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The Requirement for Viable Platelets in the Coagulation of Heparinized Blood

Karen L. Hay

Abstract

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Activated coagulation times (ACT's) of heparinized blood or plasma samples containing normal platelets were compared with ACT's of heparinized samples containing non-viable or abnormally functional platelets. The non-viable platelets were prepared by freeze/thawing, and the abnormally functional platelets resulted from the addition of adenosine or adenosine 5'-diphosphate (ADP).

The ACT's of the heparinized samples containing treated platelets were considerably longer than the ACT's performed on samples with untreated platelets. Treated platelets had no effect on unheparinized samples, however. This would indicate that, although platelet factor 3 (PF3) is sufficient to promote clotting of unheparinized blood, viable platelets, in addition, are required for clotting of heparinized blood. A possible hypothesis which explains these findings is as follows:

> In the unheparinized situation, coagulation factors are activated as clotting takes place. PF3 serves as a surface catalyst, thrombin is generated, and a fibrin clot is formed. The form in which PF3 is available is incidental to clot formation.
>
> When heparin is present, however, thrombin is neutralized as fast as it is formed. Quantities sufficient to induce fibrin formation cannot therefore form in the free plasma, and prolonged or infinite clotting times result. In order for coagulation to go to completion, an environment favorable to thrombin generation must be provided.
>
> It is probable that, even in heparinized samples, the foreign surface present and the thrombin formed are sufficient to promote platelet aggregation, even though the

thrombin levels are too low to directly affect the coagulation system.

The interstices of the platelet aggregates provide an environment favorable to thrombin formation. As the platelets aggregate, plasma containing activated clotting factors and heparin is trapped within the platelet clump. Platelet factor 4 (PF4) is released from the platelets and, in the static environment within the clump, is able to completely neutralize the heparin trapped there. This then allows thrombin generation to proceed catalytically. Fibrin formation can now go to completion, both within the platelet aggregate and in the "plasmatic atmosphere" closely surrounding the clump. The large amounts of thrombin generated overcome the heparin effect, and visible clotting occurs.

The implications of an hypothesis such as this may be summarized as follows:

1. Viable platelets are essential to the coagulation of heparinized blood and plasma samples.
2. The varying sensitivities of different individuals to comparable heparin doses may in part reflect the platelet function of those individuals.
3. The fact that variable (and sometimes considerable) numbers of platelets remain in plasma used for activated partial thromboplastin time (APTT) testing implies that the APTT results on such heparinized samples will be correspondingly variable.

4. The APTT, because it is not dependent on the patient's own platelet level, cannot truly reflect that patient's response to heparin.
5. A test such as the activated coagulation time (ACT), which is dependent on the patient's quantitative and qualitative platelet level, reflects more accurately the actual *in vivo* function of heparin than does the APTT.

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Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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KEY TO ABBREVIATIONS

ACT ..... Activated coagulation time

ADP ..... Adenosine 5'-diphosphate

APTT ..... Activated partial thromboplastin time

PF1 ..... Platelet factor 1

PF2 ..... Platelet factor 2

PF3 ..... Platelet factor 3

PF4 ..... Platelet factor 4

PPP ..... Platelet poor plasma

PRP ..... Platelet rich plasma

WBCT ..... Whole blood coagulation time

INTRODUCTION

Platelets have long been known to be intimately and actively involved in blood coagulation. The several platelet activities associated with coagulation have been designated as platelet factors 1 to 4.

Platelet factor 1 (PF1) is not a native platelet protein in the sense that it is merely adsorbed plasma coagulation factor V.[1]

Platelet factor 2 (PF2) is a true platelet protein, not adsorbed from the plasma. One of its main functions is the acceleration of the thrombin-catalyzed conversion of fibrinogen to fibrin, possibly by rendering fibrinogen a more sensitive substrate for thrombin. Other PF2 functions include its inhibitory effects on antithrombin-III, its aggregating effects on platelets from platelet rich plasma (PRP), and its potentiation of the platelet aggregating effect of ADP.[2] Aggregation of platelets does not, however, promote the release of PF2 or enhance its action.[3]

Platelet factor 3 (PF3) activity can be attributed to the phospholipid portions of a lipoprotein component of the platelet membrane.[4] The main phospholipids involved are phosphatidyl serine and phosphatidyl ethanolamine.[5] PF3 is released when platelets undergo the aggregation and release reactions and when platelets are exposed to trauma.[6,7] Rapid freezing and thawing have been used to release PF3 for assays of platelet function.[8] PF3 accelerates plasma clotting, probably by acting as a surface catalyst for the coagulation factors.[7] It is interesting to note that there is evidence indicating that plasma factors are generally present in excess, and platelet concentration may

be the limiting factor in determining the amount of thrombin formed during blood clotting.[9,10]

Platelet factor 4 (PF4), also known as anti-heparin, is another true platelet protein, a glycoprotein, localized mainly in the intracellular granule fraction.[11] It is released during normal clotting and following platelet aggregation by ADP, thrombin, epinephrine or collagen.[12-15] It may also be released by rapid freezing and thawing of platelets.[16] When released by the platelets, PF4 is in the form of a high molecular weight proteoglycan-PF4 complex. When this complex comes into contact with heparin, the carrier portion of the complex is displaced and the heparin binds to the PF4. Heparin, then, is essentially neutralized since no other glycosaminoglycan present in vascular tissue will displace it from the PF4-heparin complex.[11]

Heparin is a highly acidic mucopolysaccharide whose precise configuration is still unclear.[17] It is present in minute amounts in the normal blood, where its physiologic function is also unclear.[18] However, it has been used extensively as a therapeutic anticoagulant agent since its discovery in 1916 by Maclean.[17]

Although heparin interferes with several steps in the coagulation mechanism, one of its primary functions is its neutralization of thrombin. This action is due to its ability to enhance the effects of antithrombin-III on thrombin. Several different theories of heparin-antithrombin III-thrombin interaction have been offered to date, but the exact mechanism of action remains uncertain.[17,19,20]

Heparin therapy has long been monitored using the whole blood

clotting time (WBCT). In recent years, however, the activated partial thromboplastin time (APTT) has been increasingly used in preference to the WBCT.[21]

When it is necessary to neutralize heparin *in vivo*, protamine sulfate is often administered.[22] Protamine sulfate, a strongly basic low molecular weight protein,[17] combines with the strongly acidic heparin to form a stable salt with the loss of anticoagulant activity.[23] It may also be used to measure, by titration, the concentration of heparin in *in vitro* blood samples.[22]

OBJECTIVES

It is apparent that PF3 must be present in adequate concentration in order for blood coagulation to proceed normally. At first glance, it would seem sufficient merely to have the necessary phospholipids present, though perhaps not necessarily in the form of viable platelets. This, in essence, is what is relied on when partial thromboplastin time tests (activated or unactivated) are performed. An artificial source of phospholipid is used, making the test relatively independent of the patient's own platelet level.

It is possible, however, that in certain circumstances viable platelets, capable of going through their aggregation and release reactions, are necessary in addition to PF3 in order to promote normal clotting. An instance where this could be the case is the clotting of heparinized blood.

The present study was done to demonstrate that, although PF3 alone may be sufficient to promote normal blood coagulation in the absence of heparin, the presence of viable, functional platelets is needed to maximize clotting of heparinized samples.

METHODS AND MATERIALS

Outline of Approach

The general approach to the problem was to compare the effects of functional and abnormally or non-functional platelets on the clotting times of heparinized whole blood or plasma samples. Two main methods were used:

1. Effect of non-viable vs. normal viable platelets on the ACT of heparinized plasma:

   Samples of heparinized plasma were divided equally. One portion contained normal, untreated platelets. The platelets in the remainder were ruptured and rendered non-viable by rapid freeze/thawing. ACT's were then performed on both samples.

2. Effect of non-functional or abnormally functional vs. normal viable platelets on the ACT of heparinized whole blood:

   Samples of heparinized blood were divided into three equal portions. The first contained normal, untreated platelets. The second was treated with adenosine to inhibit platelet function. The third was treated with ADP to bring about platelet aggregation. ACT's were then performed on all three samples.

General methods

All blood was obtained from normal donors either by clean venipuncture using 20-gauge needles and plastic syringes or through the integral blood bank donor tubing.

Specimens were promptly divided into aliquots in plastic tubes and treated with sodium heparin (1000 U/ml, beef lung, Upjohn Co., Kalamazoo, Mi.) or placed in Vacutainer tubes containing EDTA (#3204, Beckton, Dickinson & Co., Division of Bioquest, Rutherford, N.J.). Baseline ACT's were performed on unheparinized samples using ACT Vacutainer tubes (#3206XF534, Beckton, Dickinson & Co., Division of Bioquest, Rutherford, N.J.) and a Type 5900 Dri-Bath (Thermolyne Corp., Dubuque, Iowa) adjusted to 37°C. Use of this type of heating block, which has plexiglas windows on the sides, made it possible to observe for clot formation without removing the tubes from the heatblock. Whole blood samples were tested using 2 ml blood per ACT tube. When plasma samples were tested, the quantity of activator in the ACT tube was altered, if necessary, to maintain approximately the same ratio of plasma to activator as would have been present in a whole blood sample. Complete details of the procedure used are listed in Appendix 2.

All other tests involving clot detection were performed using the Fibrometer Precision Coagulation Timer, Model 5 (Baltimore Biological Laboratories, Division of Bioquest, Cockeysville, Maryland). They include the protamine sulfate titration and the APTT. Details of these procedures are given in Appendices 1 and 3. Specimens and reagents were added using Oxford Samplers (Oxford Laboratories, Foster City, Ca.) of appropriate volume.

Hematocrit determinations were made using the Coulter Counter, Model S (Coulter Electronics, Hialeah, Fla.).

Platelet counts were performed on the Thrombocounter (Coulter Electronics, Hialeah, Fla.).

Platelet aggregating ability was measured using the Payton Aggregometer, single channel (Payton Associates Inc., Buffalo, New York).

Specific Methods

Initial work on the project involved performing ACT's on a series of heparinized plasma pairs, one of which contained normal, viable platelets, and the other which contained non-viable freeze/thawed platelets. It was postulated that the rapid freeze/thawing of the one set of platelets would render these non-viable, but would still permit them to contribute maximal amounts of PF3 to the clotting process. Freeze/thawing has in fact been used as a method of releasing PF3 for assay purposes and tests of platelet function.[8] It was also assumed that the freeze/thawing process would release most of the platelets' PF4 content, which in turn would affect the level of active heparin present in the plasma samples under test. So, upon completion of the clotting process, protamine sulfate titrations were performed on all sera and plasmas to determine the level of heparin remaining. The method used was a modification of that used by Bowie et al.[24] Since the protamine sulfate titration is based on a thrombin time method, test samples must contain fibrinogen (Factor I). Because many of the samples tested were sera, prothrombin-free beef plasma was used to supply

Factor I. Details of the protamine sulfate titration procedure are given in Appendix 1.

The complete procedure, from venous collection and platelet freeze/thawing to graph construction, is as follows:

1. Collect venous blood by clean venipuncture and divide into aliquots as follows:

   Aliquots #1-3 ..... Heparinize known quantities of blood with precise amounts of heparin, using plastic tubes. (heparin concentrations used ranged from 1.3 to 5.0 U/ml whole blood or 2.2 to 8.2 U/ml plasma)

   Aliquot #4 ..... Place 3-5 ml whole blood into a Vacutainer tube containing EDTA for hematocrit and platelet determinations.

2. Centrifuge aliquots #1-3 for 10 minutes @ 1000 rpm to obtain platelet rich plasma (PRP). While this is taking place, perform hematocrit and platelet determinations on aliquot #4.
3. Remove PRP's to a second set of labelled tubes.
4. Recentrifuge remaining blood in original tubes for an additional 20 minutes @ 2500 rpm to obtain additional plasma, and add this to the PRP's previously obtained. (Resultant PRP's have slightly lower platelet counts than initial PRP's, but sufficient plasma is thus made available for further testing.)

5. Divide PRP's into two equal portions and treat each as follows:

   Portion #1 ..... leave untreated

   Portion #2 ..... Centrifuge for 20 minutes @ 2500 rpm to obtain platelet poor plasma (PPP) and a platelet button. Carefully remove supernatant PPP to a second set of labelled tubes, taking care not to disturb the platelet button.

6. Perform platelet counts on both PRP's and PPP's. (Preferably, PPP's should have counts of less than $15x10^3$/cu mm, and ideally less than $10x10^3$/cu mm.)

7. Freeze/thaw the platelet buttons 6 to 8 times using an ice bath (dry ice in methanol) and a 37°C waterbath.

8. Return the PPP's to their corresponding freeze/thawed platelet buttons and resuspend.

9. Perform ACT's on all plasmas. Use approximately the same ratio of activator to plasma as would be present in a whole blood ACT. Activator from more than one ACT tube may be combined to obtain the necessary ratio. Carry timing out to at least 20 minutes before declaring any samples unclottable.

10. Calculate the heparin concentration initially present in each plasma sample as follows:

$$\frac{\text{units heparin added}}{\text{ml whole blood}} \times \frac{100}{\text{plasmacrit}} = \frac{\text{units heparin}}{\text{ml plasma}}$$

11. Construct a curve relating plasma heparin concentration to ACT times using linear graph paper. Assign ACT times to the

ordinate and heparin concentrations to the abscissa.

12. Centrifuge all samples for 5 minutes @ 2500 rpm. Remove the supernatant plasma or serum samples to a new set of labelled tubes.
13. Perform protamine sulfate titrations on all samples to determine the amount of heparin remaining in each.
14. Construct a curve relating initial heparin to residual heparin using linear graph paper. Assign initial heparin to the abscissa and residual heparin to the ordinate.

The second approach used for illustrating the need for viable platelets in the coagulation of heparinized blood was based on the effects of adenosine and ADP on platelets. ADP is known for its ability to induce platelet aggregation and release.[25] Adenosine, on the other hand, inhibits these functions.[26]

Heparinized blood was treated with adenosine (Sigma Chemical Co., St. Louis, Mo.) to render the platelets non-functional for comparison with freeze/thawed platelets. The final plasma concentrations of adenosine varied from 6 to 95 mM. Aggregometer studies indicated that in adenosine concentrations of less than 20 mM, platelets were still able to undergo at least partial aggregation before clotting occured. Concentrations of adenosine greater than 40 mM, however, were able to completely inhibit platelet aggregation, even in the presence of large amounts of thrombin sufficient to induce coagulation. The methods employed in these Aggregometer studies are detailed in Appendix 4.

ADP (sodium salt, equine muscle)(Sigma Chemical Co., St. Louis, Mo.) was added to heparinized blood to determine the effect of pre-clumped

platelets on the clotting times of these samples. ADP concentrations used were in the range of 9 to 28 mM. The ADP had been stored frozen for some time, so its actual functional concentration may have been lower. These concentrations of ADP, however, were well able to bring about platelet aggregation.

To rule out the possibility that the concentrations of ADP and adenosine used had any significant direct effect on the coagulation system, they were added to normal citrated plasma and APTT's were performed. The APTT method which was used is given in Appendix 3.

Details of the ADP-adenosine approach to the need for viable platelets are as follows:

1. Collect venous blood by clean venipuncture and divide into aliquots as follows:

   Aliquot #1 ..... Use 2 ml unheparinized blood for a baseline ACT.

   Aliquots #2-4 ..... Heparinize known quantities of blood with precise amounts of heparin. Use plastic tubes and maintain at 37°C.

   Aliquot #5 ..... Place 3-5 ml whole blood into a Vacutainer tube containing EDTA for hematocrit and platelet determinations.

2. Perform the baseline ACT on aliquot #1.
3. Divide each of aliquots #2-4 into three equal portions and treat as follows:

Portion #1 ..... Add a precise amount of adenosine. Mix and incubate at 37°C for 2 to 4 minutes.

Portion #2 ..... Add a precise amount of ADP. Mix and incubate at 37°C for 2 to 4 minutes.

Portion #3 ..... Leave untreated, but keep at 37°C until ready for further testing.

4. Perform ACT's on all samples prepared in step #3. Maintain the same ratio of blood to activator as would be used when performing a routine ACT. Carry timing out to at least 20 minutes before declaring any samples unclottable.

5. Construct a curve relating heparin concentration to ACT times using linear graph paper. Assign ACT times to the ordinate and heparin concentrations to the abscissa. (Heparin concentrations may be expressed in either U/ml whole blood or U/ml plasma, as the reaction pattern should be identical in either case. Since hematocrit values were not available for two of the individuals tested, U/ml whole blood were used for these.)

$$\frac{\text{units heparin added}}{\text{ml whole blood}} \times \frac{100}{\text{plasmacrit}} = \frac{\text{units heparin}}{\text{ml plasma}}$$

RESULTS

Response to heparin levels

Different donors showed markedly differing responses to heparin, as shown in Figure 1 (solid line) and Table 1. Plasma samples from donors 2 and 4 had very different ACT results although their heparin and platelet levels were essentially equal. Samples from donor 4 showed a much steeper slope, and hence a much greater sensitivity to heparin, than did samples from donor 2.

Effect of activation or clotting on the heparin level of plasma

Figure 2 and Table 3 show the results of tests on six individuals. Heparin levels in this series ranged from 2.2 to 8.2 U/ml plasma. Eighty-five percent of the heparin added was still active and measurable, using a modified protamine sulfate titration technique, following activation or clotting of plasmas containing viable platelets. An average of seventy-four percent of the original heparin was recovered following the activation or clotting of plasma samples containing freeze/thawed platelets.

Effect of non-viable (freeze/thawed) platelets on the ACT of heparinized PRP

Samples containing freeze/thawed platelets showed much longer ACT's than did those containing viable platelets (Tables 1 & 2, Figure 1). This effect was much more pronounced at higher heparin levels (Figure 1). Although samples with viable platelets clotted at heparin levels ranging as high as 7.2 U/ml, none of the samples with freeze/thawed platelets clotted at heparin concentrations over 2.4 U/ml.

Plasma samples which initially showed the steepest slopes on incremental addition of heparin also showed the greatest prolongation of the ACT when the platelets were rendered non-viable by freeze/thawing. For example, samples from donor 2 (Figure 1) showed initial ACT results of 6 minutes 30 seconds at a heparin concentration of 7.0 U/ml. Samples from donor 4 showed no sign of clot formation at this same heparin level, and had a 17 minute clotting time at a heparin concentration of only 4.7 U/ml, indicating the much greater sensitivity of donor 4 to heparin initially. After the platelets were freeze/thawed, samples from donor 2 had a 4 minute clotting time at a 2.3 U/ml heparin concentration, while samples from donor 4 showed no clot formation at any of the heparin concentrations tested.

Effect of adenosine on the ACT of heparinized blood

The plasma adenosine concentrations used ranged from approximately 6 to 95 mM. Increasing concentrations of adenosine progressively prolonged the ACT of heparinized blood (Figure 3, dashed lines). This effect was most apparent at the higher heparin levels. The adenosine effect decreased with decreasing heparin level, and there was no effect whatsoever in the absence of heparin, as indicated by equal ACT results at zero heparin concentration in any one donor (Figures 3 & 4). APTT's performed on normal, unheparinized plasma to which adenosine had been added gave similar results. There was no effect on coagulation of unheparinized plasma at adenosine concentrations of 25 mM or less, and only negligible effect at concentrations as high as 100 mM (Table 4).

Figure 4 gives the results of whole blood ACT's performed on samples from six donors. As in the case of the freeze/thawed platelets,

specimens initially most sensitive to heparin showed the greatest prolongation of the ACT when treated with adenosine. For instance, samples from donor 9 were initially much more sensitive to heparin than were samples from either donor 11 or 12 (solid lines). Following addition of adenosine, blood from donor 9, with a plasma adenosine concentration of only 19.8 mM, showed no clotting at heparin levels as low as 2 U/ml (dashed line), while blood from donors 11 and 12, each with adenosine concentrations greater than 80 mM, clotted at heparin levels exceeding 8 U/ml.

Effect of ADP on the ACT of heparinized blood

As was the case with adenosine, a dose-related response to ADP was very evident (Figures 3 and 4). ADP had its greatest effect on highly heparinized samples, and had negligible effect on unheparinized samples (Figure 4, Table 4).

DISCUSSION

It has long been known that PF4, an anti-heparin, is present in the granule fraction of platelets and is released following platelet aggregation and during blood coagulation. It is probable that the 15% decrease in heparin level observed following the coagulation of normal, heparinized PRP was due to the release of endogenous PF4 during the coagulation process. The more complete release of PF4 brought about during the freeze/thawing process probably accounts for the additional 11% lost from samples containing freeze/thawed platelets.

Since the freeze/thawing process releases PF3, which accelerates clotting, and PF4, which neutralizes heparin, it might be anticipated that heparinized samples containing freeze/thawed platelets would clot more readily than would samples containing viable platelets. This was not the case. This observation supports the proposal that viable platelets play a vital role in the coagulation of heparinized blood.

The results obtained using blood samples treated with adenosine provide further support. Platelets were rendered increasingly incapable of aggregating under stimulus as the adenosine concentration was raised. And likewise, increasing adenosine concentrations resulted in proportionately longer ACT's of heparinized blood.

The possibility that the adenosine was directly affecting the clotting system is unlikely since, at zero heparin concentration, the presence of adenosine did not prolong the ACT. The fact that APTT's of unheparinized plasmas were only mildly affected by high adenosine concentrations further strengthens this argument.

Although viable platelets are required for clotting of heparinized

blood, they are apparently not required for clotting of unheparinized samples. This is demonstrated by the fact that, for any one donor, ACT results at zero heparin concentration were equal, whether or not the platelets had been treated.

An hypothesis which could explain the need for viable platelets in heparinized blood samples is as follows:

> In unheparinized blood, coagulation factors are activated as clotting takes place. PF3 serves as a surface catalyst, thrombin is generated, and a fibrin clot is formed. The form in which PF3 is available is incidental to clot formation.
>
> When heparin is present, however, thrombin is neutralized as fast as it is formed. Quantities sufficient to induce fibrin formation can therefore not form in the free plasma, and prolonged or infinite clotting times result. In order for coagulation to go to completion, an environment favorable to thrombin generation must be provided.
>
> It is probable that, even in heparinized samples, the siliceous earth activity and the thrombin formed are sufficient to promote platelet aggregation, even though the thrombin levels are too low to directly affect the coagulation system.
>
> The interstices of the platelet aggregates provide an environment favorable to thrombin formation. As the platelets aggregate, plasma containing activated clotting factors and heparin is trapped within the platelet clump. PF4 is released from the platelets and, in the static environment within the

clump, is able to completely neutralize the heparin trapped there. This then allows thrombin generation to proceed catalytically. Fibrin formation can now go to completion, both within the platelet aggregate and in the plasmatic atmosphere closely surrounding the clump. The large amounts of thrombin generated overcome the heparin effect, and visible clotting occurs.

The addition of ADP to heparinized blood prior to activation produced prolonged clotting times similar to those obtained with adenosine. This would seem to indicate that, in order for a favorable environment to be provided, activated clotting factors must be incorporated into the clump at the time it is formed. It appears that, if the aggregate is formed before activation is applied, the plasma trapped within the clump is isolated and remains unactivated unless activated clotting factors can diffuse in from the surrounding free plasma.

This hypothesis has significant clinical implications. It has long been known that patients with low platelet counts are much more sensitive to heparin than are patients with normal counts. It has been thought that this is due mainly to the resultant lower PF4 levels. An even more important reason may be that, with low platelet counts, a limited number of favorable environments for thrombin generation are formed and the anticoagulant effect of heparin cannot be overcome.

Individuals vary in their response to heparin even when their platelet counts are comparable. Although this could well be attributed to variations in coagulation factor concentration and activity, it may

also be explained by the fact that malfunctioning platelets, such as those produced experimentally with adenosine and ADP, cannot undergo normal aggregation and release. An environment favorable to thrombin generation is not formed, and these individuals are more sensitive to heparin. The variation of response from individual to individual may thus be a partial expression of the function of that individual's platelets.

The implications of this hypothesis extend to the tests used in monitoring heparin therapy. The WBCT has been the method of choice for many years. The APTT, though, is being increasingly used today. Unlike the WBCT, the APTT relies on an artificial source of PF3 and has thus been thought to be independent of a patient's own platelet level. However, since platelets apparently are essential to the clotting of heparinized blood, the APTT cannot truly reflect a patient's response to heparin.

To make matters worse, the normal centrifugation procedures used for preparing plasma samples do not render these samples truly platelet free. Samples which have been centrifuged for five minutes @ 2500 rpm in preparation for APTT's may have plasma platelet counts as high as 300 x 10^3/cu mm. Attempts to run an APTT on such a sample would result in the performance of a test which more closely approximates the ACT. The variability introduced into APTT results is obvious.

For these reasons, the APTT is not the best method for monitoring the response of patients to heparin therapy. A test, such as the ACT, which is dependent on the patient's quantitative and qualitative plat platelet level, gives a more accurate indication of the actual *in vivo*

function of heparin in these patients.

TABLES

Donor #	Initial Heparin (U/ml plasma)	Platelet Count ($x10^3$/cu mm)	ACT (min, sec)
1	3.3	-	no clot @ 20'
	4.9	-	no clot @ 20'
	8.2	-	no clot @ 20'
2	2.3	536	2'30"
	4.7	550	5'00"
	7.0	468	6'30"
3	2.3	444	3'30"
	4.6	414	7'00"
	6.9	457	15-19'
4	2.3	547	5'00"
	4.7	599	17'00"
	7.0	519	no clot @ 28'
5	2.4	160	4'00"
	4.8	169	6'40"
	7.2	177	9'50"
6	2.2	172	5'00"
	4.4	189	9'25"
	6.5	119	no clot @ 23'

Table 1: ACT's of heparinized plasma samples containing viable platelets, different individuals showing differing degrees of response to heparin.

Donor #	Initial Heparin (U/ml plasma)	Initial Platelets ($x10^3$/cu mm)	Viable plts Remaining ($x10^3$/cu mm)	ACT (min, sec)
1	3.3	-	-	no clot @ 20'
	4.9	-	-	no clot @ 20'
	8.2	-	-	no clot @ 20'
2	2.3	536	9	4'00"
	4.7	550	5	no clot @ 21'
	7.0	468	5	no clot @ 21'
3	2.3	444	38	11'00"
	4.6	414	38	no clot @ 30'
	6.9	457	56	no clot @ 30'
4	2.3	547	74	no clot @ 28'
	4.7	599	57	no clot @ 28'
	7.0	519	72	no clot @ 28'
5	2.4	160	11	5'30"
	4.8	189	10	no clot @ 23'
	7.2	177	8	no clot @ 23'
6	2.2	172	14	10'00"
	4.4	189	13	no clot @ 23'
	6.5	119	11	no clot @ 23'

Table 2: ACT's of heparinized plasmas containing non-viable (freeze/thawed) platelets.

Donor #	Initial Heparin (U/ml)	VIABLE PLATELETS		NON-VIABLE PLATELETS	
		Residual Heparin (U/ml)	Residual Heparin (%)	Residual Heparin (U/ml)	Residual Heparin (%)
1	3.3	2.8	85	2.3	70
	4.9	4.2	86	3.7	76
	8.2	7.7	94	7.4	90
2	2.3	1.6	68	1.6	68
	4.7	5.5	118	2.5	53
	7.0	6.8	97	4.5	64
3	2.3	1.8	79	1.4	61
	4.6	3.2	70	3.6	79
	6.9	5.6	82	4.0	58
4	2.3	1.5	64	0.6	26
	4.7	4.0	86	3.3	71
	7.0	6.2	88	5.8	83
5	2.4	1.8	75	1.6	67
	4.8	4.4	92	4.4	92
	7.2	7.2	100	6.6	92
6	2.2	1.3	60	1.9	88
	4.4	3.2	74	3.6	83
	6.5	6.9	106	7.2	110
		Mean = 85 %		Mean = 74 %	
		S.D. = 15.1%		S.D. = 18.7%	
		C.V. = 17.8%		C.V. = 25.3%	

Table 3: Effects of viable and non-viable (freeze/thawed) platelets on sample heparin levels following activation or clotting.

Additive	APTT (secs)	INTERPRETATION
None	37	
Adenosine:		Adenosine has no direct effect on the coagulation system at concentrations of 25 mM or less, and negligible effect at concentrations of 50 mM and greater.
100 mM	44	
50 mM	43	
25 mM	39	
12.5 mM	37	
ADP:		ADP has no direct effect on the coagulation system at concentrations of 5.9 mM or less, and negligible effect at concentrations of 11.7 mM and greater.
11.7 mM	41	
5.9 mM	36	
3.0 mM	37	
1.4 mM	35	

(Platelets = 327×10^3/cu mm plasma)

Table 4: Effects of adenosine and ADP on the APTT of normal unheparinized citrated plasma

FIGURES

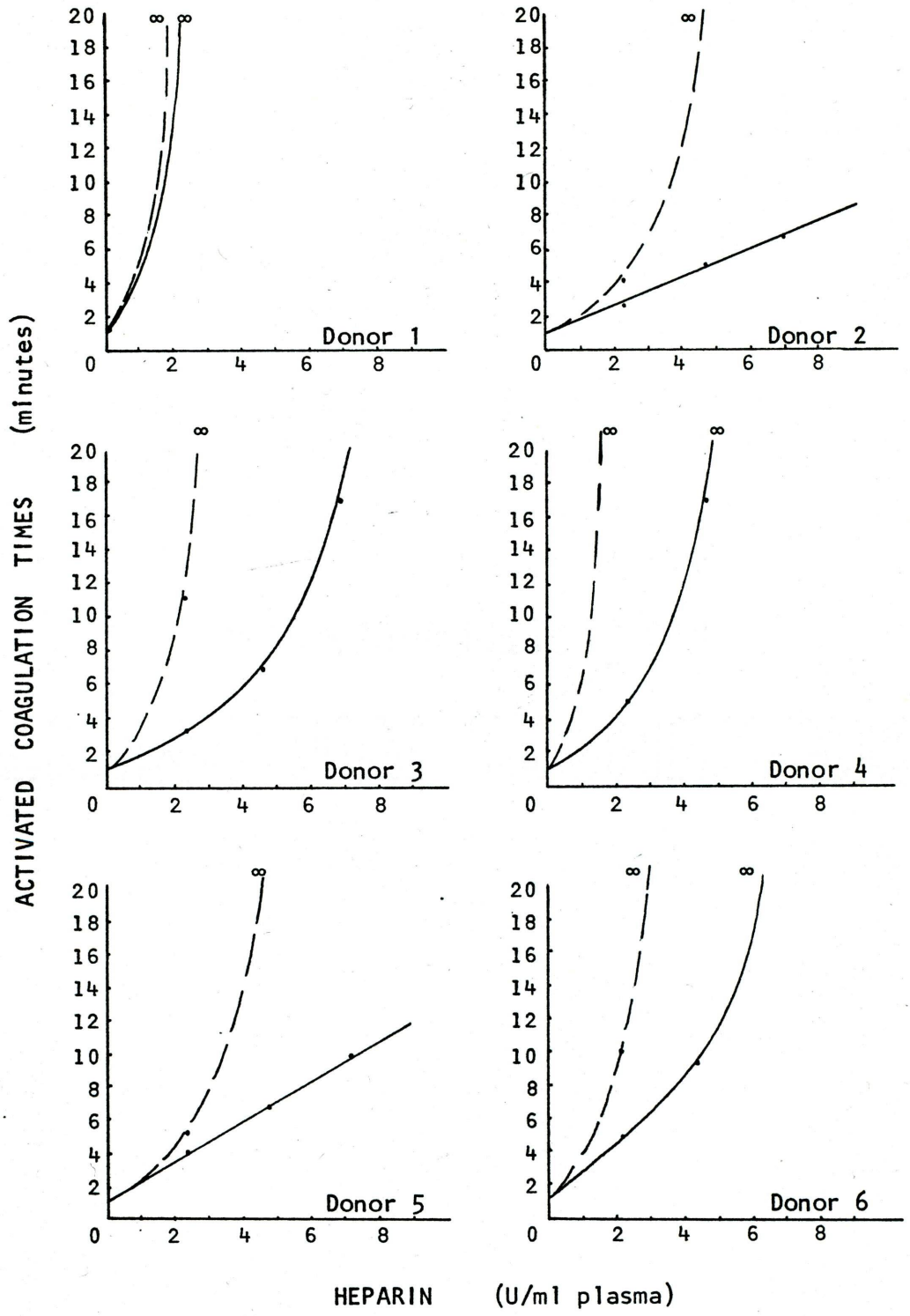

Figure 1: ACT's of heparinized plasmas containing viable platelets (———) and non-viable freeze/thawed platelets (- - -). (∞ > 20 minutes)

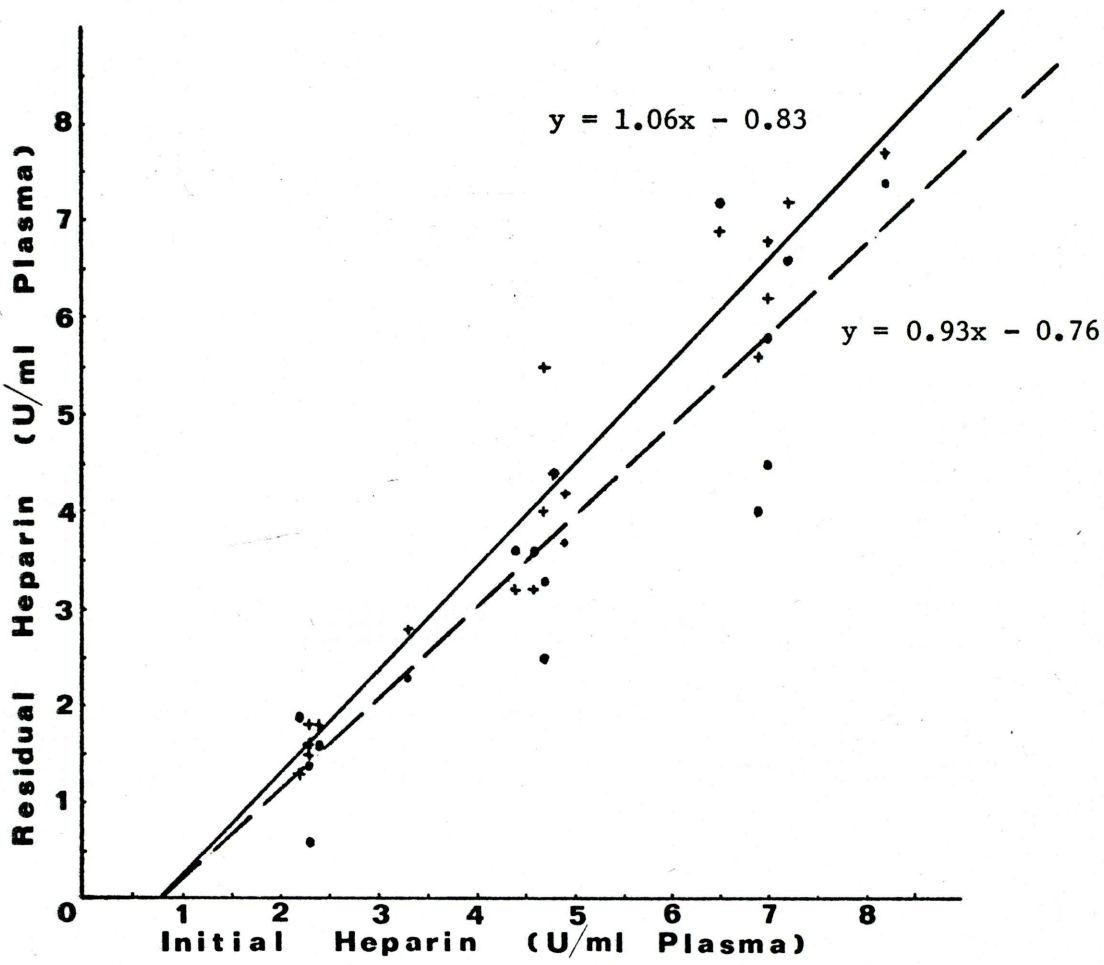

Figure 2: Relationship of initial heparin to residual heparin in a series of samples containing viable platelets (+,——) and non-viable freeze/thawed platelets (·,- -) following activation or clotting. Lines were fitted by the least mean squares technique.

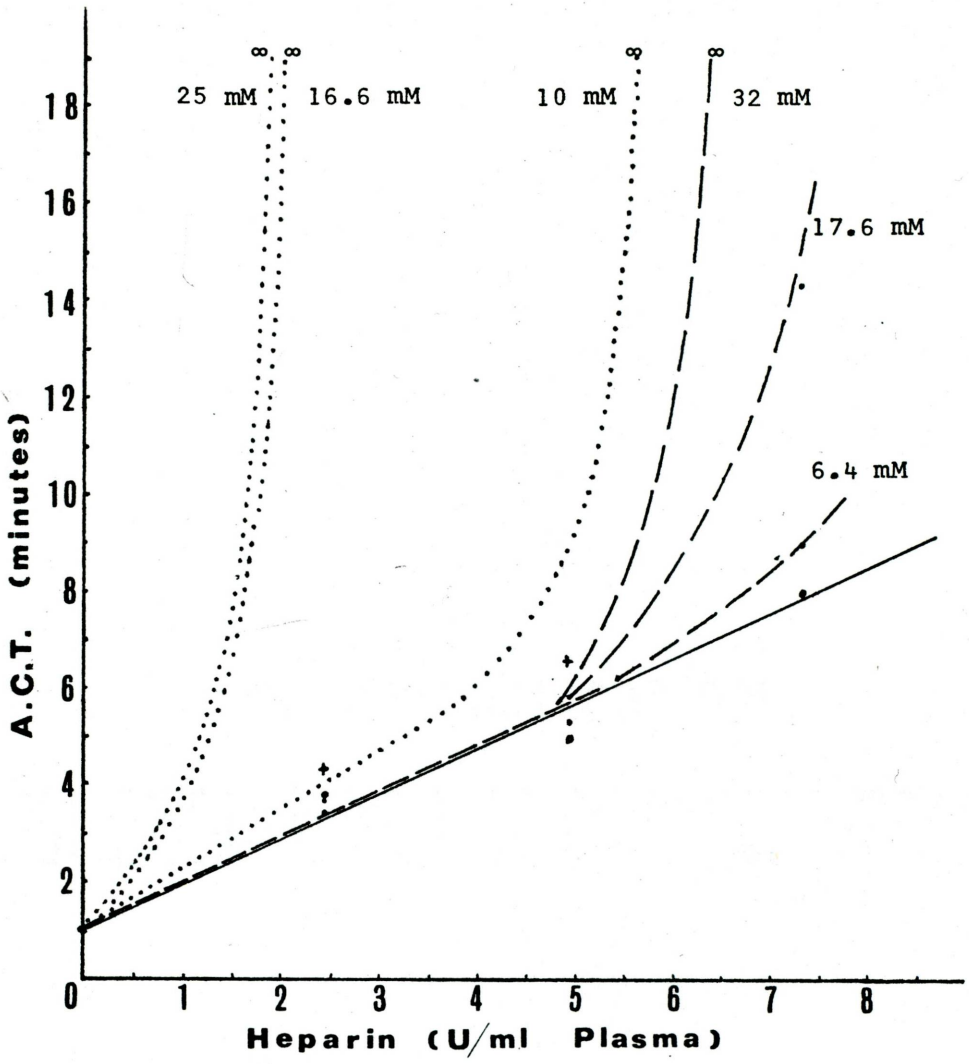

Figure 3: Dosage effect of adenosine (.,- -) and ADP (+,...) on the ACT of heparinized whole blood. Solid line (o,——) represents blood containing heparin only. Molar concentrations of ADP and adenosine are given. (∞ > 20 minutes)

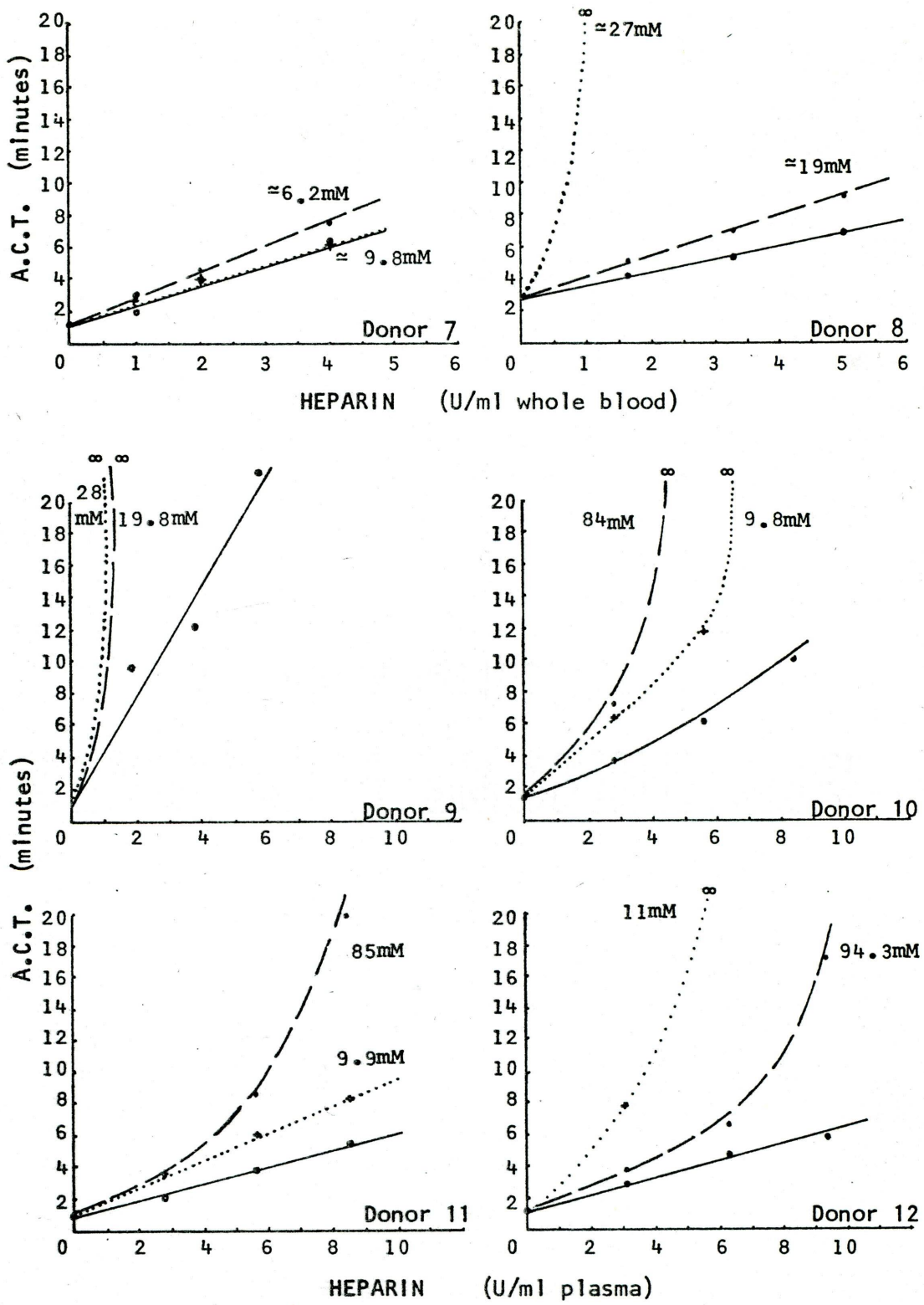

Figure 4: ACT's of blood samples containing heparin only (∘,——), heparin + adenosine (·,- -), and heparin + ADP (+,····). Molar concentrations of ADP and adenosine are given. (∞ > 20 minutes)

APPENDIX

APPENDIX 1

Protamine Sulfate Titration

Reagents

Imidazole buffer, pH 7.4 (stock solution)

1. Dissolve 1.36 gm imidazole powder (#14603, Eastman Organic Chemicals, Rochester, N.Y.) in triple-distilled water and make up to 100 ml in a volumetric flask.
2. Add 13.6 ml 0.1N HCl to 25 ml of this imidazole solution, and dilute to 100 mL with triple-distilled water.
3. Adjust to pH 7.4. Store at 1-6°C.

Imidazole-buffered saline (working solution)

Add one volume of the stock imidazole buffer (pH 7.4) to two volumes 0.85% NaCl.

Protamine sulfate, 10 mg/ml (stock solution) (Upjohn Co., Kalamazoo, Mi.)

Reconstitute the contents of one 50 mg vial of protamine sulfate with 5.0 ml bacteriostatic water in accompanying vial.

Protamine sulfate, 100 µg/ml (working stock solution)

Bring 1.0 ml stock protamine sulfate (10 mg/ml) to 100 ml with imidazole-buffered saline. Solution is stable for several months at 1-6°C.

Protamine sulfate (working serial dilutions)

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Imidazole buffered saline (ml)		1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	4.5	2.0	2.0	2.0
Protamine sulfate, 100 µg/ml (ml)	0.5	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	0.5			
Mix and transfer (ml)																				2.0	2.0	2.0
Protamine sulfate Con. (µg/ml)	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5	2.5	1

Thrombin, 100 units/ml (stock solution)

Reconstitute one bottle of Bovine Thrombin, Topical, 1000 units, (Parke, Davis & Co., Detroit, Mi.) with a mixture of 5 ml imidazole-buffered saline and 5 ml glycerol. The glycerol stabilizes the thrombin. Store at 1-6°C.

Thrombin (working solution)

Dilute an aliquot of the stock thrombin solution with imidazole-buffered saline until it gives a clotting time of 20-25 seconds when tested with the normal control plasma as shown below:[24]

1. Add 0.1 ml normal control plasma to duplicate prewarmed fibrocups.
2. Add 0.1 ml imidazole-buffered saline.
3. Warm 3 minutes.
4. Place the fibrocups under the 0.3 ml probes of a fibrometer.
5. Add 0.1 ml prewarmed thrombin dilution. START TIMER.
6. Record duplicate times to the nearest 10th of a second, and determine average time. (Duplicates should agree within one second.)

NOTE: It is important that the thrombin dilution be mixed as little as possible (no more than 3-4 inversions), and that it be made in a plastic rather than glass tube or container. If these precautions are not taken, the thrombin dilution will deteriorate at a very rapid rate.

Normal control plasma (Verify Normal Citrate, General Diagnostics, Division of Warner-Lambert Co., Morris Plains, N.J.)

Reconstitute with distilled water as directed on the vial label.

Prothrombin-free beef plasma (Hyland, Division of Travenol Laboratories Inc., Costa Mesa, Ca.)

Reconstitute with distilled water as directed on the vial label.

Procedure

The method used is a modification of that given by Bowie et al.[24] Since over half of the samples tested were sera, prothrombin-free beef plasma was used to supply fibrinogen. The procedure is performed as follows:

1. Prepare the reagents as listed above.
2. Record the times obtained when calibrating the thrombin working solution.
3. Perform protamine sulfate titrations on the test samples as follows:
    a. Add 0.1 ml of desired protamine sulfate dilution to duplicate prewarmed fibrocups.
    b. Add 0.05 ml prothrombin-free beef plasma.
    c. Add 0.05 ml test plasma or serum.
    d. Warm 3 minutes, then place fibrocups under the 0.3 ml Fibrometer probes.
    e. Add 0.1 ml prewarmed thrombin working solution and START TIMER.
    f. Record duplicate times to the nearest 0.1 second and determine the average time.

Continue testing in this manner, using various protamine sulfate concentrations, until a neutralization point for the heparin level has been obtained.

4. Retest the normal control plasma (as in calibration of the working thrombin solution) to verify that the thrombin has retained its activity. Normal control times should be within 3-4 seconds of the initial values for the thrombin working solution to be considered stable.
5. Plot the results of each titration on linear graph paper, placing clotting times on the ordinate and protamine sulfate concentrations on the abscissa.
6. Determine from the graph the most dilute concentration of protamine sulfate which is capable of completely neutralizing the heparin present in the sample (i.e. the lowest protamine sulfate concentration capable of correcting the test time to the normal control time).
7. Convert the resultant protamine sulfate concentration into its equivalent heparin concentration and multiply the results by 2 to obtain the heparin concentration in units/ml plasma or serum.

NOTE: Although 1.0 mg protamine sulfate is stated to neutralize 90 U.S.P. units of sodium heparin (beef lung), an exact relationship was obtained for each batch of reagents by heparinizing a plasma sample with a known amount of heparin, and then assaying this sample for heparin content. The resultant relationship was then used in calculating all test results.

APPENDIX 2

Activated Coagulation Times

Procedure

The method used is a modification of that given by Hattersley[27,28] and is performed as follows:

1. Place specimen into a prewarmed ACT tube and immediately start a stopwatch. A ratio of 2 ml whole blood per ACT tube is ideal and should be maintained. When plasma samples are to be tested, alter the quantity of activator in the ACT tube, if necessary, to maintain approximately the same ratio of plasma to activator as would have been present in a whole blood sample.
2. Mix well by rapid inversion and place into a 37°C heatblock.
3. Starting at 30 seconds, tilt the heatblock and tubes through a 45° angle in front of a bright light source at the following intervals:

   Clotting time < 5 minutes ..... 5-second intervals

   Clotting time = 5-10 minutes ..... 15-second intervals

   Clotting time > 10 minutes ..... 30-second intervals

   Observe for beginning clot formation. Clot detection is facilitated if the tube is rotated while it is tilted so that the blood runs down the sides of the tube. Timing should continue for at least 20 minutes before a sample is declared unclottable.

   NOTE: Tilting should be done very gently, especially when testing samples which have prolonged ACT times, as the clots are very fragile and are easily broken by too vigorous tilting.

APPENDIX 3

Activated Partial Thromboplastin Time [29,30]

Reagents

Platelin Plus Activator (General Diagnostics, Division of Warner-Lambert Co., Morris Plains, N.J.)

Reconstitute with distilled water as directed on the vial label.

Calcium chloride, 0.02M (Dade Division American Hospital Supply Corp., Miami, Fla.)

Normal control plasma (Verify Normal Citrate, General Diagnostics, Division of Warner-Lambert Co., Morris Plains, N.J.)

Reconstitute with distilled water as directed on the vial label.

Procedure

1. Prepare the reagents as listed above.
2. Pour approximately 1 ml $CaCl_2$ into a clean test tube, and place it in a 37°C heatblock to warm.
3. Add 0.1 ml well-shaken Platelin Plus Activator to duplicate fibrocups. Let warm 3-4 minutes.
4. Add 0.1 ml plasma (control or test). Start stopwatch.
5. Incubate 5 minutes at 37°C, mixing well at the beginning and again at the end of the incubation period, then place under the 0.3 ml Fibrometer probes.
6. Add 0.1 ml prewarmed $CaCl_2$. START TIMER.
7. Record duplicate times to the nearest 10th of a second, and report average time to the nearest second.

APPENDIX 4

Aggregometer Check of Adenosine Potency

Reagents

Thrombin, Topical, 1000 units (Parke, Davis & Co., Detroit, Mi.)

Adenosine (Sigma Chemical Co., St. Louis, Mo.)

Procedure

To determine the amount of adenosine sufficient to prevent clumping of platelets, even in the presence of high concentrations of thrombin, the following was performed, using a Payton Aggregometer.

1. Calibrate the Aggregometer output to least transmittance with PRP, and to greatest transmittance with PPP.
2. Perform the following steps with different concentrations of thrombin to determine the concentration of thrombin sufficient to induce complete and irreversible platelet aggregation without inducing coagulation (approximately 0.18 U/ml should be sufficient).
    a. Place 0.5 ml PRP into a suitable cuvette, and insert the cuvette into the Aggregometer slot.
    b. Add a magnetic stirrer and set mixing speed to 1100 rpm. Wait 2-3 minutes until temperature and graph plot have stabilized.
    c. Add 0.05 ml thrombin of known concentration and observe the effect on the graph.

    When the desirable thrombin concentration has been determined, continue with the following steps.
3. Place 0.5 ml fresh PRP in Aggregometer and wait until temperature

and graph plot have stabilized.

4. Add 0.05 ml adenosine of known concentration and observe the effect on the graph. Wait until the graph reading has stabilized.

5. Add 0.05 ml thrombin in a concentration sufficient to induce aggregation but not coagulation, and observe the effect on the graph. If there is no change in the graph reading, continue adding increasing amounts of thrombin until either the platelets aggregate or the plasma clots.

Interpretation

If the platelets aggregate before the plasma clots, the adenosine concentration is insufficient. Steps 3-5 must then be repeated with increasing concentrations of adenosine.

If the plasma clots before aggregation takes place, repeat steps 3-5 using lower concentrations of adenosine to determine if adenosine is present in just adequate amounts or in large excess.

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