and 3 minute observations in test samples ($p > 0.05$), but there was a significant decrease in mean aggregate size ($p < 0.05$) and cumulative volume ($p < 0.005$) for test samples by 5 minutes. However, cumulative populations in the test samples are the same over 5 minutes ($p > 0.05$). Therefore, (0.048 wt.%) glutaraldehyde fixed the aggregates successfully, in PRP aggregated with 20 μM ADP, for 3 minutes. A possible explanation of the shrinkage in the mean aggregate size and cumulative volume (on the order of 15%) observed

5 minutes after "completion of aggregation" (fixation) could be that fusion and contraction of platelet aggregates, characteristic of irreversible aggregation, has taken place.

For this final experimental phase, the aggregate size distribution was determined for channels 9 through 0 on the 280 µm aperture. For the aggregate size distribution, the differential volume in each channel was represented as a percentage of the cumulative volume to channel 9 (CV9). Recall, channels 9 through 0 cover size range (13-101 µm in equivalent spherical diameter). With PRP, this means platelet aggregates 13-101 µm in equivalent spherical diameter which implies that each channel represents a different sized platelet aggregate between 13 and 101 µm in equivalent spherical diameter. These aggregate size distributions were plotted for observations immediately, 3, and 5 minutes after aggregation on the vortex, for all ADP strengths (0.5 µM, 2 µM, and 20 µM). See Table V-7 and Figure V-5.

For all 3 ADP levels (0.5 µM, 2.0 µM, and 20 µM), the numbers for each channel is the same for both the control samples and fixed test samples immediately after aggregation on the vortex. As stated previously, (0.048 wt.%) glutaraldehyde is not introducing artifacts into the system (platelet aggregates).

In PRP aggregated with 0.5 µm ADP, platelet aggregates in control samples disaggregate within 3 minutes after aggregation. This is demonstrated by increased proportions of cumulative volume in smaller sized channels (12-16 µm) and decreased proportions (percentages) in larger sized channels (20 µm and up). In fixed samples, percentages were distributed similarly for immediate, 3, and 5 minute observations. For all

Table V-7: Effect of Glutaraldehyde (0.048 wt.%) in Diluent and PRP Prior to Dilution on the Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter. (' denotes minute)

(mean ± 1 s.e.m., n=12; 0.5 μM ADP)--for top half of data

Channel #	Partice Diameter (μm)	Control			Test (Fixed with Glutaraldehyde)		
		0'	3'	5'	0'	3'	5'
0	101.6	0.59±0.31	0.77±0.77	0.0 ±0.0	0.40±0.27	1.16±0.36	0.61±0.45
1	80.6	0.64±0.21	2.91±2.44	7.95±2.94	0.68±0.21	0.10±0.10	1.28±0.55
2	64.0	0.89±0.35	0.63±0.31	6.07±2.61	0.30±0.13	0.64±0.15	1.77±0.56
3	50.8	1.42±0.66	4.43±2.41	10.03±2.84	2.42±0.98	2.14±1.07	2.87±0.98
4	40.3	9.56±3.62	9.83±3.77	8.31±1.94	9.46±3.69	10.09±4.01	9.43±3.63
5	32.0	23.78±5.16	6.85±1.63	6.65±1.60	22.53±4.78	20.02±4.40	18.51±4.26
6	25.4	27.43±2.57	10.46±2.26	5.96±1.04	25.94±2.83	24.03±3.41	23.28±3.42
7	20.2	20.58±3.91	15.18±2.45	8.78±2.08	20.94±3.67	19.78±3.21	19.83±3.00
8	16.0	10.37±2.73	21.31±3.41	15.68±3.54	11.45±2.94	13.52±3.54	13.64±3.15
9	12.7	4.75±1.49	27.65±5.86	30.54±7.55	5.90±1.86	8.53±2.95	8.79±2.78
0	101.6	0.54±0.29	0.86±0.37	2.14±0.95	1.43±0.84	0.57±0.30	0.0 ±0.0
1	80.6	2.11±0.80	6.80±2.18	7.18±2.51	1.78±0.49	1.40±0.45	1.28±0.61
2	64.0	14.25±2.02	23.20±4.98	19.43±4.78	10.87±1.67	12.18±2.09	12.73±3.04
3	50.8	36.63±1.08	29.91±3.03	24.22±2.98	37.98±1.78	34.06±3.46	31.43±3.47
4	40.3	33.04±2.09	22.41±3.43	20.62±3.24	33.88±2.09	31.93±2.10	32.80±2.42
5	32.0	9.94±1.05	9.38±2.44	13.12±2.88	10.51±1.18	13.28±2.34	13.36±2.45
6	25.4	2.18±0.21	4.16±1.41	6.92±2.09	2.28±0.34	4.13±1.07	4.98±1.42
7	20.2	0.66±0.07	1.79±0.59	3.45±1.13	0.70±0.07	1.39±0.32	2.03±0.56
8	16.0	0.38±0.03	0.94±0.32	1.83±0.58	0.38±0.03	0.64±0.11	0.93±0.21
9	12.7	0.26±0.02	0.57±0.16	1.08±0.33	0.24±0.02	0.42±0.05	0.50±0.09

(mean ± 1 s.e.m., n=12; 2.0 μM ADP)--for bottom half of data

Continued

Table V-7: Effect of Glutaraldehyde (0.048 wt.%) in Diluent and PRP Prior to Dilution on the Stability of Platelet Aggregates 13-101 μm in equivalent spherical diameter. (' denotes minute)

(mean ± 1 s.e.m., n=11; 20 μM ADP)

Channel #	Particle Diameter (μm)	Control			Test (Fixed with Glutaraldehyde)		
		0'	3'	5'	0'	3'	5'
0	101.6	0.19±0.19	9.26±2.05	14.16±3.19	2.35±0.90	0.90±0.49	1.21±0.55
1	80.6	6.56±1.36	31.57±2.78	38.67±2.21	9.61±2.70	10.75±2.73	8.69±1.64
2	64.0	24.81±1.70	37.79±2.41	28.00±2.35	28.63±1.32	34.75±1.78	32.94±2.15
3	50.8	41.44±1.11	14.66±1.98	11.91±1.18	39.83±2.47	36.36±1.99	37.01±2.20
4	40.3	20.00±1.51	4.26±0.45	3.57±0.46	14.62±1.69	13.24±1.23	14.35±1.33
5	32.0	4.32±0.41	1.19±0.11	1.86±0.21	3.17±0.37	2.76±0.38	3.25±0.34
6	25.4	1.06±0.10	0.66±0.07	0.81±0.08	0.84±0.09	0.81±0.07	1.21±0.11
7	20.2	0.80±0.39	0.31±0.04	0.50±0.07	0.48±0.06	0.41±0.03	0.65±0.07
8	16.0	0.47±0.21	0.23±0.02	0.29±0.04	0.30±0.03	0.29±0.03	0.43±0.07
9	12.7	0.33±0.19	0.14±0.02	0.23±0.04	0.21±0.01	0.20±0.02	0.26±0.02

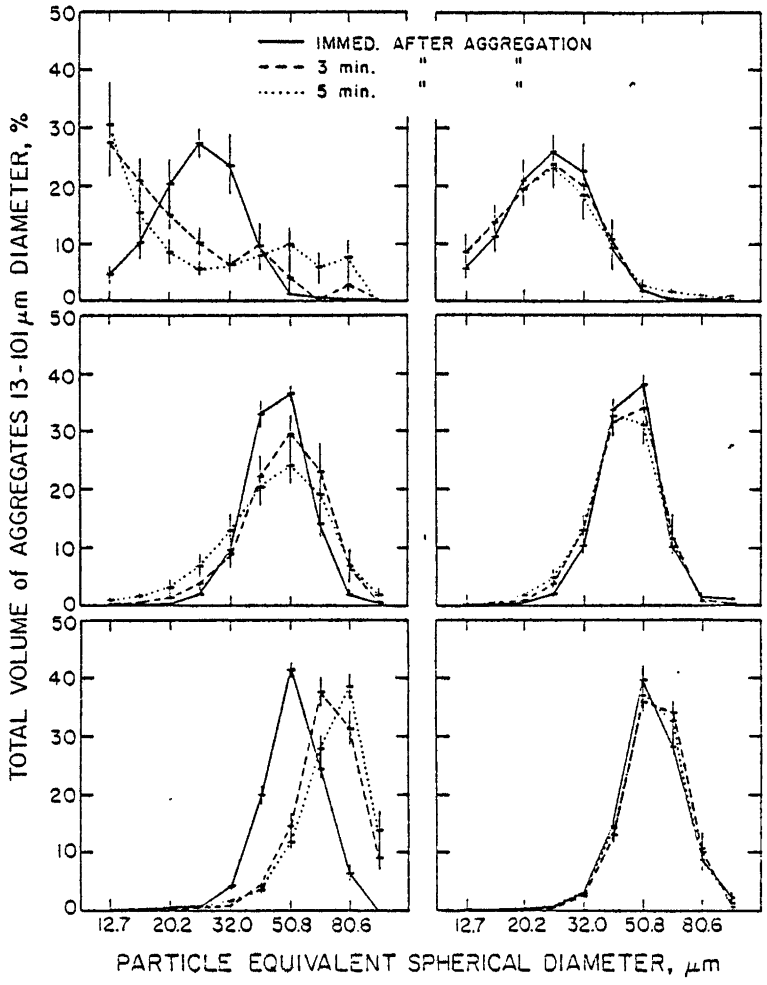

Figure V-5. Effect of glutaraldehyde (0.048 wt.% concentration) in diluent and PRP prior to dilution on stability of volume size distribution of platelet aggregates 13-101 μm in equivalent spherical diameter induced by ADP (final concentration: 0.5, 2.0, and 20 μM). Measurements were made immediately, at 3, and 5 minutes after aggregation and glutaraldehyde was added to test PRP sample immediately after aggregation. Note, particle size analysis was carried out immediately after dilution in isoton-glutaraldehyde counting solution (glutaraldehyde concentration: 0.048 wt.%) in all cases. Data are represented as a percentage of the cumulative volume of aggregates 13-101 μm in equivalent spherical diameter for each of the particle size channels in that size range. Test PRP samples are exhibited on the right; control samples are exhibited on the left.

Top - 0.5 μM ADP (mean ± 1 s.e.m., n=12)
Middle - 2.0 μM ADP (mean ± 1 s.e.m., n=12)
Bottom - 20 μM ADP (mean ± 1 s.e.m., n=11)

fixed samples and controls immediately after aggregation, most of the cumulative volume was in size range from 20-32 μm in equivalent spherical diameter. Glutaraldehyde (0.048 wt.%) fixed aggregates in aggregated PRP (0.5 μM ADP) for 5 minutes after aggregation, as shown previously.

In PRP aggregated with 2.0 μM ADP, platelet aggregates in control samples either disaggregated or continued to aggregate further. Therefore, relative proportions of cumulative volume increases in outer size ranges, i.e. 64-101 μm in diameter as well as 32 μm and less. The size range from 40-64 μm in diameter progressively decreases in percentage of cumulative volume over the 5 minutes. In fixed samples, percentages were distributed similarly for immediate, 3, and 5 minute observations. For all fixed samples and controls immediately after aggregation, most of cumulative volume was in size range from 40-64 μm in equivalent spherical diameter. Glutaraldehyde (0.048 wt.%) fixed aggregates in aggregates PRP (2.0 μM ADP) for 5 minutes, but not as well as with 0.5 μM ADP.

In PRP aggregated with 20 μM ADP, platelet aggregates in control samples continue to aggregate further after removal of agitation. This is demonstrated by the increased percentages in larger sized channels (64-101 μm) and decreased percentages in smaller sized channels (13-50 μm). For fixed samples, percentages were distributed similarly for immediate, 3, and 5 minute observations. For all fixed samples and controls immediately after removal from vortex, most of the cumulative volume was in size range from 40-80 μm in equivalent spherical diameter. Glutaraldehyde (0.048 wt.%) fixed aggregates in aggregated PRP (20 μM) for 3 minutes, certainly. For 5 minutes, this glutaraldehyde maintained the relative distribution, but not the cumulative volume.

VI. DISCUSSION

From the preliminary findings, fixation of platelet aggregates in PRP, aggregated with 2.0 μM ADP, for 24 hours appeared unfeasible, regardless of fixative agent, or resuspension method used. All 24 hour results indicated swelling, disaggregation, or both plus sedimentation of aggregates to bottom of test tube. For one hour fixation, resuspension method did not matter, since 2 second vortex, 2 and 8 inversions gave the same results, except continuous rotation on turntable accelerated disaggregation within one hour. Also for one hour fixation, 0.048 wt.% glutaraldehyde in aggregated PRP proved to be the most promising fixative. 0.024 wt.% Glutaraldehyde and 0.048 wt.% formalin permitted disaggregation within an hour, whereas 0.095 wt.% glutaraldehyde, 0.190 wt.% formalin, and the Wu-Hoak EDTA/formalin fixative (0.8 wt.% formalin) resulted in apparent swelling. Results could not be obtained with higher glutaraldehyde concentrations than 0.23 wt.% in the sample, because the sample gelled. Such gelling presumably was caused by cross-linking of plasma proteins,[18] since higher glutaraldehyde concentrations in the isoton counting solution did not result in gelling. Glutaraldehyde, in concentrations up to 0.619 wt.% in isoton counting solution, introduced virtually no artifacts in free platelets nor background counts on the 280 μm aperture in the isoton counting solution.

Platelet aggregates in PRP (0.5 and 2 μM ADP) were found to disaggregate shortly after dilution in isoton. Since 10 seconds elapse between isoton dilution and the end of the counting process, there is

considerable disaggregation occurring as the count is taken. Hence, the necessity of adding glutaraldehyde to the isoton counting solution. Isoton counting solution containing glutaraldehyde (concentration: 0.143-1.190 wt.%) produced results for 30-90 minutes after dilution with no significant differences statistically from the initial measurements ($p > 0.05$), and all produced slight swelling initially. An isoton counting solution with 0.048 wt.% glutaraldehyde produced similar results to those with more glutaraldehyde, except for slightly lower cumulative volumes and mean aggregated sizes for observations made immediately 1,2, and 3 minutes after dilution in the counting solution. The counting solution with 0.048 wt.% glutaraldehyde also caused significant swelling by 30-90 minutes after dilution. Isoton counting solutions with glutaraldehyde (concentration: 0.048-1.190 wt.%) showed the same cumulative populations statistically for observations made immediately, 1,2,3, and (30-90) minutes after dilution in counting solution. Therefore, 0.048 wt.% glutaraldehyde was used in the isoton counting solution as well as the aggregated PRP itself.

Other advantages in using 0.048 wt.% glutaraldehyde in the isoton counting solution are its osmolality and applicability to whole blood. The osmolality is very close to that of pure isoton (see Section C of Materials and Methods Section). Sutera and Mehrjardi[40,41] used 0.048 wt.% glutaraldehyde to fix red blood cells. Furthermore, red blood cells in specimens fixed with 0.048% glutaraldehyde can be lysed with zap isoton.[42] Higher glutaraldehyde levels fix the red blood cells in such a way that they cannot be lysed. Lysis of red cells is

important in studies on platelet aggregates in whole blood.

The set procedure with 0.048 wt.% glutaraldehyde in both the isoton counting solution and the aggregated PRP prior to dilution, stabilized the platelet aggregate size distribution for 5 minutes after aggregation with either 0.5 or 2.0 μm ADP. This procedure also successfully stabilized the platelet aggregate size distribution for 3 minutes (but not 5 minutes) after aggregation with 20 μM ADP. Perhaps fusion and contraction of irreversible aggregates was not prevented for 5 minutes with the very high ADP concentration of 20 μM.

Although relatively little work has been carried out involving fixation of platelets and platelet aggregates, a few studies are related to the present work. Borne and Hume[9] used formalin (1 wt.%) to arrest aggregation after certain time intervals subsequent to ADP addition. Optical methods were used to quantitate aggregation. Wu and Hoak[45,46] later developed another means of quantifying platelet aggregation involving platelet counts of blood samples drawn into a buffered EDTA solution, in comparison to counts in samples drawn into buffered EDTA/formalin solution. The ratio of the platelet count of the EDTA/formalin solution to that of the EDTA solution was designated as the platelet aggregate ratio. This method makes no direct measurements of platelet aggregate sizes or volumes.

Recently, other fixation studies used glutaraldehyde for platelet aggregates. Seamen used glutaraldehyde to fix platelet aggregates in PRP (final glutaraldehyde concentration: 0.5 wt.%)[6] and blood microaggregate particles in whole blood (0.06 wt.%).[42] They defined a

platelet count ratio, as the ratio of the platelet count, at a specified time after addition of an aggregating agent, to the original platelet count in PRP prior to aggregation.[6] This group used a Payton aggregation module for light transmission analyses, as well as an electronic particle counter in studies over a range of ADP concentrations in order to cover both reversible and irreversible aggregation. Like Wu and Hoak, they were concerned with platelet counts (populations), not aggregate sizes and cumulative volumes. Tamblyn, Nordt, Swank, Zukorski, and Seamen[42] used 0.06 wt.% glutaraldehyde in 0.15 M NaCl solution to stabilize microaggregate counts in whole blood. They used electronic particle size distribution and screen filtration pressure measurements, in studies on blood filters.

The closest work to the present work was carried out at the University of Rochester by Nichols and Bosman in 1978 and 1979.[23] They worked with formaldehyde and glutaraldehyde as fixative agents for platelet aggregates in PRP, and concluded that glutaraldehyde was more promising, as was the case in the preliminary studies of the present work. They varied the ADP concentration to study both reversible and irreversible aggregation, but only use 5 μM ADP as their strongest ADP, whereas the present work went as high as 20 μM ADP. As in the present work, they used the Coulter Counter (Model TAII, 70 μm and 280 μm aperture),[38] a slightly different model. They also found that aggregates started to break up immediately upon isoton dilution (with no fixative). Another finding was that in irreversible aggregation (high ADP levels), the largest aggregates exceed the size of the largest particle detectable with the 280 μm aperture. There are a number of similarities

between the work of Nichols, and Bosman and the present work and several fundamental differences. Nichols and Bosman primarily concerned themselves with platelet aggregate counts (populations), even though aggregate sizes and cumulative volumes were also analyzed. They used a final glutaraldehyde concentration of 1 wt.% in the isoton counting solution, whereas the present work involved a final glutaraldehyde concentration of 0.048 wt.% in counting solution. Finally, Nichols and Bosman only concern themselves with how long glutaraldehyde will stabilize the platelet aggregates in isoton counting solution. The present work addresses both this problem and how long glutaraldehyde will fix the aggregates in the PRP sample prior to dilution in the isoton-glutaraldehyde counting solution.

Nichols and Bosman assert that "glutaraldehyde preserves the state of platelets and aggregates in suspension unchanged for up to 48 hours." Their conclusion is based on several measurements of the total concentration of particulates (platelets and aggregates) in each diluted sample over a particular time interval. Such measurements were made over a 30 min interval (n=3, 2 μM ADP-induced irreversible aggregation with samples taken for 1:800 dilution after 10 sec), 60 min interval (n=1, 2.8 μM ADP-induced irreversible aggregation with samples taken for 1:5000 dilution after 30 sec), and 3 hour interval (n=3, 0.7 μM ADP-induced reversible aggregation with samples taken for 1:40 dilution after 20 sec), where the first measurement was taken 45 seconds after dilution in all cases. In every case, there were no significant differences between any pair of values in the intervals. Under these conditions, glutaraldehyde prevents both

aggregation and disaggregation processes from altering the state of platelet aggregates. In similar experiments (n=8), total concentrations of particulates in the samples diluted (1:50) in Isoton II containing 1 wt.% glutaraldehyde were not significantly different from values obtained during repeat analyses 48 hours later. Note that Nichols and Bosman are taking particulate concentration, i.e. cumulative population, but deemphasizing the aggregate sizes and cumulative volume. Furthermore, they state that, "the distributions of platelet and aggregate sizes in these samples show no major differences." They merely demonstrate that such distributions are similar for 3 and 48 hours after dilution in the isoton-glutaraldehyde counting solution (diluent; final glutaraldehyde concentration: 1 wt.%), whereas no results are demonstrated for observations made immediately after dilution. The present work demonstrated apparent swelling of the aggregates within 30 to 90 minutes after dilution in isoton-glutaraldehyde counting diluent (final glutaraldehyde concentration: 0.143 to 1.190 wt.%). Such swelling was on the order of 10% (See Figure V-2).

Interpreting the present study as well as previous studies of platelet aggregate fixation for electronic particle size analysis can be difficult. A major problem in interpreting particle size distributions noted immediately after dilution in isoton alone and isoton containing agents which "fix" platelet aggregates (glutaraldehyde in present work) is that an independent means of assessing the particle size distribution of platelet aggregates does not exist, i.e. there is no standard. This makes interpretation of data diffucult, because

previous studies with red blood cells[36] showed that fixation of particles may change their shape and deformability. Such alterations can affect the path and orientation of the particle as they go through the orifice of the Coulter Counter, thus changing the results.[28,36] In other words, fixation always poses the danger of introducing an artifact. Furthermore, the question arises as to whether dilution in isoton alone accelerates disaggregation further than that which takes place in "undisturbed" aggregated PRP, i.e. not diluted. Therefore, one can not know unequivocally in which situation the true aggregate size distribution of the sample is revealed.

For platelet aggregation induced by a final ADP concentration of 2 μM, the higher glutaraldehyde concentration (0.619 wt.%) in the isoton diluent appeared to cause an initial increase in the mean size of platelet aggregates, which is partially reversible within 3 minutes after dilution (See Figure V-1). Zap isoton shows some stabilizing effect on the aggregate size distribution when added to the isoton diluent, although disaggregation gradually sets in. The lower glutaraldehyde concentration (0.048 wt.%) in the isoton diluent stabilizes the aggregate size distribution even more than zap isoton, but also gradually leads to some apparent increase in size of platelet aggregates, i.e. 30 to 90 minutes after dilution the mean aggregate size had increased by 15% (See Table V-1). Yet, observations made immediately after dilution in the counting diluent were similar for isoton alone, isoton with zap isoton, and isoton with 0.048 wt.% glutaraldehyde, except that the initial mean aggregate size using the diluent with 0.048 wt.% glutaraldehyde was approximately 10% higher

than that observed in the isoton diluents with no glutaraldehyde added (See Figure V-1). These 3 diluents were considerably different from the diluent with 0.619 wt.% glutaraldehyde immediately after dilution. Since the mean aggregate size is changing for the first 3 minutes after dilution in the diluent with 0.619 wt.% glutaraldehyde and the cumulative volume of the aggregates exceeds volume available for aggregation from free platelets (measured prior to platelet aggregation), the possibility of glutaraldehyde introducing a measurement artifact can not be dismissed (See Table V-1). Therefore, the diluent with 0.048 wt.% glutaraldehyde seems to be most promising. A possible explanation for the apparent increase in aggregate size or cumulative volume is that aggregates can trap plasma, which would then be interpreted by the instrument as particle mass, not plasma. Hence, the apparent cumulative volume of the platelet aggregates is increased. Glutaraldehyde can possibly cause larger aggregates to cross-link with each other which would result in increased mean aggregate size and decreased cumulative population.

For platelet aggregation induced by a final ADP concentration of 0.5 μM, the aggregates are presumably less firmly held together than those induced by 2 μM ADP. All diluents with glutaraldehyde (0.048 to 1.190 wt.%) gave statistically similar results, except that the diluent with 0.048 wt.% gave an initial mean aggregate size and cumulative volume about 10% less than the diluents with more glutaraldehyde (See Figures V-2 and V-3). However, there was a gradual and significant increase in mean size of platelet aggregates

and cumulative volume of the aggregates, i.e. 30 to 90 minutes after dilution, the mean aggregate size had increased by 29% and the cumulative volume by 26% (See Table V-3), when using the diluent with 0.048 wt.% glutaraldehyde. Again, the possibility of a measurement artifact caused by glutaraldehyde can not be ruled out, and with high glutaraldehyde concentrations, the artifact can be incorporated more rapidly, if not initially after dilution in the counting diluent. As with 2 μM ADP, the diluent with 0.048 wt.% is preferred with aggregates induced by 0.5 μM ADP in PRP. As previously mentioned, other advantages of using 0.048 wt.%, in the counting diluent, include its osmolality and applicability to whole blood, i.e. red blood cells can be lysed with zap isoton, as Seamen did in presence of 0.06 wt.% glutaraldehyde.[42]

The value of the present work lies in the demonstration that the cumulative volume and mean size of platelet aggregates in PRP can be fixed during the process of platelet aggregation (See Section C of the Principal Results Section) induced by widely varying final ADP concentrations (0.5, 2, and 20 μM). A logical progression beyond the present work would be to extend the study to whole blood instead of PRP. The results show promise of value in studies <u>in vitro</u> as well as <u>in vivo</u> (clinical).

VII. APPENDICES

APPENDIX A

THE COULTER COUNTER

The general principles of the Coulter Counter are discussed in Section A of the Materials and Methods section. Methods of treatment of the raw data from the tape printed out will be discussed in this appendix.

There are 5 apertures used on the Coulter Counter, i.e., 70 μm, 100 μm, 200 μm, 280 μm, and 400 μm apertures. The 70 μm aperture draws 0.5 ml of solution and takes 25 second for particle size analysis. The 200 μm, 280 μm, and 400 μm apertures all draw 2.0 ml of solution and take 12.4, 6.4, and 3.4 seconds for particle size analysis, respectively. The geometric mean volume for channel 7 serves as the aperture volume conversion factor to μm^3. For the 70 μm aperture, this factor is 94.78, for the 100 μm aperture, it is 189.6, for the 200 μm aperture, it is 3033, for the 280 μm aperture, it is 6066, and for the 400 μm aperture, it is 24,270. The 70 μm aperture was used for free platelets, and the 280 μm aperture for platelet aggregates.

The size ranges for each aperture are given in Table A, at the end of this appendix. For example, the 70 μm aperture covers particles 1.0-25.4 μm in equivalent spherical diameter. For free platelets, channels 12 through 8 are used, i.e., particles 1.59-4.0 μm in equivalent spherical diameter. The 280 μm aperture covers particles 4.0-101.6 μm in equivalent spherical diameter. For platelet aggregates

13-101 µm in equivalent spherical diameter, channels 9 through 0 are used.

An example will now be illustrated with 70 µm and 280 µm aperture data. With each aperture, one can print out cumulative volume and population as well as differential volume and population. Cumulative implies all volume or population counted from channel 0 to the particular channel, whereas differential denotes the volume or population of that particular channel. In the usual procedure with the 280 µm aperture, the differential volume was printed on tape, whereas cumulative population and volume to channel 9 was displayed. With the 70 µm aperture, the cumulative population and volume were printed. The cumulative population and volume to channels 9 and 12 were directly read off the tape.

Example:

280 µm aperture:

Channel	Differential Volumes	Cumulative Volumes	
0	0	0	
1	0	0	
2	32	32	
3	1536	1568	
4	2720	4288	
5	1272	5560	
6	322	5882	
7	66	5948	
8	26	5974	
9	16	5990	CV9
10	16	6006	
11	44	6050	
12	61	6111	
13	44	6155	
14	61	6216	

Note that one can calculate cumulative quantities by adding the differential quantities.

cumulative volume to channel 9 (CV9) = 5990

cumulative population to channel 9 (CP9) = 1098

One could print out differential populations and cumulative population, and these numbers are handled in the same way as the volume numbers.

To convert to actual cumulative volume and population, one has to take into account all the dilutions of the sample (PRP here) as well as the 280 μm aperture conversion factor and the fact that the 280 μm aperture draws 2 ml (2000 μl) of solution for particle size analysis.

Case 1: nothing added to the aggregated sample.

	Dilution factor
Dilutions:	
i) 0.9 ml of PRP + 0.1 ml of ADP (1 ml of aggregated PRP)	$\frac{10}{9} = 1.11$
ii) 0.2 ml of aggregated PRP + 31.5 ml of diluent	$\frac{31.5 + 0.2}{0.2} = \frac{31.7}{0.2} = 158.5$

280 μm aperture volume conversion factor = 6066 μm^3

280 μm aperture draws 2000 μl for particle size analysis

Actual cumulative volume of platelet aggregates

$$CV = CV9 \times \frac{10}{9} \times \frac{31.7}{0.2} \times \frac{6066\ \mu m^3}{2000\ \mu l \text{ of sample}}$$

$$= 5990 \times 1.11 \times 158.5 \times \frac{3.033\ \mu m^3}{\mu l \text{ of sample}}$$

$$CV = 5990 \times 534.145\ \frac{\mu m^3}{\mu l \text{ of sample}} = 3.200 \times 10^6\ \frac{\mu m^3}{\mu l \text{ of sample}}$$

Actual cumulative population of platelet aggregates

$$CP = CP9 \times \frac{10}{9} \times \frac{31.7}{0.2} \times \frac{1 \text{ aggregate}}{2000 \text{ µl of sample}}$$

$$= 1098 \times 1.11 \times 158.5 \times \frac{1 \text{ aggregate}}{2000 \text{ µl of sample}}$$

$$CP = 1098 \times 0.0881 \frac{\text{aggregate}}{\text{µl of sample}} = 96.7 \frac{\text{aggregate}}{\text{µl of sample}}$$

Mean Aggregate Size ÷ M.A.S.

$$\frac{CV}{CP} = \frac{3.200 \times 10^6 \frac{\mu m^3}{\text{µl of sample}}}{96.7 \frac{\text{aggregates}}{\text{µl of sample}}} = 3.310 \times 10^4 \frac{\mu m^3}{\text{aggregate}}$$

Case 2: 50 µl of 1% glutaraldehyde, isoton, or 3% glutaraldehyde added to the aggregated sample.

Dilutions:

	Dilution factor
i) (1 ml of aggregated PRP) 0.9 ml of PRP + 0.1 ml of ADP	$\frac{10}{9} = 1.11$
(50 µl = 0.05 ml) (1.05 ml of fixed aggregated PRP)	
ii) 50 µl of glutaraldehyde or isoton + 1 ml of aggregated PRP	$\frac{1+.05}{1} = 1.05$
iii) 0.2 ml of fixed aggregated PRP + 31.5 ml of diluent	$\frac{31.5+0.2}{0.2} = \frac{31.7}{0.2}$ = 158.5

280 µm aperture volume conversion factor = 6066 μm^3

280 µm aperture draws 2000 µl for particle size analysis

$$CV = CV9 \times \frac{10}{9} \times 1.05 \times \frac{31.7}{0.2} \times \frac{6066\ \mu m^3}{2000\ \mu l \text{ of sample}}$$

$$= 5990 \times 1.11 \times 1.05 \times 158.5 \times 3.033\ \frac{\mu m^3}{\mu l \text{ of sample}}$$

$$CV = 5990 \times 560.852\ \frac{\mu m^3}{\mu l \text{ of sample}} = 3.360 \times 10^6\ \frac{\mu m^3}{\mu l \text{ of sample}}$$

$$CP = CP9 \times \frac{10}{9} \times 1.05 \times \frac{31.7}{0.2} \times \frac{\mu l \text{ aggregate}}{2000\ \mu l \text{ of sample}}$$

$$= 1098 \times 1.11 \times 1.05 \times 158.5 \times \frac{\mu l \text{ aggregate}}{2000\ \mu l \text{ of sample}}$$

$$CP = 1098 \times 0.0925\ \frac{\text{aggregate}}{\mu l \text{ of sample}} = 101.5\ \frac{\text{aggregates}}{\mu l \text{ of sample}}$$

$$M.A.S. = \frac{CV}{CP} = \frac{3.360 \times 10^6\ \frac{\mu m^3}{\mu l \text{ of sample}}}{101.5\ \frac{\text{aggregates}}{\mu l \text{ of sample}}} = 3.31 \times 10^4\ \frac{\mu m^3}{\text{aggregate}}$$

Note that the mean aggregate size is the same in both cases, since this is just the quotient of the cumulative volume divided by the cumulative population. If one closely follows the numerical conversions of the volume and population data to actual volumes and population data to actual volumes and populations in $\frac{\mu m^3}{\mu l \text{ of sample}}$ and $\frac{\text{aggregates}}{\mu l \text{ of sample}}$ respectively, one can see that both quantities use the identical dilution factors as well as the draw quantity of 2000 µl. Therefore the only difference between the cumulative volume and population, aside from CV9 and CP9 is the aperture volume conversion factor, which for the 280 µm aperture is 6066 μm^3. Hence, M.A.S. = $\frac{CV9}{CP9} \times 6066\ \frac{\mu m^3}{\text{aggregate}}$. Usually, $\frac{CV9}{CP9}$ gives numbers in the range 0 to 30. Therefore, it is convenient to report mean aggregate size data as $\frac{CV9}{CP9}$ and specify that

the actual mean aggregate size is $\frac{CV9}{CP9} \times 6066$. In this example, $\frac{CV9}{CP9} = 5.46$. A summary of the conversion factor for getting actual cumulative volumes and populations, and mean aggregate size from the CV9 and CP9 on the 280 μm aperture is given below.

	Case 1	Case 2
50 μl added to 1 ml of aggregated PRP	no	yes
cumulative volume	CV9 x 534.145	CV9 x 560.852
cumulative population	CP9 x 0.0881	CP9 x 0.0925
(M.A.S.) mean aggregate size	$\frac{CV9}{CP9} \times 6066$	$\frac{CV9}{CP9} \times 6066$

To further illustrate how the Coulter Counter works, consider analysis of platelet aggregates, larger than white blood cell size, ranging up to 161 μm in equivalent spherical diameter (instead of 101 μm, as in the case discussed above). In this case the 400 μm aperture would be used since the 280 μm aperture would not cover the larger particles. In this case, one would use CV11 and CP11, since channel 11 on the 400 μm pertains to the same size particles as channel 9 on the 280 μm aperture. It would only take 3.4 seconds for particle size analysis (instead of 6.4 seconds on the 280 μm aperture), but with the larger aperture, one may lose some accuracy. As another example, an investigator was interested in platelet aggregates only up to 81 μm in equivalent spherical diameter and larger than white blood size. He would use the 200 μm aperture, and take CV8 and CP8. It would take 12.4 seconds for particle size analysis. It is important to

note that for any aperture, the time is specified and critical. For the 280 μm aperture, it must be in the range 6.3-6.4 seconds. For the 70 μm aperture, it must be in the range 23.5-25 seconds. If time specifications are not met, then the results are not valid, and particle size analysis should be repeated. For any aperture, reliability of the results are compromised if cumulative population, CP9 on the 280 μm aperture, exceeds 10,000, since many particles would be missed in the counts due to coincidence.[35,38] For any aperture, actual mean aggregate size is cumulative volume to the respective channel divided by cumulative population to the same channel, multiplied by the volume conversion factor of the respective aperture.

As part of the illustrative example, 70 μm aperture data will be compared with the 280 μm aperture data presented earlier in this appendix. Clearly, one handles tapes from the 70 μm aperture the same way as from the 280 μm aperture, i.e. one can obtain cumulative populations and volumes as well as differential populations and volumes from print out. Consider the example data given below:

	CV8 = 147	CP8 = 62
	CV12 = 665	CP12 = 4870

Note, that platelets range from 1.59-4.0 μm in equivalent spherical diameter, i.e. channels 12 through 8 on the chart for 70 μm aperture. One also has to subtract out the background isoton diluent counts for both the volume and population.

Background:	CV8 = 165	CP8 = 6
	CV12 = 170	CP12 = 114

70 µm aperture used — Dilution factor

Dilution: 0.5 µl (0.0005 ml) into 20 ml of isoton;

$$\frac{20 + 0.0005}{0.0005} = \frac{20.0005}{0.0005} = 40{,}001$$

70 µm aperture conversion factor = 94.78 μm^3

70 µm aperture draws 0.5 ml (500 µl) of solution for particle size analysis.

total volume of free platelets = TV

total population of free platelets = platelet count = PC

$$TV = (CV12 - CV8) \times \frac{20.0005}{0.0005} \times \frac{94.78\ \mu m^3}{500\ \mu l \text{ of sample}}$$

$$PC = (CP12 - CP8) \times \frac{20.0005}{0.0005} \times \frac{1 \text{ platelet}}{500\ \mu l \text{ of sample}}$$

$$TV = (CV12 - CV8) \times 7582.6 \frac{\mu m^3}{\mu l \text{ of sample}}$$

$$PC = (CP12 - CP8) \times 80.002 \frac{\text{platelet}}{\mu l \text{ of sample}}$$

Before going to actual data, we have to subtract the background counts from sample counts.

sample (CV12 - CV8) = 518 — sample (CP12 - CP8) = 4808

background (CV12 - CV8) = 5 — background (CP12 - CP8) = 108

(CV12 - CV8) = 513 — (CP12 - CP8) = 4700

TV = 513 x 7582.6 = 3.890 x 10^6 $\mu m^3/\mu l$ of sample

PC = 4700 x 80.002 = 376,009 platelets/µl of sample

$$\text{Mean Platelet Size} = \frac{3.890 \times 10^6\ \mu m^3/\mu l \text{ of sample}}{376{,}009 \text{ platelets}/\mu l \text{ of sample}} = 10.345 \frac{\mu m^3}{\text{platelet}}$$

Note: Mean Platelet Size $= \frac{CV12 - CV8}{CP12 - CP8} \times 94.78 \frac{\mu m^3}{platelet}$

$$= \frac{513}{4700} \times 94.78 \frac{\mu m^3}{platelet}$$

$$\text{Mean Platelet Size} = 10.345 \frac{\mu m^3}{platelet}$$

In most cases, using the 70 μm aperture for total volume and population, 4 or 5 readings were averaged to give the TV (Vavail) and PC.

It should be pointed out that the conversion factors would be slightly different if we are dealing with the preliminary study designed to determine whether glutaraldehyde induces artifacts in free platelets. In this case, we had 21 ml of diluent, since 1 ml of glutaraldehyde was used to 20 ml of isoton.

Therefore dilution factor is $\frac{21 + 0.0005}{0.0005} = \frac{21.0005}{0.0005} = 42{,}001$

Volume Conversion factor $= 42{,}001 \times \frac{94.75}{500} = 7961.7$

Population Conversion factor $= 42{,}001 \times \frac{1}{500} = 84.002$

Of course, mean platelet size is always $(\frac{CV12 - CV8}{CP12 - CP8}) \times 94.78$, regardless of dilutions. For the most part, our conversion factors to obtain total volume, platelet count, and mean platelet size, from 70 μm aperture data is as follows:

$$\text{total volume} = TV = (CV12 - CV8) \times 7582.6 \frac{\mu m^3}{\mu l \text{ of sample}}$$

$$\text{platelet count} = PC = (CP12 - CP8) \times 80.002 \frac{\text{platelets}}{\mu l \text{ of sample}}$$

$$\text{mean platelet size} = (\frac{CV12 - CV8}{CP12 - CP8}) \times 94.78 \frac{\mu m^3}{platelet}$$

In our study, we always had 20 ml of isoton as the diluent, except in the experiment designed to decide whether glutaraldehyde introduces artifacts in free platelets or not.

Finally, to get % of available volume for aggregated particles, one takes the ratio of the cumulative volume of platelet aggregates on the 280 μm aperture to the total volume of free platelets on the 70 μm prior to aggregation. In the example discussed in this appendix;

CV = 3.20×10^6 $\mu m^3/\mu l$ of sample if nothing is added to aggregated sample--Case 1

CV = 3.36×10^6 $\mu m^3/\mu l$ of sample if 50 μl of 1% glutaraldehyde isoton, or 3% glutaraldehyde is added to the 1 ml of aggregated sample--Case 2

available volume = TV = 3.89×10^6 $\mu m^3/\mu l$ of sample.

Therefore in the 1st case;

% of available volume for aggregated particles =

$$\frac{CV}{TV} = \frac{3.20 \times 10^6 \ \mu m^3/\mu l \text{ of sample}}{3.89 \times 10^6 \ \mu m^3/\mu l \text{ of sample}} \times 100\% = 82.3\%$$

Analogously in the 2nd case;

% of available volume for aggregated particles =

$$\frac{CV}{TV} = \frac{3.36 \times 10^6 \ \mu m^3/\mu l \text{ of sample}}{3.89 \times 10^6 \ \mu m^3/\mu l \text{ of sample}} \times 100\% = 86.4\%$$

Conversion of volume data from the 70 μm aperture to actual volume in $\mu m^3/\mu l$ of sample is accomplished by taking (CV12 - CV8) and multiplying it by 7582.6. Also, one can convert this data to a 280 μm aperture

volume, by dividing this actual volume by 534.145 if nothing added to the sample, or by 560.852 if 50 µl of a solution (such as glutaraldehyde or isoton) is added to 1 ml of aggregated PRP. Here, TV = Vavail = 3.89×10^6 $\mu m^3/\mu l$ of sample

$$\text{Case 1, TV} = \frac{3.89 \times 10^6}{534.145} = 7282.7$$

$$\text{Case 2, TV} = \frac{3.89 \times 10^6}{560.852} = 6935.9$$

One can then take these available volume as 280 µm aperture volume, and calculate the % of Vavail for aggregated particles for the cumulative volumes directly from the 230 µm aperture data.

Here, CV9 = 5990,

for Case 1: % of Vavail for aggregated particles = $\frac{5990}{7282.7} = 82.3\%$

for Case 2: % of Vavail for aggregated particles = $\frac{5990}{6935.9} = 86.4\%$

Same percentages as calculated with the actual volumes.

Geometric Mean μ^3	Volume μ^3	Diameter μ	Channel (W)				
.00575	.004091	.198					
.0115	.008181	.250					
.0231	.01636	.315					
.0462	.03272	.397					
.0925	.06545	.500					
.1851	.1309	.630					
.3702	.2618	.794					
.7405	.5236	1.00	14				
1.481	1.047	1.26	13				14
2.962	2.094	1.59	12				13
5.924	4.189	2.00	11				12
11.85	8.378	2.52	10				11
23.70	16.76	3.17	9	14			10
47.39	33.51	4.00	8	13	14		9
94.78	67.02	5.04	7	12	13		8
189.6	134.0	6.35	6	11	12	14	7
379.1	268.1	8.00	5	10	11	13	6
758.3	536.2	10.08	4	9	10	12	5
1516.	1072.	12.7	3	8	9	11	4
3033.	2145.	16.0	2	7	8	10	3
6066.	4289.	20.2	1	6	7	9	2
12.13×10^3	8579.	25.4	0	5	6	8	1
24.27×10^3	17.16×10^3	32.0		4	5	7	0
48.54×10^3	34.31×10^3	40.3		3	4	6	
97.18×10^3	68.63×10^3	50.8		2	3	5	
194.4×10^3	137.3×10^3	64.0		1	2	4	
388.7×10^3	274.5×10^3	80.6		0	1	3	
777.4×10^3	549.0×10^3	101.6			0	2	
1.555×10^6	1.098×10^6	128.				1	
3.109×10^6	2.196×10^6	161.				0	
6.219×10^6	4.392×10^6	203					
12.44×10^6	8.784×10^6	256.					
24.88×10^6	17.57×10^6	322.					
49.75×10^6	35.14×10^6	406.					
99.50×10^6	70.27×10^6	512.					
199.0×10^6	140.6×10^6	645.					
398.0×10^6	281.1×10^6	812.					
795.0×10^6	562.2×10^6	1024.					
			1	2	3	4	

TABLE A: Particle Volumes and Equivalent Spherical Diameters for the Coulter Counter (model T, Coulter Electronics, Hialeah, Fla.)

Column 1 shows the channels and consequently the size range pertinent to the 70 µm aperture, whose sampling time is 25 seconds and sampling volume is 0.5 ml.

TABLE A (continued)

Column 2 shows the channels and consequently the size range pertinent to the 200 µm aperture, whose sampling time is 12.4 seconds and sampling volume is 2.0 ml.

Colume 3 shows the channels and consequently the size range pertinent to the 280 µm aperture, whose sampling time is 6.4 seconds and sampling volume is 2.0 ml.

Colume 4 shows the channels and consequently the size range pertinent to the 400 µm aperture, whose sampling time is 3.4 seconds and sampling volume is 2.0 ml.

APPENDIX B

STATISTICAL ANALYSIS

To determine the meaning of a set of numerical data, one performs a statistical analysis that includes calculating the arithmetic mean ($\overline{X}$), standard deviation (s.d.), and standard error of the mean (s.e.m.). The arithmetic mean is an indication of the average or central tendency of the data. For n samples, each with a value Xi, the mean ($\overline{X}$) is given by

$$\overline{X} = \frac{\sum_{i=1}^{n} Xi}{n}$$

The standard deviation is calculated by the formulae

$$\text{s.d.} = \sqrt{\frac{\sum_{i=1}^{n} (Xi - \overline{X})^2}{n - 1}} = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - (\sum_{i=1}^{n} Xi)^2/n}{n - 1}}$$

for a small number of samples (small n). As n gets very large, the s.d. can be given by

$$\text{s.d.} = \sqrt{\sum_{i=1}^{n} (Xi - \overline{X})^2/n}$$

For values in the interval $\overline{X} \pm$ s.d., 68% of the population (samples) can be expected to occur, 95% of the population can be found in the

interval $\overline{X} \pm 2$ s.d., and 99% of the population can be found in the interval $\overline{X} \pm 3$ s.d.

For all parameters measured in this thesis, the standard error of the mean (s.e.m.) was used to denote the error. The s.e.m. is given by s.e.m. = $^{s.d.}/_{\sqrt{n}}$. The s.e.m. is more desirable to the s.d., since it places less importance on extreme values and estimates the distribution for the "parent population" from which the sample was derived. The calculations for individual response variables could include algebraic additions, subtractions, multiplications, and divisions. To calculate s.e.m. for u + v, u - v, uv, or $^{u}/_{v}$,

$$\text{s.e.m.} = [\text{s.e.m.}^2(u) + \text{s.e.m.}^2(v)]^{1/2}.$$

This accounts for larger variance in the mean aggregate size (M.A.S.) than in the cumulative population (CP) or cumulative volume (CV), since M.A.S. = $^{CV}/_{CP}$.

To determine whether 2 sample means (each with its own s.e.m.) are significantly different, a student t test is carried out. The t-value is given by

$$t = \frac{\overline{X}_{(u)} - \overline{X}_{(v)}}{[\text{s.e.m.}^2(u) + \text{s.e.m.}^2(v)]^{1/2}}$$

In particular, the paired t test, which was used in this thesis, the t-value is given by

$$t = \frac{\overline{X}_{(u)} - \overline{X}_{(v)}}{\text{s.e.m.}_{(u-v)}} = \frac{\overline{X}_{(u)} - \overline{X}_{(v)}}{\text{s.d.}_{(u-v)}/\sqrt{n}}$$

where n is the sample size of both u and v. Standard charts are available listing the significance level (p-value) as a function of t and n. Generally, if $t < 2$ then the difference is not significant. When the difference between means is less than or equal to the sum of the standard errors of the mean, the $t < 1.5$ and the difference is not significant. Therefore overlapping error bars for the data plotted can be interpreted as no significant difference between the means.

The statistical comparisons here used the paired t test. The test determines whether the difference observed between sets of data can be attributed to random variation alone. Since there always exists the possibility that chance variations account for even large observed differences, a significance level is chosen corresponding to the t-test. A significance level of $p < 0.05$ means that there is less than a 5 percent chance that the observed difference is due merely to random variation, i.e. 95 out of 100 such comparisons will have authentic (true) data differences.

APPENDIX C

SURVEY OF DATA FOR PRINCIPAL RESULTS

The following data were obtained during a series of experiments using various isoton-glutaraldehyde counting solutions. The PRP was aggregated with an ADP concentration of 2 μM. No fixative was added to the aggregated PRP prior to dilution. The sample numbers correspond to repeated runs (starting with ADP addition) on the same PRP specimen. The time denotes time after dilution in isoton containing the indicated amount of fixative. The volume available for aggregation figures are from platelet counts with the 70 μm aperture on unaggregated PRP.

Treatment	Final Glutaraldehyde Concentration (wt.%)
a) 31.5 ml of isoton	0%
b) 31.5 ml of isoton + 3 drops of zap isoton	0%
c) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde	0.048%
d) 30 ml of isoton + 1.5 ml of 13% glutaraldehyde	0.619%

Donor DKG Date: 1/29/80

CUMULATIVE VOLUMES TO CHANNEL 9(CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	mean ± s.e.m., n=4 CV9	$CV(\frac{\mu m^3}{\mu l})X10^{-6}$
a	0	3573	3698	3530	3621	3606±36	1.926±0.019
	1	1782	plug	2688	2236	(n=3) 2235±262	1.194±0.14
	2	--	--	--	--	--	--
	3	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--
b	0	3016	5125	3381	3775	3824±461	2.043±0.246
	1	3058	3557	2777	3406	3200±176	1.709±0.094
	2	3570	3761	3006	3347	3421±162	1.827±0.087
	3	3219	2890	3207	3869	3296±206	1.761±0.110
	(30-90)Late	914	1232	1389	1075	1153±102	0.616±0.054
c	0	3633	4230	4053	4402	4080±165	2.179±0.088
	1	3911	3776	4164	3232	3771±197	2.014±0.105
	2	3563	3374	3568	4054	3640±146	1.944±0.078
	3	3843	3877	4032	3817	3892±46	2.079±0.025
	(30-90)Late	4172	4484	3578	4453	4172±210	2.228±0.112
d	0	4752	4043	5271	4000	4517±305	2.413±0.163
	1	4420	4103	3490	3531	3886±227	2.076±0.121
	2	4261	3547	3809	3884	3875±148	2.070±0.079
	3	3965	3983	4584	3732	4066±182	2.172±0.097
	(30-90)Late	5116	4717	4438	4451	4681±159	2.500±0.085

Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l} X10^{-6}$) → (2.290±0.089)

Actual Cumulative Volume = CV = CV9 X 534.145.

Donor: DKG Date: 1/29/80

CUMULATIVE POPULATIONS TO CHANNEL 9 (CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	mean ± s.e.m., n=4 CP9	CP(Aggregates/µl)
a	0	564	699	577	723	641±41	56.5±3.6
	1	3133	plug	2284	2462	2626±259(n=3)	231.4±22.8
	2	--	--	--	--	--	--
	3	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--
b	0	549	749	619	729	662±47	58.3±4.2
	1	741	826	680	867	779±42	68.6±3.7
	2	864	836	733	828	815±29	71.8±2.5
	3	794	774	740	856	791±25	69.7±2.2
	(30-90)Late	519	631	619	579	587±26	51.7±2.3
c	0	515	689	634	763	650±52	57.3±4.6
	1	597	567	615	591	593±10	52.2±0.9
	2	509	568	548	640	566±28	49.9±2.4
	3	563	595	691	632	621±28	54.7±2.4
	(30-90)Late	598	589	491	598	569±26	50.1±2.3
d	0	412	442	495	443	448±18	39.5±1.6
	1	427	404	379	417	407±11	35.9±1.0
	2	457	471	439	421	447±11	39.4±1.0
	3	466	582	553	468	517±30	45.5±2.6
	(30-90)Late	603	614	547	571	583±16	51.5±1.4

Actual cumulative population = CP = CP9 x 0.0881.

Donor: DKG Date: 1/29/80

$$\frac{CV9}{CP9} = M.A.S. \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	mean s.e.m., n=4 ($^{CV9}/_{CP9}$)
a	0	6.34	5.29	6.12	5.01	5.69±0.32
	1	0.57	plug	1.18	0.91	0.89±0.18(n=3)
	2	--	--	--	--	--
	3	--	--	--	--	--
	(30-90)Late	--	--	--	--	--
b	0	5.49	6.84	5.46	5.18	5.74±0.37
	1	4.13	4.31	4.08	3.93	4.11±0.08
	2	4.13	4.50	4.10	4.04	4.19±0.11
	3	4.05	3.73	4.33	4.52	4.16±0.17
	(30-90)Late	1.76	1.95	2.24	1.86	1.95±0.11
c	0	7.05	6.14	6.39	5.77	6.34±0.27
	1	6.55	6.66	6.77	5.47	6.36±0.30
	2	7.00	5.94	6.51	6.33	6.44±0.22
	3	6.83	6.52	5.84	6.04	6.31±0.23
	(30-90)Late	6.98	7.61	7.29	7.45	7.33±0.14
d	0	11.53	9.15	10.65	9.03	10.09±0.61
	1	10.35	10.16	9.21	8.47	9.55±0.44
	2	9.32	7.53	8.68	9.23	8.69±0.41
	3	8.51	6.84	8.29	7.97	7.90±0.37
	(30-90)Late	8.48	7.68	8.11	7.80	8.02±0.18

Actual mean aggregate size = M.A.S. = $\frac{CV9}{CP9}$ X 6066.

Donor: DKG Date: 1/29/80

70 μm aperture data

Available Volumes (Vavail) and Platelet Counts (PC)
Note: Δ = CP12-CP8 or CV12-CV8 and the backgrounds are subtracted out.
$\overline{\Delta}$ = Δ average, where there were 2 readings for that sample (not the same sample on the 280 μm aperture).
$\overline{\Delta}_b$ denotes $\overline{\Delta}$ for background counts.
*See Appendix A.

	CP9	CP12	Δ	CV8	CV12	Δ
Background	6	260	254	39	49	10
	5	130	125	169	175	6

$\overline{\Delta}_b$ = 190 $\overline{\Delta}_b$ = 8

	CP8	CP12	Δ	$\Delta-\overline{\Delta}_b$	CV8	CV12	Δ	$\Delta-\overline{\Delta}_b$
Sample 1	32	3590	3558	3368	26	354	328	320
Sample 2	25	3503	3478	3288	284	598	314	306
Sample 3	31	3223	3192	3002	172	460	288	280
(mean ± s.e.m., n=3)				3219.3±110.1				302.0±11.7

PC = (3219.3±110.1) X *30.002 = 257,553±8,888 platelets/μl of sample.
Vavail = (302.0±11.7) X *7582.6 = (2.290±0.089) X 10^6 μm^3/μl of sample.

The following data were obtained during a series of experiments using various isoton-glutaraldehyde counting solutions (diluents). The PRP was aggregated with a final ADP concentration of 0.5 μM. No fixative was added to aggregated PRP prior to dilution. The sample numbers correspond to repeated runs (starting with ADP addition) on the same PRP specimen, except as indicated, different donors were used on different days. The time denotes time after dilution in isoton containing the indicated amount of fixative. The volume available for aggregation figures are from platelet counts with the 70 μm aperture on unaggregated PRP.

Treatment	Final Glutaraldehyde Concentration (wt.%)
a) 31.5 ml of isoton	0%
b) 30 ml of isoton + 1.5 ml of 6% glutaraldehyde	0.286%
c) 30 ml of isoton + 1.5 ml of 10.5% glutaraldehyde	0.500%
d) 30 ml of isoton + 1.5 ml of 13% glutaraldehyde	0.619%
e) 30 ml of isoton + 1.5 ml of 21% glutaraldehyde	1.000%
f) 30 ml of isoton + 1.5 ml of 25% glutaraldehyde	1.190%

Donors: DKG (Samples 1-3 on 2/7/80); DKG (Samples 4-6 on 2/14/80); TKB (Samples 7-9 on 2/21/80)

Cumulative Volumes to Channel 9(CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	mean ± s.e.m., n=9 CV9	mean ± s.e.m., n=9 $CV(\frac{\mu m^3}{\mu l})X10^{-6}$	mean ± s.e.m., *n=5 CV9	mean ± s.e.m., *n=5 $CV(\frac{\mu m^3}{\mu l})X10^{-6}$
a	0	5131	4889	4578	5366	5310	5137	3805	4175	3694	4676±215	2.498±0.115	4424±337	2.363±0.180
	1	2459	1637	2142	2140	2450	1595	2864	2114	1375	2086±160	1.114±0.085	2080±272	1.111±0.145
	2	--	--	--	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--	--	--	--
b	0	6521	6078	5965	6225	6360	6375	4257	5034	4791	5734±274	3.063±0.146	5363±429	2.865±0.229
	1	6434	6138	5690	6519	6086	6253	4845	4910	5319	5799±213	3.098±0.114	5483±293	2.929±0.157
	2	6195	5944	5606	6391	6242	6462	5242	4795	5102	5775±204	3.085±0.109	5569±330	2.975±0.176
	3	6298	6036	5808	5846	6247	6547	5455	5230	5174	5849±162	3.124±0.087	5731±280	3.061±0.150
	(30-90)Late	*	*	*	*	6446	7262	5730	5289	5186	*	*	6083±430	3.249±0.230
c	0	6383	6129	5917	6409	6440	6393	5234	5212	5199	5924±186	3.164±0.099	5696±294	3.042±0.157
	1	5821	5791	5761	6869	6347	6309	4911	5086	5230	5792±215	3.094±0.115	5577±311	2.979±0.166
	2	6096	6061	5929	6533	6318	6503	5106	4932	5361	5871±199	3.136±0.106	5644±322	3.015±0.172
	3	5920	6913	6334	6474	6352	6406	4977	4833	5328	5949±244	3.178±0.130	5579±336	2.980±0.179
	(30-90)Late	*	*	*	*	7333	6917	5254	5300	5313	*	*	6023±455	3.217±0.243
	Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l} X 10^{-6}$) →										(2.907±0.112)		(2.698±0.140)	

Actual Cumulative Volume = CV = CV9 X 534.145.

*This is the 5 out of the 9 samples, where the late readings were taken, i.e. Samples 5-9.

Donors: DKG (Samples 1-3 on 2/1/80); DKG (Samples 4-6 on 2/14/80); TKB (Samples 7-9 on 2/21/80).

Treatment	Time (min.)	Cumulative Volumes to Channel 9(CV9) Sample Number 1	2	3	4	5	6	7	8	9	mean ± s.e.m., n=9 CV9	mean ± s.e.m., n=9 $CV(\frac{\mu m^3}{\mu l})X10^{-6}$	mean ± s.e.m., n=5* CV9	mean ± s.e.m., n=5* $CV(\frac{\mu m^3}{\mu l})X10^{-6}$
d	0	6788	6167	5846	6432	6742	6241	5120	4895	5316	5950±233	3.178±0.124	5663±354	3.025±0.189
	1	6292	6084	5793	6467	6371	6226	5393	4884	5376	5876±183	3.139±0.098	5650±281	3.018±0.150
	2	6297	5996	5611	6057	6282	6408	5224	5458	4698	5781±192	3.088±0.103	5614±323	2.999±0.173
	3	6105	6624	5576	6578	6612	6290	5553	4791	5213	5927±224	3.166±0.120	5692±336	3.040±0.179
	(30-90)Late	*	*	*	*	6786	6777	5407	4914	5453	*	*	5867±385	3.134±0.206
e	0	6678	5588	6417	6344	6670	6506	5057	5312	4755	5925±250	3.165±0.134	5660±390	3.023±0.208
	1	6111	5820	5521	6638	6298	6416	4894	4738	4967	5711±238	3.051±0.127	5463±368	2.918±0.197
	2	6206	5795	5765	6317	6288	6447	4716	4904	4973	5712±226	3.051±0.121	5466±372	2.920±0.199
	3	6229	5490	5599	6596	6968	6225	4597	5492	5041	5804±253	3.100±0.135	5665±423	3.026±0.226
	(30-90)Late	*	*	*	*	6912	6632	5332	5424	5590	*	*	5774±370	3.084±0.198
f	0	6089	5767	5901	6589	6705	6617	5244	5223	4927	5896±221	3.149±0.118	5743±379	3.068±0.202
	1	6289	5712	5771	6713	6427	6371	5343	5166	4655	5827±227	3.112±0.121	5592±348	2.987±0.186
	2	6171	5982	5782	6798	6525	6465	5141	4861	5146	5875±231	3.138±0.123	5628±358	3.006±0.191
	3	6474	5617	5786	6340	7103	6725	4926	5444	5023	5937±254	3.171±0.136	5844±449	3.122±0.240
	(30-90)Late	*	*	*	*	6872	6627	5532	5642	5169	*	*	5968±330	3.188±0.176
Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l}$ X 10^{-6} →												(2.907±0.112)		(2.698±0.140)

Actual Cumulative Volume = CV = CV9 X 534.145.

*This is the 5 out of the 9 samples, where the late readings were taken, i.e. Samples 5-9.

Donors: DKG (Samples 1-3 on 2/7/80); DKG (Samples 4-6 on 2/14/80); TKB (Samples 7-9 on 2/21/80).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	mean ± s.e.m., n=9 CP9	CP (aggregates/µl)	mean ± s.e.m., n=5* CP9	CP (aggregates/µl)
a	0	3107	4675	7094	4191	4802	6407	3470	2759	3983	4499±484	396±43	4284±627	377±55
	1	5467	4505	5547	5573	2450	4785	7082	4825	4042	4920±423	433±37	4637±747	409±66
	2	--	--	--	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--	--	--	--
b	0	3961	3905	6040	3475	3866	4786	1385	1821	2516	3528±484	311±43	2875±636	253±56
	1	3678	3979	5828	3549	3887	4779	1356	1693	2576	3481±474	307±42	2858±650	252±57
	2	3724	3911	5829	3471	3847	4807	1371	1703	2557	3469±473	306±42	2857±648	252±57
	3	3761	3898	5829	3380	3840	4919	1502	1770	2668	3507±464	304±41	2940±642	259±57
	(30-90)Late	*	*	*	*	3855	4854	1473	1782	2599	*	*	2913±637	257±56
c	0	2274	4121	5563	3065	3626	4543	1515	2268	2576	3283±430	289±38	2906±531	256±47
	1	2206	4034	5599	3122	3611	4559	1429	2312	2609	3276±436	289±38	2904±541	256±48
	2	2235	4120	5547	3024	3606	4569	1468	2255	2702	3281±432	289±38	2920±538	257±47
	3	2242	4256	5103	3000	3658	4541	1391	2222	2620	3226±413	284±36	2886±551	254±49
	(30-90)Late	*	*	*	*	3935	4470	1440	2231	2561	*	*	2927±558	258±49

Actual Cumulative Population = CP = CP9 X 0.0881.

*This is the 5 out of the 9 samples, where the late readings were taken, i.e. Samples 5-9.

Donors: DKG (Samples 1-3 on 2/7/80); DKG (Samples 4-6 on 2/14/80); TKB (Samples 7-9 on 2/21/80).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	mean ± s.e.m., n=9 CP9	CP (aggregates/µl)	mean ± s.e.m., n=5* CP9	CP (aggregates/µl)
d	0	2802	4415	7296	3912	4780	5361	1618	1700	2093	3775±636	333±56	3110±809	274±71
	1	2677	4506	7316	3864	4644	5392	1659	1756	2049	3763±637	332±56	3100±795	273±70
	2	2700	4381	6950	3885	4477	5483	1624	1788	1964	3697±611	326±54	3071±801	271±71
	3	2678	4569	7148	3932	4787	5664	1671	1723	2071	3805±639	335±56	3183±848	280±75
	(30-90)Late	*	*	*	*	4589	5375	1586	1699	2062	*	*	3062±797	270±70
e	0	3280	4353	5318	3117	4449	5222	1882	2226	3680	3725±407	328±36	3292±638	290±56
	1	3124	4419	4961	3246	4373	5172	1821	1974	3710	3644±404	321±36	3230±622	285±55
	2	3057	4520	5180	3181	4346	5199	1853	2062	3517	3657±414	322±36	3202±615	282±54
	3	3192	4332	4993	3294	4390	5134	1806	2199	3665	3667±388	323±34	3240±607	285±54
	(30-90)Late	*	*	*	*	4419	5260	1815	2356	3691	*	*	3298±615	290±54
f	0	3518	5578	5297	3617	4092	5095	1874	1890	2170	3681±488	324±43	3024±662	266±58
	1	3324	5517	5072	3711	4005	5180	1957	1946	2065	3642±477	321±42	3031±665	267±59
	2	3488	5505	5040	3710	4055	5058	1948	1896	2236	3660±464	322±41	3039±642	268±57
	3	3622	5368	5047	3740	4167	5239	1912	2000	2196	3699±465	326±41	3103±677	273±60
	(30-90)Late	*	*	*	*	3928	4994	1946	1952	2196	*	*	3003±620	265±55

Actual Cumulative Population = CP = CP9 X 0.0881.

*This is the 5 out of the 9 samples, where the late readings were taken, i.e. Samples 5-9.

Donors: DKG (Samples 1-3 on 2/7/80); DKG (Samples 4-6 on 2/14/80); TKB (Samples 7-9 on 2/21/80).

CV9/CP9 = M.A.S. (μm^3) ÷ 6066

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	mean ± s.e.m., n=4	mean ± s.e.m., n=5*
a	0	1.65	1.05	0.65	1.28	1.11	0.80	1.10	1.51	0.93	1.12±0.11	1.09±0.12
	1	0.45	0.36	0.39	0.38	0.42	0.33	0.40	0.44	0.34	0.39±0.01	0.39±0.02
	2	--	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--	--
b	0	1.65	1.56	0.99	1.79	1.65	1.33	3.07	2.76	1.90	1.86±0.22	2.14±0.33
	1	1.75	1.54	0.98	1.84	1.57	1.31	3.57	2.90	2.06	1.95±0.27	2.28±0.42
	2	1.66	1.52	0.96	1.84	1.62	1.34	3.82	2.82	2.00	1.95±0.29	2.32±0.45
	3	1.67	1.55	1.00	1.73	1.63	1.33	3.63	2.95	1.94	1.94±0.28	2.30±0.43
	(30-90)Late	*	*	*	*	1.80	1.50	3.89	2.97	2.00	*	2.43±0.44
c	0	2.81	1.49	1.06	2.09	1.78	1.41	3.45	2.30	2.02	2.05±0.25	2.19±0.35
	1	2.64	1.44	1.03	2.20	1.76	1.38	3.44	2.20	2.00	2.01±0.24	2.16±0.35
	2	2.73	1.47	1.07	2.16	1.75	1.42	3.48	2.19	1.98	2.03±0.25	2.16±0.35
	3	2.64	1.62	1.24	2.16	1.74	1.41	3.58	2.18	2.03	2.07±0.24	2.19±0.37
	(30-90)Late	*	*	*	*	1.86	1.55	3.65	2.38	2.07	*	2.30±0.36

Actual mean aggregate size = M.A.S. = $\frac{CV9}{CP9}$ X 6066

*This is the 5 out of the 9 samples, where the late readings were taken, i.e. Samples 5-9.

Donors: DKG (Samples 1-3 on 2/7/80); DKG (Samples 4-6 on 2/14/80); TKB (Samples 7-9 on 2/21/80).

$CV9/CP9 = M.A.S.\ (\mu m^3) \div 6066$

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	mean ± s.e.m., n=9	mean ± s.e.m., n=5*
d	0	2.42	1.40	0.80	1.64	1.41	1.16	3.16	2.88	2.54	1.93±0.28	2.23±0.40
	1	2.35	1.35	0.79	1.67	1.37	1.15	3.25	2.78	2.62	1.93±0.28	2.23±0.41
	2	2.33	1.37	0.81	1.56	1.40	1.17	3.22	3.05	2.39	1.92±0.29	2.25±0.42
	3	2.28	1.45	0.78	1.67	1.38	1.11	3.32	2.78	2.52	1.92±0.28	2.22±0.42
	(30-90)Late	*	*	*	*	1.48	1.26	3.41	2.89	2.64	*	2.34±0.42
e	0	2.04	1.28	1.21	2.04	1.50	1.25	2.69	2.39	1.29	1.74±0.19	2.03±0.30
	1	1.96	1.32	1.11	2.04	1.44	1.24	2.69	2.40	1.34	1.73±0.19	2.02±0.29
	2	2.03	1.28	1.11	1.99	1.45	1.24	2.55	2.38	1.41	1.72±0.18	1.99±0.27
	3	1.95	1.27	1.12	2.00	1.59	1.21	2.55	2.50	1.38	1.73±0.18	2.04±0.27
	(30-90)Late	*	*	*	*	1.56	1.26	2.94	2.30	1.29	*	2.05±0.31
f	0	1.73	1.03	1.11	1.82	1.64	1.30	2.80	2.76	2.27	1.83±0.22	2.15±0.30
	1	1.89	1.04	1.14	1.81	1.60	1.23	2.73	2.65	2.25	1.82±0.21	2.09±0.30
	2	1.77	1.09	1.15	1.83	1.61	1.28	2.64	2.56	2.30	1.80±0.20	2.08±0.27
	3	1.79	1.05	1.15	1.70	1.70	1.28	2.58	2.72	2.29	1.81±0.20	2.11±0.27
	(30-90)Late	*	*	*	*	1.75	1.33	2.84	2.89	2.35	*	2.23±0.30

Actual mean aggregate size = $M.A.S. = \frac{CV9}{CP9} \times 6066.$

*This is the 5 out of the 9 samples, where the late readings were taken, i.e. Samples 5-9.

Donors: DKG, DKG, TKB
Dates: 2/7/80, 2/14/80, 2/21/80

70 μm aperture data

Available Volumes (Vavail) and Platelet Counts (PC)

Note: Δ = CP12-CP8 or CV12-CV8 and the backgrounds are subtracted out. $\overline{\Delta}$ = Δ average, where there were 2 readings for that sample (not the same sample on the 280 μm aperture). *See Appendix A. $\overline{\Delta}_b$ denotes $\overline{\Delta}$ for the background counts.

2/7/80 - 1 reading per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	5	60	55	5	8	3
	2	29	27	2	4	2

$\overline{\Delta}_b$ = 41 $\overline{\Delta}_b$ = 2.5

	CP8	CP12	Δ	$\Delta-\overline{\Delta}_b$	CV8	CV12	Δ	$\Delta-\overline{\Delta}_b$
Sample 1	82	5518	5436	5395	63	539	476	473.5
Sample 2	61	5788	5727	5686	115	589	474	471.5
Sample 3	112	4489	4377	4336	110	511	401	398.5
Sample 4	85	4675	4590	4549	56	469	413	410.5
Sample 5	233	4221	3988	3947	586	951	365	362.5
(mean ± s.e.m., n=5)				4783±327				423.3±21.6

PC = (4783 ± 327) X *80.002 = 382,650 ± 26,189 platelets/μl of sample.

Vavail = (423.3 ± 21.6) X *7582.6 = (3.210 ± 0.164) X 10^6 μm^3/μl of sample.

2/14/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	5	159	154	3	14	11
	10	88	78	16	21	5

$\overline{\Delta}_b$ = 116 $\overline{\Delta}_b$ = 8

2/14/80 (continued)

	CP8	CP12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$	CV8	CV12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$
Sample 1	40	4547	4507	4522.5	4406.5	302	719	417	416.5	408.5
	38	4576	4538			104	520	416		
Sample 2	62	3929	3867	3889	3773	354	721	367	367	359.0
	47	3958	3911			185	552	367		
Sample 3	48	4803	4755	4808	4692	81	524	443	444	436.0
	57	4918	4861			180	625	445		
(mean ± s.e.m., n=3)					4290.5±271.6					401.2±22.5

PC = (4290.5±271.6) X *80.002 = 343,249±21,728 platelets/µl of sample.

Vavail = (401.2±22.5) X *7582.6 = (3.042±0.171) X $10^6 \mu m^3/\mu l$ of sample.

2/21/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	4	34	30	3	5	2
	5	24	19	21	22	1
		$\bar{\Delta}_b$ = 24.5			$\bar{\Delta}_b$ = 1.5	

	CP8	CP12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$	CV8	CV12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$
Sample 1	90	3158	3068	3131.5	3107	76	383	307	315	313.5
	67	3212	3195			194	517	323		
Sample 2	108	3189	3081	3055	3030.5	490	792	302	297.5	296.0
	99	3128	3029			219	512	293		
Sample 3	217	3629	3412	3428.5	3404	1627	1970	343	348.5	347.0
	138	3583	3445			145	499	354		
Sample 4	112	3458	3346	3391	3366.5	481	820	339	347.5	346.0
	94	3530	3436			208	564	356		
(mean ± s.e.m., n=4)					3227±93					325.6±12.6

PC = (3227±93) X *80.002 = 258,166±7,440 platelets/µl of sample.

Vavail = (325.6±12.6) X *7582.6 = (2.469±0.096) X $10^6 \mu m^3/\mu l$ of sample.

Average Available Volumes for Comparison with Average Cumulative Volumes of Aggregates on the 280 µm Aperture:

**late readings were included

9 sample average	5 sample average**
2/7/80 3.210×10^6 (n=3)	2/14/80 3.042×10^6 (n=2)
2/14/80 3.042×10^6 (n=3)	2/21/80 2.469×10^6 (n=3)
2/21/80 2.469×10^6 (n=3)	(mean ± s.e.m., n=5)
(mean ± s.e.m., n=9)	$(2.698 \pm 0.140) \times 10^6 \mu m^3$ / µl of sample
$(2.907 \pm 0.112) \times 10^6$ $\mu m^3/\mu l$ of sample	

These available volumes from each day are averaged in, according to their relative proportion of the total sample size of the 280 µm aperture data. The daily standard errors of available volumes serve the sole purpose of demonstrating the reliability of that particular volume, but plays no part in the standard error of the average available volume.

The following data were obtained during a series of experiments using various isoton-glutaraldehyde counting solutions (diluents). A final ADP concentration of 0.5 μM was used to aggregate PRP. No fixative was added to aggregated PRP prior to dilution. The sample numbers correspond to repeated runs (starting with ADP addition) on the same PRP specimen, except as indicated, different donors were used on different days. The time denotes time after dilution in isoton continuing the indicated amount of fixative. The volume available for aggregation figures are from platelet counts with the 70 μm aperture on unaggregated PRP.

Treatment	Final Glutaraldehyde Concentration (wt.%)
a) 31.5 ml of isoton	0
b) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde	0.048%
c) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde	0.143%
d) 30 ml of isoton + 1.5 ml of 6% glutaraldehyde	0.286%

Donors: DKG (Samples 1-5 on 2/28/80); AR (Samples 6-9 on 3/7/80); RAH (Samples 10-15 on 3/13/80).

Cumulative Volumes to Channel 9 (CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9
a	0	4335	3650	1967	1522	1870	2593	2900	3098	2845
	1	962	505	171*	147*	99*	639	1233	1371	1041
	2	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--
b	0	4548	3715	4142	2768	2668	2955	3424	3421	3326
	1	4782	3945	3885	3048	2767	3024	3469	3274	3301
	2	4906	4950	4000	2873	2718	3159	3288	3530	3295
	3	4862	4390	4297	2797	2732	3406	3616	3478	3257
	(30-90)Late	6700	5067	5119	3399	3299	3815	4404	4085	3903
c	0	5331	4702	3399	3317	2064	3505	3659	3951	3492
	1	5323	4698	3343	3206	1894	3815	3643	3848	3547
	2	5288	4738	3343	3435	2068	3942	3703	3896	3689
	3	5231	4777	3252	3355	1848	4002	3618	4488	3628
	(30-90)Late	5968	5405	3685	3509	1971	4104	3930	3906	3650
d	0	5029	4966	3843	2813	2574	3889	3886	3759	3765
	1	5013	5082	3780	2787	2777	3840	3722	3632	3584
	2	5038	5185	3859	2789	2792	3656	3721	3919	3685
	3	5245	4963	4090	2792	2478	3769	3991	3679	3696
	(30-90)Late	5502	5544	4208	3183	2557	4004	4434	3706	3815

Cumulative Volumes to Channel 9(CV9) (continued)

Treatment	Time (min.)	Sample Number 10	11	12	13	14	15	mean ± s.e.m., n=15 CV9	$CV(\frac{\mu m^3}{\mu l}) \times 10^{-6}$
a	0	5482	5770	5518	5678	5988	5446	3911±418	2.089±0.223
	1	2038	2504	2084	2049	2641	2022	1300±227	0.694±0.121
	2	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--
b	0	6095	6380	6080	6125	6301	6645	4573±391	2.443±0.209
	1	6022	5999	6169	5885	6319	6155	4536±361	2.423±0.193
	2	6359	6260	6275	6337	6565	6216	4715±389	2.518±0.208
	3	6595	6549	6710	6233	6549	6680	4810±407	2.569±0.217
	(30-90)Late	8543	8303	8606	8203	--	7811	5804±563(n=14)	3.100±0.301
c	0	6237	6515	6444	6487	6564	6633	4840±411	2.585±0.220
	1	7171	6788	6845	6901	6928	7014	4998±464	2.670±0.248
	2	7032	6847	6737	7007	6743	6767	5016±439	2.679±0.234
	3	6600	6969	7165	6748	6992	7460	5075±463	2.711±0.247
	(30-90)Late	too cloudy	7802	8004	7407	7997	7546	5349±553(n=14)	2.857±0.295
d	0	7241	7104	7222	6887	6736	7345	5137±456	2.744±0.244
	1	7232	7115	7223	7070	6994	6846	5113±458	2.731±0.245
	2	7156	7250	7316	6962	6948	6910	5146±455	2.749±0.243
	3	7032	7347	7343	6974	6975	6821	5146±458	2.749±0.245
	(30-90)Late	8130	8391	8267	7927	7543	7938	5677±550	3.032±0.294

Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l} \times 10^{-6}$) → (2.805±0.220)

*In these situations, mean aggregate size is meaningless.

Actual Cumulative Volume = CV = CV9 X 534.145.

Donors: DKG (Samples 1-5 on 2/28/80); AR (Samples 6-9 on 3/7/80); RAH (Samples 10-15 on 3/13/80).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9
a	0	5250	7328	5568	4629	5405	3373	2864	3561	3568
	1	2335	1779	*235	*146	*225	1615	3220	3404	2913
	2	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--
b	0	6123	8406	8805	7792	7587	2150	2830	3123	3052
	1	6088	8431	8570	7641	7632	2117	2842	3103	3054
	2	6048	8446	8501	7702	7685	2100	2747	3077	3082
	3	6024	8517	8603	7764	7862	2161	2862	3061	3050
	(30-90)Late	6409	9161	9338	8917	8620	2088	2862	3139	3176
c	0	6170	8125	8637	8683	6026	2633	2518	2685	3246
	1	5942	8029	8618	8658	6106	2677	2439	2664	3269
	2	5979	7969	8490	8667	6124	2726	2481	2737	3202
	3	5927	8097	8474	8708	6034	2821	2517	2844	3241
	(30-90)Late	6089	8039	8726	8891	6159	2635	2535	2732	3327
d	0	6995	7535	8783	7927	7606	2394	2365	3544	3089
	1	6873	7746	8623	7965	7776	2434	2161	3489	2935
	2	6858	7873	8564	7872	7782	2267	2241	3581	3062
	3	6864	7637	8646	7790	7521	2454	2407	3633	3046
	(30-90)Late	6990	7817	8869	8241	7451	2425	2512	3564	3131

Cumulative Populations to Channel 9(CP9)(continued)

Treatment	Time (min.)	Sample Number 10	11	12	13	14	15	mean s.e.m., n=15 CP9	CP($\frac{\text{aggregates}}{\mu l}$)
a	0	6603	6247	6237	7329	8316	8861	5676±476	500±42
	1	5872	6804	5969	5955	7287	6062	3588±656	316±58
	2	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--
b	0	6208	6242	5819	7026	7659	7682	6033±569	532±50
	1	6251	5380	5813	6712	7671	7715	5935±563	523±50
	2	6253	5308	5586	6667	7766	7543	5901±566	520±50
	3	6071	5273	5729	6611	7543	7669	5920±568	522±50
	(30-90)Late	6157	5369	5857	6814	--	7758	6119±671(n=14)	539±59
c	0	4770	5009	5043	8071	6879	7747	5749±583	506±51
	1	4612	4980	5034	8051	6797	7960	5722±585	504±52
	2	4382	5103	4890	7999	6778	7652	5679±574	500±51
	3	4382	5079	5005	8057	6745	7814	5716±573	504±51
	(30-90)Late	too cloudy	5172	5150	8191	7035	7654	5881±624(n=14)	518±55
d	0	6032	4956	5078	5629	6374	7914	5748±548	506±48
	1	6021	5018	5040	5576	6475	7750	5725±557	504±49
	2	5901	4940	5068	5599	6342	7779	5715±553	503±49
	3	5963	4994	5084	5483	6214	7812	5703±534	502±47
	(30-90)Late	6088	5397	5281	5843	6410	7887	5860±548	516±48

Actual Cumulative Population = CP = CP9 X 0.0881.

*In these situations, mean aggregate size is meaningless.

Donors: DKG (Samples 1-5 on 2/28/80); AR (Samples 6-9 on 3/7/80); RAH (Samples 10-15 on 3/13/80).

$$CV9/CP9 = M.A.S.\ (\mu m^3) \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	mean ± s.e.m., n=15 CV9/CP9
a	0	0.83	0.50	0.35	0.33	0.35	0.77	1.01	0.87	0.80	0.83	0.92	0.88	0.77	0.72	0.61	0.70±0.06
	1	0.41	0.28	0.73*	1.00*	0.44*	0.40	0.38	0.40	0.36	0.35	0.37	0.35	0.34	0.36	0.33	0.36±0.01
	2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
b	0	0.74	0.44	0.47	0.36	0.35	1.37	1.21	1.10	1.09	0.98	1.02	1.04	0.87	0.82	0.87	0.85±0.08
	1	0.79	0.47	0.45	0.40	0.36	1.43	1.22	1.06	1.08	0.96	1.11	1.06	0.88	0.82	0.80	0.86±0.08
	2	0.81	0.59	0.47	0.37	0.35	1.50	1.20	1.15	1.07	1.02	1.18	1.12	0.95	0.85	0.82	0.89±0.09
	3	0.81	0.52	0.50	0.36	0.35	1.58	1.26	1.14	1.07	1.09	1.24	1.17	0.94	0.87	0.87	0.92±0.09
	(30-90)Late	1.05	0.55	0.55	0.38	0.38	1.83	1.54	1.30	1.23	1.39	1.55	1.47	1.20	--	1.01	(n=14) 1.10±0.13
c	0	0.86	0.58	0.39	0.38	0.34	1.33	1.45	1.47	1.08	1.31	1.30	1.28	0.80	1.00	0.86	0.96±0.10
	1	0.90	0.59	0.39	0.37	0.31	1.43	1.49	1.44	1.09	1.55	1.36	1.36	0.86	1.02	0.88	1.00±0.11
	2	0.88	0.59	0.39	0.40	0.34	1.45	1.49	1.42	1.15	1.60	1.34	1.38	0.88	0.99	0.88	1.01±0.11
	3	0.88	0.59	0.38	0.39	0.31	1.42	1.44	1.58	1.12	1.51	1.37	1.43	0.84	1.04	0.95	1.02±0.11
	(30-90)Late	0.98	0.67	0.42	0.39	0.32	1.56	1.55	1.43	1.10	too cloudy	1.51	1.55	0.90	1.14	0.99	(n=14) 1.04±0.12
d	0	0.72	0.66	0.44	0.35	0.34	1.62	1.64	1.06	1.22	1.20	1.43	1.42	1.22	1.06	0.93	1.02±0.11
	1	0.73	0.66	0.44	0.35	0.36	1.58	1.72	1.04	1.22	1.20	1.42	1.43	1.27	1.08	0.88	1.03±0.11
	2	0.73	0.66	0.45	0.35	0.36	1.61	1.66	1.09	1.20	1.21	1.47	1.44	1.24	1.10	0.89	1.03±0.11
	3	0.76	0.65	0.47	0.36	0.33	1.54	1.66	1.01	1.21	1.18	1.47	1.44	1.27	1.12	0.87	1.02±0.11
	(30-90)Late	0.79	0.71	0.47	0.39	0.34	1.65	1.77	1.04	1.22	1.34	1.55	1.57	1.36	1.18	1.01	1.09±0.12

Actual mean aggregate size = M.A.S. = $\frac{CV9}{CP9}$ X 6066

*In these situations, mean aggregate size is meaningless.

Donors DKG, AR, RAH
Dates: 2/28/80, 3/7/80, 3/13/80

70 μm aperture data

Available Volumes (Vavail) and Platelet Counts (PC)

Note: Δ = CP12-CP8 or CV12-CV8 and the backgrounds are subtracted out. $\overline{\Delta}$ = Δ average, where there were 2 readings for that sample (not the same sample on the 280 μm aperture).

*See Appendix A. $\overline{\Delta}_b$ denotes $\overline{\Delta}$ for background counts.

2/28/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	23	166	143	50	59	9
	23	160	137	65	74	9

$\overline{\Delta}_b$ = 140 $\overline{\Delta}_b$ = 9

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	69	2787	2712	2805.5	2665.5	51	326	275	284.5	275.5
	60	2959	2899			116	410	294		
2	94	3051	2957	3113.0	2973.0	110	419	309	327.5	318.5
	93	3362	3269			246	592	346		
3	157	3339	3182	3522.5	3382.5	128	478	350	365.0	356.0
	110	3973	3863			169	549	380		
4	90	3089	2999	3194.0	3054.0	197	521	324	340.0	331.0
	93	3482	3389			340	696	356		
	85	2962	2877	2804.5	2664.5	206	514	308	298.0	289.0
	74	2806	2732			149	437	288		
(mean ± s.e.m., n=5)					2947.9±134.3					314.0±14.5

PC = (2947.9±134.3) X *80,002 = 235,838 ± 10,748 platelets/μl of sample.

Vavail = (314.0±14.5) X *7582.6 = (2.381±0.110) X $10^6 \mu m^3/\mu l$ of sample.

3/7/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	27	211	184	170	184	14
	24	172	148	36	47	11

$\overline{\Delta}_b$ = 166 $\overline{\Delta}_b$ = 12.5

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	107	2320	2213	2362	2196	388	609	221	234.5	222.0
	71	2582	2511			221	469	248		
2	120	2702	2582	2658	2492	212	457	245	257.0	244.5
	103	2837	2734			135	404	269		
3	117	2312	2195	2370	2204	114	335	221	237.5	225.0
	78	2623	2545			350	604	254		
4	130	3059	2929	3008	2842	163	458	295	308.0	295.5
	113	3200	3087			176	497	321		
(mean ± s.e.m., n=4)					2433.5±152.6					246.8±17.0

$\dot{PC}$ = (2433.5±152.6) X *80.002 = 194,685±12,207 platelets/µl of sample.

Vavail = (246.8±17.0) X *7582.6 = (1.871±0.129) X $10^6 \mu m^3/\mu l$ of sample.

3/13/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	6	114	108	165	170	5

No $\overline{\Delta}_b$ for this one.

Sample	CP8	CP12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\Delta_b$	CV8	CV12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\Delta_b$
1	101	4801	4700	4755.0	4647	342	854	512	512.5	507.5
	81	4891	4810			61	574	513		
2	124	4816	4692	4613.0	4505	504	1005	501	493.0	488.0
	109	4643	4534			560	1045	485		
3	62	4870	4808	4636.5	4528.5	147	665	518	497.0	492.0
	84	4549	4465			104	580	476		
4	90	4467	4377	4358.5	4250.5	416	885	469	469.5	464.5
	73	4413	4340			813	1283	470		
5	72	4533	4461	4443.5	4335.5	495	969	474	470.5	465.5
	76	4502	4426			66	533	467		
6	99	5495	5386	5447.5	5339.5	886	1464	578	580.5	575.5
	94	5603	5509			318	901	583		
(mean ± s.e.m., n=6)				4601.0±158.7					498.8±16.7	

PC = (4601.0±158.7) X *80.002 = 368,089±12,695 platelets/μl of sample.

Vavail = (498.8±16.7) X *7582.6 = (3.782±0.127) X $10^6 \mu m^3/\mu l$ of sample.

Average Available Volume for Comparison with Average Cumulative Volumes of Aggregates on the 280 μm Aperture:

total n=15	
2/28/80	2.381 X 10^6 (n=5)
3/7/80	1.871 X 10^6 (n=4)
3/13/80	3.782 X 10^6 (n=6)

(mean ± s.e.m., n=15) (2.805±0.220) X $10^6 \mu m^3/\mu l$ of sample.

These daily available volumes are averaged in, according to their relative proportion of the total sample size of the 280 μm aperture data. The daily variation (standard errors) of available volumes serve the sole purpose of demonstrating the reliability of that particular volume, but plays no part in the standard error of the average available volume.

The following data were obtained during a series of experiments in which the ADP concentration used to aggregate PRP was varied from 0.2 to 1.0 μM final concentration. Various glutaraldehyde concentrations in the diluent below 0.143 wt.% were used. In addition, in half the samples, glutaraldehyde was added (final concentration of 0.048 wt. %) to the samples prior to dilution. In the other samples, the same volume of isoton was added. The sample numbers correspond to repeated runs (starting with ADP addition) on the same PRP specimen, except as indicated, different donors were used on different days. The time denotes time after dilution in isoton containing the indicated amount of fixative. The volume available for aggregation figures are from platelet counts with the 70 μm aperture on unaggregated PRP.

Treatment	Final Glutaraldehyde Concentrations, wt.%	
	Diluent	PRP Sample
a) 31.5 ml of isoton, 50 μl of 1% glutaraldehyde added to 1 ml of aggregated PRP	0%	0.048%
b) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde; 50 μl of 1% glutaraldehyde added to 1 ml of aggregated PRP	0.048%	0.048%
c) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde; 50 μl of 1% glutaraldehyde added to 1 ml of aggregated PRP	0.143%	0.048%
d) 31.5 ml of isoton; 50 μl of isoton added to 1 ml of aggregated PRP	0%	0%
e) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde; 50 μl of isoton added to 1 ml of aggregated PRP	0.048%	0%
f) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde; 50 μl of isoton added to 1 ml of aggregated PRP	0.143%	0%

Donors: DKG (Samples 1-3 on 3/20/80 all with 1.0 μM ADP); AR (Samples 4-6 on 3/2180 all with 0.5 μM ADP); NL (Samples 7-9 on 3/25/80; 7 and 9 with 0.5 μM ADP; 8 with 0.2 μM ADP).

Treatment	Time (min.)	Cumulative Volumes to Channel 9(CV9) Sample Number 1	2	3	4	5	6	7	8	9	(mean ± s.e.m., n=9) CV9	$CV(\frac{\mu m^3}{\mu l}) \times 10^{-6}$
a	0	4119	4593	4568	3538	2239	2520	4969	3908	4950	3934±333	2.206±0.187
	1	3201	3460	4214	3156	1956	2565	5004	4071	4953	3620±345	2.030±0.193
	2	2504	2313	3840	3620	2506	2447	4594	3667	4383	3319±297	1.861±0.167
	3	2214	1342	3337	2520	2200	2437	4410	2803	4171	2826±329	1.585±0.185
	(30-90)Late	3364	999	2415	1804	873	2459	4820	1569	3639	2438±435	1.367±0.244
b	0	4813	5048	4831	3640	3653	2935	5322	4069	5101	4379±277	2.456±0.155
	1	4671	4836	4783	3525	3322	3131	5574	4019	5193	4339±290	2.434±0.163
	2	5049	4912	5069	3490	3617	3042	5756	4101	5464	4500±320	2.524±0.179
	3	5081	5137	5092	3541	3535	3256	6455	4246	5563	4662±360	2.615±0.202
	(30-90)Late	6111	6376	5155	4388	4432	3582	7118	5248	6417	5425±387	3.043±0.217
c	0	5211	5028	4803	4030	3332	3301	5295	4282	5883	4574±299	2.565±0.168
	1	5471	5152	4808	3950	3216	3304	5864	4460	6059	4698±349	2.635±0.196
	2	5217	5225	5231	4034	3337	2730	6084	4421	6132	4712±392	2.643±0.220
	3	5487	5182	5270	4088	3852	3682	5634	4510	6399	4900±306	2.748±0.172
	(30-90)Late	5744	5316	5031	4585	3513	3816	6431	4722	6704	5096±361	2.858±0.202

Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l} \times 10^{-6}$) → (2.757±0.120)

Actual Cumulative Volume = CV = CV9 X 560.852.

Cumulative Volumes to Channel 9(CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	(mean ± s.e.m., n=9) CV9	CV($\frac{\mu m^3}{\mu l}$) X 10^{-6}
d	0	4472	4747	4261	3064	2996	1113	4692	2352	4868	3899±301	2.187±0.169
	1	1587	1898	1914	1317	1362	433	3415	430	2762	1937±322	1.086±0.181
	2	--	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--	--
e	0	4735	4714	5007	3711	2655	2088	5241	3449	5426	4114±397	2.307±0.223
	1	4628	4857	4830	3561	3474	1725	5730	3755	5464	4225±412	2.370±0.231
	2	4833	4866	4870	3613	3484	2771	5615	3596	5307	4328±326	2.427±0.183
	3	5118	5253	4771	3622	3463	2799	5973	3609	5517	4458±368	2.500±0.206
	(30-90)Late	6429	5828	5377	4690	4574	3160	6587	4768	6587	5333±384	2.991±0.215
f	0	5194	5141	4964	3683	2552	1220	5717	3902	5401	4197±502	2.354±0.282
	1	5116	5027	4865	3839	3558	2748	5653	3989	5647	4494±335	2.520±0.188
	2	5290	5227	4941	4083	3549	3036	6805	4127	5835	4766±394	2.673±0.221
	3	5244	5196	5030	3947	3341	2995	6263	3965	5891	4652±379	2.609±0.213
	(30-90)Late	5569	5528	4707	4425	3929	3208	4248	4290	6367	4697±322	2.634±0.181

Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l}$ X 10^{-6}) → (2.757±0.120)

Actual Cumulative Volume = CV = CV9 X 560.852.

Donors: DKG (Samples1-3 on 3/20/80 all with 1.0 μM ADP); AR (Samples 4-6 on 3/21/80 all with 0.5 μM ADP); NL (Samples 7-9 on 3/25/80; 7 and 9 with 0.5 μM ADP, 8 with 0.2 μM ADP).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	(mean ± s.e.m., n=9) CP9	CP ($\frac{\text{aggregates}}{\mu l}$)
a	0	5552	4987	4548	4395	5175	6065	1542	8125	2911	4817±625	446±58
	1	6779	6454	5115	5032	5190	6213	2061	8450	3733	5447±614	504±57
	2	6171	5695	6148	5008	6220	6019	2635	8125	4821	5652±492	523±46
	3	5889	3799	6478	5534	5833	6083	3858	7692	6256	5714±410	529±38
	(30-90)Late	6998	2977	6361	4790	2717	6336	5260	4587	7387	5268±556	487±51
b	0	3885	5138	5916	5290	6050	6391	1962	8787	3368	5199±657	481±61
	1	3745	5127	5850	5227	5635	6686	2033	8658	3349	5146±649	476±60
	2	3630	5049	5900	5082	5814	6462	2102	8658	3360	5117±644	473±60
	3	3754	5222	5957	5084	5771	6564	2337	8566	3392	5183±620	479±57
	(30-90)Late	3744	5249	5877	5490	6144	6850	2095	9203	3267	5324±703	492±65
c	0	4772	4853	4116	3575	6654	5838	1121	8466	2814	4690±720	434±67
	1	4693	4865	3952	3591	6419	5713	1089	8607	2742	4630±725	428±67
	2	4750	4891	4178	3591	6554	4908	1059	8545	2814	4588±712	424±66
	3	4746	4792	4061	3653	6484	5438	1049	8570	2972	4641±712	429±66
	(30-90)Late	4786	4738	4025	3811	6674	5858	1106	8670	2982	4739±728	438±67

Actual Cumulative Population = CP = CP9 X 0.0925.

Donors: DKG (Samples 1-3 on 3/20/80 all with 1.0 μM ADP); AR (Samples 4-6 on 3/21/80 all with 0.5 μM ADP); NL (Samples 7-9 on 3/25/80; 7 and 9 with 0.5 μM ADP, 8 with 0.2 μM ADP).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	(mean ± s.e.m., n=9) CP9	CP ($\frac{\text{aggregates}}{\mu l}$)
d	0	5049	4720	6202	4397	5028	3386	1859	6782	4329	4639±483	429±45
	1	4578	5101	4424	3777	4061	683	5825	1222	6748	4047±660	374±61
	2	--	--	--	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--	--
e	0	4169	5185	4842	3991	4457	5304	1612	8447	2717	4525±631	419±58
	1	4062	5245	4681	3810	5096	4750	1769	8659	2739	4535±640	419±59
	2	4137	5191	4600	3733	4911	6254	1715	8422	2641	4623±655	428±61
	3	4231	5324	4609	3861	4861	6137	1734	8488	2644	4654±654	430±61
	(30-90)Late	4184	5187	4602	3812	5103	6607	1398	9588	2611	4788±783	443±72
f	0	4715	5310	5284	3975	4915	3602	1500	8407	2927	4515±638	418±59
	1	4525	5148	5274	3986	5668	6270	1378	8330	2887	4830±665	447±62
	2	4594	5248	5379	4027	5568	6557	1615	8452	2892	4926±665	456±62
	3	4642	5288	5279	4069	5618	6556	1408	8390	2930	4909±673	454±62
	(30-90)Late	4640	5255	5097	4138	5855	6757	1349	8477	3103	4963±685	459±63

Actual Cumulative Population = CP = CP9 X 0.0925.

Donors: DKG (Samples 1-3 on 3/20/80 all with 1.0 μM ADP); AR (Samples 4-6 on 3/21/80 all with 0.5 μM ADP); NL (Samples 7-9 on 3/25/80; 7 and 9 with 0.5 μM ADP, 8 with 0.2 μM ADP).

$$CV9/CP9 = M.A.S.\ (\mu m^3) \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	(mean ± s.e.m., n=9) CV9/CP9
a	0	0.74	0.92	1.00	0.81	0.43	0.42	3.22	0.48	1.70	1.08±0.30
	1	0.47	0.54	0.82	0.63	0.38	0.41	2.43	0.48	1.33	0.83±0.22
	2	0.41	0.41	0.62	0.72	0.40	0.41	1.74	0.45	0.91	0.67±0.15
	3	0.38	0.35	0.52	0.46	0.38	0.40	1.14	0.36	0.67	0.52±0.09
	(30-90)Late	0.48	0.34	0.38	0.38	0.32	0.39	0.92	0.34	0.49	0.45±0.06
b	0	1.24	0.98	0.82	0.69	0.60	0.46	2.71	0.46	1.51	1.05±0.24
	1	1.25	0.94	0.82	0.67	0.59	0.47	2.74	0.46	1.55	1.05±0.24
	2	1.39	0.97	0.86	0.69	0.62	0.47	2.74	0.47	1.63	1.09±0.24
	3	1.35	0.99	0.85	0.70	0.61	0.50	2.76	0.50	1.64	1.10±0.24
	(30-90)Late	1.63	1.21	0.88	0.80	0.72	0.52	3.40	0.57	1.96	1.30±0.31
c	0	1.09	1.04	1.17	1.13	0.50	0.57	4.72	0.51	2.09	1.42±0.45
	1	1.17	1.06	1.22	1.10	0.50	0.58	5.38	0.52	2.21	1.53±0.51
	2	1.10	1.07	1.25	1.12	0.51	0.56	5.75	0.52	2.18	1.56±0.55
	3	1.16	1.08	1.30	1.12	0.59	0.68	5.37	0.53	2.15	1.55±0.51
	(30-90)Late	1.20	1.12	1.25	1.20	0.53	0.65	5.81	0.54	2.25	1.62±0.55

Actual mean aggregate size = M.A.S. = $\frac{CV9}{CP9}$ X 6066.

Donors: DKG (Samples 1-3 on 3/20/80 all with 1.0 μM ADP); AR (Samples 4-6 on 2/21/80 all with 0.5 μM ADP); NL (Samples 7-9 on 3/25/80; 7 and 9 with 0.5 μM ADP, 8 with 0.2 μM ADP).

$$CV9/CP9 = M.A.S.\ (\mu m^3) \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	(mean ± s.e.m., n=9) CV9/CP9
d	0	0.89	1.01	0.69	0.70	0.60	0.33	2.52	0.35	1.12	0.91±0.22
	1	0.35	0.37	0.43	0.35	0.34	*0.63	0.59	0.35	0.41	0.42±0.04
	2	--	--	--	--	--	--	--	--	--	-
	3	--	--	--	--	--	--	--	--	--	--
	(30-90)Late	--	--	--	--	--	--	--	--	--	--
e	0	1.14	0.91	1.03	0.93	0.60	0.39	3.25	0.41	2.00	1.18±0.30
	1	1.14	0.93	1.03	0.93	0.68	0.36	3.24	0.43	1.99	1.19±0.30
	2	1.17	0.94	1.06	0.97	0.71	0.44	3.27	0.43	2.01	1.22±0.30
	3	1.21	0.99	1.04	0.94	0.71	0.46	3.44	0.43	2.09	1.26±0.32
	(30-90)Late	1.54	1.12	1.17	1.23	0.90	0.48	4.71	0.50	2.52	1.57±0.44
f	0	1.10	0.97	0.94	0.93	0.52	0.34	3.81	0.46	1.85	1.21±0.36
	1	1.13	0.98	0.92	0.96	0.63	0.44	4.10	0.48	1.96	1.29±0.38
	2	1.15	1.00	0.92	1.01	0.64	0.46	4.21	0.49	2.02	1.32±0.39
	3	1.13	0.98	0.95	0.97	0.59	0.46	4.45	0.47	2.01	1.33±0.42
	(30-90)Late	1.20	1.05	0.92	1.07	0.67	0.47	3.15	0.51	2.05	1.23±0.29

$$\text{Actual mean aggregate size} = M.A.S. = \frac{CV9}{CP9} \times 6066$$

*This mean aggregate size has questionable significance, since the cumulative volume and population was already low (not far from background levels).

Donors: DKG, AR, NL
Dates: 3/20/80, 3/21/80, 3/25/80

70 μm aperture data

Available Volumes (Vavail) and Platelet Counts (PC)

Note: Δ = CP12-CP8 or CV12-CV8 and the backgrounds are subtracted out.

$\overline{\Delta}$ = Δ average, where there were 2 readings for that sample (not the same sample on the 280 μm aperture).

*See Appendix A $\overline{\Delta}_b$ denotes $\overline{\Delta}$ for the background counts.

3/20/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ		
Background	3	20	17		49	50	1		

No $\overline{\Delta}_b$ for this one.

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	80 69	4216 4215	4136 4146	4141.0	4124.0	441 249	834 648	393 399	396.0	395.0
2	54 41	3981 4069	3927 4028	3977.5	3960.5	98 201	464 575	366 374	370.0	369.0
3	53 51	3847 3847	3794 3796	3795.0	3778.0	81 236	438 601	357 365	361.0	360.0
4	73 43	4177 4183	4104 4140	4122.0	4105.0	536 173	921 570	385 397	391.0	390.0
(mean ± s.e.m., n=4)					3991.9±80.1					378.5±8.4

PC = (3991.9±80.1) X *80.002 = 319,358±6,408 platelets/μl of sample.

Vavail = (378.5±8.4) X *7582.6 = (2.870±0.064) X $10^6 \mu m^3/\mu l$ of sample

3/21/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ
Background	9	97	89		15	21	6
	13	86	73		13	18	5
		$\bar{\Delta}$ = 81				$\bar{\Delta}$ = 5.5	

Sample	CP8	CP12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$	CV8	CV12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$
1	45	3227	3182	3245.5	3164.5	465	755	290	291.5	286.0
	36	3345	3309			343	636	293		
2	57	3775	3718	3681.5	3600.5	103	436	333	328.5	323.0
	50	3695	3645			179	503	324		
3	31	3356	3325	3364.0	3283.0	674	975	301	304.5	299.0
	37	3440	3403			221	529	308		
(mean ± s.e.m., n=3)					3349.3±130.1					302.6±10.9

PC = (3349.3±130.1) X *80.002 = 267,953±10,413.platelets/µl of sample.

Vavail = (302.6±10.9) X *7582.6 = (2.295±0.082) X 10^6 μm^3/µl of sample.

3/25/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ
Background	17	183	167		277	288	11
	19	131	112		95	103	8
		$\bar{\Delta}$ = 139.5				$\bar{\Delta}$ = 9.5	

Sample	CP8	CP12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$	CV8	CV12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$
1	96	4006	3910	3880.0	3740.5	791	1216	425	420.0	410.5
	75	3925	3850			185	600	415		
2	155	3740	3585	3648.5	3509.0	676	1059	383	389.0	379.5
	158	3870	3712			585	780	395		
3	173	4277	4104	4227.5	4088.0	1757	2195	438	448.0	438.5
	118	4469	4351			221	679	458		
(mean ± s.e.m., n=3)					3779.2±16.8					409.5±17.0

PC = (3779.2±16.8) X *80.002 = 302,341±13,461 platelets/µl of sample.
Vavail = (409.5±120) X *7582.6 = (3.105±0.129) X $10^6 \mu m^3$/µl of sample.

Average Available Volume for Comparison with Average Cumulative Volumes of Aggregates on the 280 μm Aperture:

	total n=9
3/20/80	2.870×10^6 (n=3)
3/21/80	2.295×10^6 (n=3)
3/25/80	3.105×10^6 (n=3)

(mean ± s.e.m., n=9)(2.757 0.120) $\times 10^6$ $\mu m^3/\mu l$ of sample

These daily available volumes are averaged in, according to their relative proportion of the total sample size of the 280 μm aperture data. The daily variation (standard errors) of available volumes serve the sole purpose of demonstrating the rdliability of that particular volume, but plays no part in the standard error of the average available volume.

The following data were obtained during a series of experiments with a final glutaraldehyde concentration of 0.143 wt.% in the sample prior to dilution. PRP was aggregated with a final ADP concentration of 0.5 μM. The diluent had various glutaraldehyde concentrations. The sample numbers correspond to repeated runs (starting with ADP addition) on the same PRP specimen, except as indicated, different donors were used on different days. The time denotes time after glutaraldehyde platelet counts with the 70 μm aperture on unaggregated PRP.

	Final Glutaraldehyde Concentrations, wt.%	
	Diluent	PRP Sample
a) 31.5 ml of isoton; 50 μl of 3% glutaraldehyde added to 1 ml of aggregated PRP	0%	0.143%
b) 30 ml of isoton + 1.5 ml of 1% glutaraldehyde; 50 μl of 3% glutaraldehyde added to 1 ml of aggregated PRP	0.048%	1.143%
c) 30 ml of isoton + 1.5 ml of 3% glutaraldehyde; 50 μl of 3% glutaraldehyde added to 1 ml of aggregated PRP	0.143%	0.143%

Donors: DKG (Sample 1 on 4/15/80); SN (Samples 2-4 on 4/17/80).

Cumulative Volumes to Channel 9(CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	mean ± s.e.m., n=4 CV9	CV($\frac{\mu m^3}{\mu l}$) X 10-6
a	0	4712	5258	5718	5115	5201±208	2.917±0.117
	3	4722	5944	4951	5476	5273±274	2.957±0.154
	5	3827	4968	4925	5013	4683±286	2.626±0.160
	10	4100	4240	3554	4271	4041±167	2.266±0.094
b	0	4934	5616	5310	4949	5202±163	2.918±0.091
	3	4840	5533	5819	4803	5249±254	2.944±0.142
	5	4553	4555	5019	4883	4753±118	2.666±0.066
	10	3575	3899	4396	3644	3879±186	2.176±0.104
c	0	4920	5634	5906	5417	5469±209	3.067±0.117
	3	5291	5345	5655	5883	5544±139	3.109±0.078
	5	4403	4871	4571	4837	4671±111	2.620±0.062
	10	4338	4028	3939	3979	4071± 91	2.283±0.051

Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l}$ X 10^{-6}) → (2.780±0.009)

Actual Cumulative Volume = CV = CV9 X 560.852.

Donors: DKG (Sample 1 on 4/15/80); SN (Samples 2-4 on 4/17/80).

Treatment	Time (min.)	Cumulative Populations to Channel 9(CP9) Sample Number 1	2	3	4	mean ± s.e.m., n=4 CP9	CP(aggregates/µl)
a	0	3306	2912	2055	2073	2587±312	239±29
	3	2793	2237	1366	1552	1987±327	184±30
	5	2346	1957	1313	1489	1776±234	164±22
	10	2440	1664	980	1213	1574±322	146±30
b	0	3376	2607	2726	2299	2752±227	255±21
	3	2901	1916	2175	1475	2117±299	196±28
	5	2703	1633	1936	1459	1933±275	179±25
	10	2193	1437	1562	1116	1577±226	146±21
c	0	3781	2696	2371	2534	2846±319	263±30
	3	3550	1919	1812	1746	2258±433	209±40
	5	2495	1779	1471	1524	1942±357	180±33
	10	2939	1407	1330	1275	1738±401	161±37

Actual Cumulative Population = CP = CP9 X 0.0925.

Donors: DKG (Sample 1 on 4/15/80); SN (Samples 2-4 on 4/17/80).

$$CV9/CP9 = M.A.S.\ (\mu m^3) \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	mean ± s.e.m., n=4 (CV9/CP9)
a	0	1.43	1.81	2.78	2.47	2.12±0.31
	3	1.69	2.66	3.62	3.53	2.88±0.45
	5	1.63	2.54	3.75	3.37	2.82±0.47
	10	1.68	2.55	3.66	3.52	2.85±0.46
b	0	1.46	2.15	1.95	2.15	1.93±0.16
	3	1.67	2.89	2.68	3.26	2.63±0.34
	5	1.68	2.79	2.59	3.35	2.60±0.35
	10	1.63	2.71	2.81	3.27	2.61±0.35
c	0	1.30	2.09	2.49	2.14	2.01±0.25
	3	1.49	2.79	3.12	3.37	2.69±0.42
	5	1.47	2.74	3.11	3.17	2.62±0.40
	10	1.48	2.86	2.96	3.12	2.61±0.38

Actual mean aggregate size = M.A.S. = $\frac{CV9}{CP9}$ X 6066.

Available Volumes (Vavail) and Platelet Counts (PC) - 70 μm aperture data.

Note: Δ = CP12-CP8 or CV12-CV8 and the backgrounds are subtracted out. $\overline{\Delta}$ = Δ average where there were 2 readings for that sample (not the same sample on the 280 μm aperture).

*See Appendix A $\overline{\Delta}_b$ denotes $\overline{\Delta}$ for the background counts.

4/15/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ	
Background	7	21	14		23	23	0	
	5	13	8		24	24	0	
			$\overline{\Delta}_b$ = 11				$\overline{\Delta}_b$ = 0	

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	50	3961	3911	3957.5	3946.5	553	922	369	370.0	370.0
	46	4050	4004			223	594	371		
2	101	3571	3470	3691.5	3680.5	433	778	345	363.5	363.5
	93	4006	3913			620	1002	382		
3	126	4223	4097	4056.0	4045.0	1115	1536	421	426.5	426.5
	89	4104	4015			599	1031	432		
4	162	4292	4130	4103.0	4092.0	1109	1518	409	407.5	407.5
	205	4281	4076			1224	1630	406		
5	110	3252	3142	3248.5	3237.5	622	942	320	328.5	328.5
	108	3463	3355			344	681	337		
6	90	3139	3049	3174.0	3163.0	588	905	317	326.0	326.0
	76	3375	3299			89	424	335		
(mean ± s.e.m., n=6)					3694.1±166.9					370.3±16.6

PC = (3694.1±166.9) X *80.002 = 295,534±13,354platelets/μl of sample.

Vavail = (370.3±16.6) X *7582.6 = (2.808±0.126) X $10^6 \mu m^3/\mu l$ of sample.

4/17/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ
Background	11	87	76		81	86	5
	4	64	60		2	6	4
		$\bar{\Delta}_b$ = 68				$\bar{\Delta}_b$ = 4.5	

Sample	CP8	CP12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$	CV8	CV12	Δ	$\bar{\Delta}$	$\bar{\Delta}-\bar{\Delta}_b$
1	130	3032	2902	2856.5	2788.5	354	708	354	350.5	346.0
	140	2951	2811			248	595	347		
2	129	3097	2968	2970.5	2902.5	146	504	358	363.5	359.0
	156	3129	2973			298	667	369		
3	131	2997	2866	2982.5	2914.5	184	533	349	363.0	358.5
	107	3206	3099			353	730	377		
4	200	3257	3057	3105.0	3037.0	271	647	376	381.5	377.0
	198	3351	3153			413	800	387		
5	221	3294	3073	3083.0	3015.0	223	615	392	391.0	386.5
	195	3288	3093			379	769	390		
(mean ± s.e.m., n=5)					2931.5±44.5					365.4±7.2

PC = (2931.5±44.5) x *80.002 = 234,526 ± 3,560 platelets/µl of sample.

Vavail = (365.4±7.2) X *7582.6 = (2.771±0.055) X 10^6 μm^3/µl of sample

Average Available Volume for Comparison with Average Cumulative Volumes of Aggregates on the 280 µm Aperture:

	total n=4
4/15/80	2.808 X 10^6 (n=1)
4/17/80	2.771 X 10^6 (n=3)
(mean ± s.e.m., n=4)	(2.780±0.009) X 10^6 μm^3/µl of sample

These daily available volumes are averaged in, according to their relative proportion of the total sample size of the 280 μm aperture data. The daily variation (standard errors) of available volumes serve the sole purpose of demonstrating the reliability of that particular volume, but plays no part in the standard error of the average available volume.

The following data were obtained during a series of experiments that attempted to fix platelet aggregates in PRP prior to dilution, for 3-5 minutes, i.e. prevent breakup as well as stop the aggregation process. PRP samples were aggregated with final ADP concentrations of 0.5 μM, 2.0 μM, and 20 μM. In half the samples, 50 μl of 1% glutaraldehyde was added to 1 ml of aggregated PRP for a final glutaraldehyde concentration of 0.048 wt.%. In the other (control) samples, nothing was added to the aggregated PRP prior to dilution. In all cases, the isoton-glutaraldehyde diluent had a glutaraldehyde concentration of 0.048 wt.% and the count was taken immediately on dilution. The time columns give the ellapsed time after addition of the fixative to the aggregated sample (prior to dilution for counting).

Treatment	In Sample: Glutaraldehyde Level (wt.%)	In Sample: ADP Level
a) 0.9 ml of PRP + 0.1 ml of ADP (5 μM) → 1.0 ml of aggregated PRP (0.5 μM)		0.5 μM
nothing added to aggregated PRP*	0%	
a') 0.9 ml of PRP + 0.1 ml of ADP (5 μM) → 1.0 ml of aggregated PRP (0.5 μM)		
50 μl of 1% glutaraldehyde added*	0.048%	0.5 μM
b) 0.9 ml of PRP + 0.1 ml of ADP (20 μM) → 1.0 ml of aggregated PRP (2.0 μM)		
nothing added to aggregated PRP*	0%	2.0 μM
b') 0.9 ml of PRP + 0.1 ml of ADP (20 μM) → 1.0 ml of aggregated PRP (2.0 μM)		
50 μl of 1% glutaraldehyde added*	0.048%	2.0 μM
c) 0.9 ml of PRP + 0.1 ml of ADP (200 μM) → 1.0 ml of aggregated PRP (20 μM)		
nothing added to aggregated PRP*	0%	20 μM
c') 0.9 ml of PRP + 0.1 ml of ADP (200 μM) → 1.0 ml of aggregated PRP (20 μM)		
50 μl of 1% glutaraldehyde added*	0.048%	20 μM

*prior to dilution in the isoton-glutaraldehyde diluent.

Donors: DKG (Sample 1 for a, a', c, c', and Samples 1-2 for b, b' on 4/24/80).
JS (Sample 2 for a, a', c, c', and Sample 3 for b, b', on 4/29/80).
DKG (Samples 3-7 for a, a', c, c', and Samples 4-8 for b, b' on 5/1/80).
MN (Samples 8-10 for a, a', Samples 9-10 for b, b', and Samples 8-9 for c, c' on 5/6/80).
MH (Samples 11-12 for a, a', b, b', and Samples 10-11 for c, c' on 5/8/80).

Cumulative Volumes to Channel 9(CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	(mean ± s.e.m., n=12) CV9	CV($\frac{\mu m^3}{\mu l}$) X 10^{-6}
a	0	5082	6486	6196	5704	5856	5959	4899	5119	5730	5448	5215	5268	5580±141	2.981±0.075
	3	401	2781	1140	50	105	217	156	3044	2698	2094	3530	2543	1563±387	0.835±0.207
	5	290	1809	194	350	195	275	232	1619	1238	945	3123	2915	1099±308	0.587±0.165
a'	0	5102	6952	5953	5911	5762	5006	4080	5829	5073	5911	4803	5107	5455±216	3.059±0.121
	3	4989	6444	5456	4986	5476	4050	3540	8312	5515	5836	4644	5026	5359±349	3.006±0.196
	5	4643	6475	5078	5742	4895	5022	3416	4890	5420	5664	4882	4680	5067±214	2.842±0.120
Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l}$ X 10^{-6})														→	(3.149±0.164)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	(mean ± s.e.m., n=12) CV9	CV($\frac{\mu m^3}{\mu l}$) X 10^{-6}
b	0	5507	5983	6524	5863	6196	6497	5616	6750	6165	5930	4452	5772	5938±173	3.172±0.092
	3	5614	4728	off 8180	5978	5886	5372	5597	6008	5951	5877	5978	5307	5871±237	3.136±0.127
	5	4578	4455	off 6904	4081	5057	4423	4525	4170	off 4793	off 4687	4369	4996	4753±213	2.539±0.114
b'	0	6500	5355	6384	6431	6444	6019	6221	6078	5090	6102	4836	4983	5870±180	3.292±0.101
	3	4969	4940	6166	5856	5506	6044	6256	6690	4761	4413	4472	4761	5403±225	3.030±0.126
	5	4295	5113	5344	5781	5827	5006	5976	5862	4317	4436	3890	3595	4954±240	2.778±0.135
Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l}$ X 10^{-6})														→	(3.188±0.146)

(Continued)

Donors: DKG (Sample 1 for a, a', c, c', and Samples 1-2 for b, b' on 4/24/80).
JS (Sample 2 for a, a', c, c', and Sample 3 for b, b', on 4/29/80).
DKG (Samples 3-7 for a, a', c, c', and Samples 4-8 for b, b' on 5/1/80).
MN (Samples 8-10 for a, a', Samples 9-10 for b, b', and Samples 8-9 for c, c' on 5/6/80).
MH (Samples 11-12 for a, a', b, b', and Samples 10-11 for c, c' on 5/8/80).

Cumulative Volumes to Channel 9(CV9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	CV9 (mean ± s.e.m., n=11)	$CV(\frac{\mu m^3}{\mu l}) \times 10^{-6}$ (mean ± s.e.m., n=11)
c	0	6224	5831	6166	6152	7071	6838	6320	5777	5438	4428	4384	5875±259	3.138±0.138
	3	off	off	off	off	off	off	off	off	off	off	off	6680±388	3.568±0.207
		7485	7933	7170	8554	5972	5316	7962	7330	5258	5291	5209		
	5	off	off	off	off	off	off	off	off	off	off	off	4115±361	2.198±0.193
		6513	3527	4711	4494	5437	4891	3026	2773	3277	3749	2868		
c'	0	4863	6991	6849	5445	6878	6156	4478	4805	5983	5354	4370	5652±295	3.170±0.165
	3	4517	6044	5583	5824	4487	5567	5213	5697	4724	4874	3919	5132±203	2.878±0.114
	5	3574	5926	4000	4234	4785	5010	4923	3777	5483	4058	2892	4424±269	2.481±0.151
Vavail (available volume for aggregation from free platelets in $\frac{\mu m^3}{\mu l} \times 10^{-6}$)													→	(3.212±0.158)

a,b,c Actual Cumulative Volume = CV = CV9 X 534.145.

a', b', c' Actual Cumulative Volume = CV = CV9 X 560.852.

off =>distribution went off the 280 μm aperture.

Donors: DKG (Sample 1 for a, a', c, c', and Samples 1-2 for b, b' on 4/24/80).
JS (Sample 2 for a, a', c, c', and Sample 3 for b, b' on 4/29/80).
DKG (Samples 3-7 for a, a', c, c' and Samples 4-8 for b, b' on 5/1/80).
MN (Samples 8-10 for a, a', Samples 9-10 for b, b', and Samples 8-9 for c, c' on 5/6/80).
MH (Samples 11-12 for a, a', b, b', and Samples 10-11 for c, c' on 5/8/80).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	(mean ± s.e.m., n=12) CP9	CP(aggregates/µl)
a	0	3155	7972	2862	6472	7465	6835	8182	1317	1952	3070	2548	3003	4570±743	403±65
	3	1282	7187	2171	57	69	68	143	6214	6011	4949	6042	5638	3319±841	292±74
	5	1004	5522	277	100	74	109	100	3830	3462	1944	5453	5477	2279±671	201±59
a'	0	3892	7999	5501	6946	5830	7483	7722	1409	1682	2765	2175	2905	4692±721	434±67
	3	3380	6830	5570	7934	6286	8088	7783	1930	1675	2936	2571	3264	4854±714	449±66
	5	3732	6427	6733	7543	6422	7840	7755	1249	1751	3062	2694	3536	4895±710	453±66

Treatment	Time (min).	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	(mean ± s.e.m., n=12) CP9	CP(aggregates/µl)
b	0	756	712	764	600	799	837	776	1111	616	698	671	884	769± 40	68± 3
	3	764	660	off 471	740	866	1373	1581	2581	496	415	567	464	915±184	81±16
	5	909	1171	off 418	637	1229	1879	2218	2440	off 282	375	405	555	1043±219	92±19
b'	0	833	677	740	703	922	1006	965	1040	483	693	616	675	779± 50	72± 5
	3	585	725	636	909	969	1300	1801	1663	472	496	674	688	910±129	84±12
	5	537	715	556	1052	1333	1321	1379	2309	397	511	643	546	941±162	87±15

(Continued)

Donors: DKG (Sample 1 for a, a', c, c', and Samples 1-2 for b, b' on 4/24/80).
JS (Sample 2 for a, a', c, c', and Sample 3 for b, b' on 4/29/80).
DKG (Samples 3-7 for a, a', c, c' and Samples 4-8 for b, b' on 5/1/80).
MN (Samples 8-10 for a, a', Samples 9-10 for b, b', and Samples 8-9 for c, c' on 5/6/80).
MH (Samples 11-12 for a, a', b, b', and Samples 10-11 for c, c' on 5/8/80).

Cumulative Populations to Channel 9(CP9)

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	(mean ± s.e.m., n=11) CP9	CP ($\frac{\text{aggregates}}{\mu l}$)
c	0	614	532	576	479	650	516	535	467	612	481	474	540±20	48±1.7
	3	off	off	off	off	off	off	off	off	off	off	off		
		426	366	361	280	282	299	421	324	287	298	361	337±16	30±1.4
	5	off	off	off	off	off	off	off	off	off	off	off		
		309	150	273	176	322	219	228	177	195	223	213	226±17	20±1.5
c'	0	299	618	596	467	415	424	389	444	598	560	446	478±31	44±2.7
	3	299	509	476	464	323	416	384	401	437	506	378	418±21	39±1.8
	5	256	466	464	384	423	440	475	298	522	435	329	408±25	38±2.3

a,b,c, Actual Cumulative Population = CP = CP9 X 0.0881.

a',b',c' Actual Cumulative Population = CP = CP9 X 0.0925.

off ⇒ distribution went off the 282 μm aperture.

Donors: DKG (Sample 1 for a, a', c, c', and Samples 1-2 for b, b' on 4/24/80).
JS (Sample 2 for a, a', c, c', and Sample 3 for b, b' on 4/29/80)
DKG (Samples 3-7 for a, a', c, c', and Samples 4-8 for b, b' on 5/1/80).
MN (Samples 8-10 for a, a', Samples 9-10 for b, b', and Samples 8-9 for c, c' on 5/6/80).
MH (Samples 11-12 for a, a', b, b', and Samples 10-11 for c, c' on 5/8/80).

$$CV9/CP9 = M.A.S. (\mu m^3) \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	(mean ± s.e.m., n=12)
a	0	1.61	0.81	2.16	0.88	0.78	0.87	0.60	3.89	2.94	1.77	2.05	1.75	1.68±0.26
	3	0.31	0.39	0.53	*0.88	*1.52	*3.19	*1.09	0.49	0.45	0.42	0.58	0.45	0.45±0.02
	5	0.29	0.33	*0.70	*3.50	*2.64	*2.52	*2.32	0.42	0.36	0.49	0.57	0.53	0.43±0.03
a'	0	1.31	0.87	1.08	0.85	0.99	0.67	0.52	4.14	3.02	2.14	2.21	1.76	1.63±0.31
	3	1.48	0.94	0.98	0.63	0.87	0.50	0.45	4.31	3.29	1.99	1.81	1.54	1.57±0.34
	5	1.24	1.01	0.75	0.76	0.76	0.64	0.44	3.92	3.10	1.85	1.81	1.32	1.47±0.31

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	12	(mean ± s.e.m., n=12)
b	0	7.28	8.40	8.54	9.77	7.75	7.76	7.24	6.08	8.50	10.00	6.63	6.53	7.87±0.36
	3	7.35	7.16	off	8.08	6.80	3.91	3.53	2.33	14.16	12.00	10.54	11.44	8.72±1.30
				17.37										
	5	5.04	3.80	off	6.41	4.11	2.35	2.04	1.71	off	off	10.79	9.00	7.61±1.58
				16.52						12.50	17.00			
b'	0	7.80	7.91	8.63	9.15	6.99	5.98	6.45	5.84	8.81	10.54	7.85	7.38	7.78±0.40
	3	8.49	6.81	9.69	6.44	5.68	4.65	3.47	4.02	8.90	10.09	6.64	6.92	6.82±0.62
	5	8.00	7.15	9.61	5.50	4.37	3.79	4.33	2.54	8.68	10.87	6.05	6.58	6.46±0.73

Donors: DKG (Sample 1 for a, a', c, c', and Samples 1-2 for b, b' on 4/24/80).
JS (Sample 2 for a, a', c, c', and Sample 3 for b, b' on 4/29/80).
DKG (Samples 3-7 for a, a', c, c', and Samples 4-8 for b, b' on 5/1/80).
MN (Samples 8-10 for a, a', Samples 9-10 for b, b', and Samples 8-9 for c, c' on 5/6/80).
MH (Samples 11-12 for a, a', b, b', and Samples 10-11 for c, c' on 5/8/80).

$$CV9/CP9 = M.A.S.\ (\mu m^3) \div 6066$$

Treatment	Time (min.)	Sample Number 1	2	3	4	5	6	7	8	9	10	11	(mean ± s.e.m., n=11)
c	0	10.14	10.46	10.70	12.84	10.88	13.25	11.81	12.37	8.89	9.21	9.25	10.94±0.45
	3	off	off	off	off	off	off	off	off	off	off	off	20.06±1.25
		17.57	21.67	19.86	30.55	21.18	17.78	18.91	22.62	18.32	17.76	14.43	
	5	off	off	off	off	off	off	off	off	off	off	off	18.42±1.23
		21.08	23.51	17.26	25.53	16.89	22.33	13.27	15.66	16.81	16.81	13.46	
c'	0	16.26	11.31	11.49	11.66	16.57	14.52	11.51	10.82	10.00	9.56	9.80	12.14±0.75
	3	15.11	11.87	11.73	12.55	13.89	13.38	13.58	14.20	10.81	9.63	10.37	12.46±0.52
	5	13.96	12.72	8.62	11.03	11.31	11.39	10.36	12.67	10.50	9.33	8.79	10.97±0.51

Actual mean aggregate size = M.A.S. = $\frac{CV9}{CP9}$ X 6066.

off => distribution went off the 280 μm aperture.

*Here, mean aggregate size is meaningless, since the volume and population were very low.

Donors: DKG, JS, DKG, MM, MH
Dates: 4/24/80, 4/29/80, 5/1/80, 5/6/80, 5/8/80

70 μm aperture data

Available Volumes (Vavail) and Platelet Counts (PC)

Note: Δ = CP12-CP8 or CV12-CV8 and the backgrounds are subtracted out.

$\overline{\Delta}$ = Δ average, where there were 2 readings for that sample (not the same sample on the 280 μm aperture).

*See Appendix A $\overline{\Delta}_b$ denotes $\overline{\Delta}$ for the background counts.

4/24/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ	
Background	8	53	45		166	169	3	
	2	37	35		64	66	2	
			$\overline{\Delta}_b$ = 40				$\overline{\Delta}_b$ = 2.5	

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	49	3861	3812	3953.5	3913.5	190	556	366	378.5	376.0
	22	4117	4095			25	416	391		
2	51	4080	4029	4043.0	4003.0	96	487	391	389.5	387.0
	41	4098	4057			150	538	388		
3	117	4395	4278	4261.0	4221.0	1130	1573	443	425.5	423.0
	116	4360	4244			897	1305	408		
4	29	4055	4026	4114.0	4074.0	483	866	383	390.0	387.5
	49	4251	4202			277	674	397		
5	49	3864	3815	3859.0	3819.0	189	548	359	363.5	361.0
	52	3955	3903			277	645	368		
(mean ± s.e.m., n=5)					4006.2±68.6					386.8±10.2

PC = (4006.2±68.6) X *80.002 = 320,504±5,492 platelets/μl of sample.

Vavail = (386.8±10.2) X *7582.6 = (2.933±0.078) X 10^6 μm^3/μl of sample.

4/29/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ
Background	38	173	135		206	217	11

No $\overline{\Delta}_b$ for this one.

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	29	5423	5394	5441.5	5306.5	88	553	465	467.0	456.0
	28	5517	5489			153	622	469		
2	35	5419	5384	5514	5379.0	123	589	466	481.0	470.0
	36	5680	5644			68	564	496		
3	36	5001	4965	5368.5	5233.5	155	602	447	475.0	464.0
	48	5820	5772			34	537	503		
4	82	5365	5283	5335.5	5220.5	274	763	489	485.0	474.0
	41	5429	5388			102	583	481		
5	121	5488	5367	5493.0	5358.0	517	1032	515	521.5	510.5
	72	5691	5619			95	623	528		
(mean ± s.e.m., n=5)					5295.5±34.5					474.9±9.4

PC = (5295.5±34.5) X *80.002 = 423,651±2,764 platelets/µl of sample.

Vavail = (474.9±9.4) X *7582.6 = (3.601±0.071)X 10^6 μm^3/µl of sample.

5/1/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	15	109	94	318	324	6
	9	82	73	71	76	5
		Δ_b = 83.5			Δ_b = 5.5	

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\Delta_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\Delta_b$
1	39	5448	5409	5463.0	5379.5	466	968	502	499.5	494.0
	35	5552	5517			59	556	497		
2	39	5368	5329	5360.0	5276.5	78	560	482	486.5	481.0
	31	5422	5391			150	641	491		
3	49	5175	5126	5188.0	5104.5	238	703	465	473.5	468.0
	29	5279	5250			46	528	482		
4	45	5573	5528	5466.5	5383.0	201	704	503	495.0	489.5
	28	5433	5405			43	530	487		
5	41	5425	5384	5318.0	5234.5	48	539	491	487.0	481.5
	30	5282	5252			25	508	483		
(mean ± s.e.m., n=5)					5276.0±51.6					483.0±4.5

PC = (5276.0±51.6) X *80.002 = 422,091±4,126 platelets/µl of sample.

Vavail = (483.0±4.5) X *7582.6 = (3.662±0.034) X 10^6 μm^3/µl of sample.

5/6/80 - 2 readings per sample

	CP8	CP12	Δ	CV8	CV12	Δ
Background	8	112	104	43	49	6
	0	20	20	0	1	1

Δ_b = 62 Δ_b = 3.5

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\Delta_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\Delta_b$
1	187	3140	2953	3144.0	3082.0	658	990	332	352.0	348.5
	187	3522	3335			791	1163	372		
2	233	3160	2927	3000.0	2938.0	290	618	328	339.0	335.5
	174	3247	3073			203	553	350		
3	126	2934	2808	2893.0	2831.0	431	747	316	326.5	323.0
	129	3107	2978			108	445	337		
4	171	2650	2479	2556.0	2494.0	470	748	278	293.0	289.5
	125	2758	2633			159	467	308		
(mean ± s.e.m., n=4)					2836.3±125.1					324.1±12.2

PC = (2836.3±125.1) X *80.002 = 226,906±10,011 platelets/µl of sample.

Vavail = (324.1±12.2) X *7582.6 = (2.461±0.096) X 10^6 μm^3/µl of sample.

5/8/80 - 2 readings per sample

	CP8	CP12	Δ		CV8	CV12	Δ
Background	8	111	103		59	66	7
	4	60	56		22	26	4

$\overline{\Delta}_b = 79.5$ $\overline{\Delta}_b = 5.5$

Sample	CP8	CP12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$	CV8	CV12	Δ	$\overline{\Delta}$	$\overline{\Delta}-\overline{\Delta}_b$
1	118	3977	3859	3770.0	3690.5	346	762	416	405.0	399.5
	92	3773	3681			164	558	394		
2	78	3541	3463	3461.0	3381.5	363	733	370	373.5	368.0
	67	3526	3459			70	447	377		
3	75	3362	3287	3316.5	3237.0	56	415	359	362.5	357.0
	94	3440	3346			97	463	366		
4	75	3362	3287	3256.0	3176.5	107	457	350	347.5	342.0
	67	3292	3225			64	409	345		
(mean ± s.e.m., n=4)				3371.4±114.7					366.8±12.3	

PC = (3371.4±114.7) X *80.002 = 269,687±9,176 platelets/μl of sample.

Vavail = (366.8±12.3) X *7582.6 = (2.787±0.093) X 10^6 $\mu m^3/\mu l$ of sample.

Average Available Volume for Comparison with Average Cumulative Volumes of Aggregates on the 280 μm Aperture:

	a, a' (total n=12)	b, b' (total n=12)	c, c' (total n=11)
4/24/80	2.933 X 10^6 (n=1)	2.933 X 10^6 (n=2)	2.933 X 10^6 (n=1)
4/29/80	3.601 X 10^6 (n=1)	3.601 X 10^6 (n=1)	3.601 X 10^6 (n=1)
5/1/80	3.662 X 10^6 (n=5)	3.662 X 10^6 (n=5)	3.662 X 10^6 (n=5)
5/6/80	2.461 X 10^6 (n=3)	2.461 X 10^6 (n=2)	2.461 X 10^6 (n=2)
5/8/80	2.781 X 10^6 (n=2)	2.781 X 10^6 (n=2)	2.781 X 10^6 (n=2)
(mean±s.e.m)	(3.149±0.164) X 10^6	(3.188±0.146) X 10^6	(3.212±0.158) X 10^6

As usual these volume units are $\mu m^3/\mu l$ of sample.

These daily available volumes are averaged in, according to their relative proportion of the total sample size of the 280 μm aperture data. The daily variation (standard errors) of available volumes serve the sole purpose of demonstrating the reliability of that particular volume, but plays no part in the standard error of the average available volume.

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