

OFFICE OF NAVAL RESEARCH  
CONTRACT N00014-88-C-0118

TECHNICAL REPORT 92-06

THE PLATELET FUNCTION DEFECT OF CARDIOPULMONARY BYPASS

BY

A.S. KESTIN, C.R. VALERI, S.F. KHURI, J. LOSCALZO, P.A. ELLIS,  
H. MACGREGOR, V. BIRJINIUK, H. OUMET, B. PASCHE, M.J. GIOMBETTI,  
S.E. BENOIT, L.J. RODINO, M.R. BARNARD, AND A.D. MICHELSON

NAVAL BLOOD RESEARCH LABORATORY  
BOSTON UNIVERSITY SCHOOL OF MEDICINE  
615 ALBANY STREET  
BOSTON, MA 02118

24 NOVEMBER 1992

Reproduction in whole or in part is permitted for any purpose of  
the United States Government.

Distribution of this report is unlimited.

19990225185

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                       | READ INSTRUCTIONS<br>BEFORE COMPLETING FORM                 |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|-------------------------------------------------------------|
| 1. REPORT NUMBER<br>NBRL, BUSM 92-06                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | 2. GOVT ACCESSION NO. | 3. RECIPIENT'S CATALOG NUMBER                               |
| 4. TITLE (and Subtitle)<br>THE PLATELET FUNCTION DEFECT OF CARDIO-PULMONARY BYPASS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                       | 5. TYPE OF REPORT & PERIOD COVERED<br>Technical Report      |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                       | 6. PERFORMING ORG. REPORT NUMBER                            |
| 7. AUTHOR(s)<br>Anita S. Kestin, C. Robert Valeri, Shukri F. Khuri, Joseph Loscalzo, Patricia A. Ellis, Hollace MacGregor, Vladimir Birjiniuk, Helene Ouimet                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                       | 8. CONTRACT OR GRANT NUMBER(s)<br>N00014-88-C-0118          |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS<br>Naval Blood Research Laboratory<br>Boston University School of Medicine<br>615 Albany St., Boston, MA 02118                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                       | 10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS |
| 11. CONTROLLING OFFICE NAME AND ADDRESS<br>Naval Medical Research and Development Command<br>Bethesda, MD 20814                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                       | 12. REPORT DATE<br>24 November 1992                         |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                       | 13. NUMBER OF PAGES<br>53                                   |
| 14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)<br>Bureau of Medicine and Surgery<br>Department of the Navy<br>Washington, D.C. 20372                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                       | 15. SECURITY CLASS. (of this report)<br>Unclassified        |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                       | 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE                  |
| 16. DISTRIBUTION STATEMENT (of this Report)<br>Approved for public release and sale. Distribution unlimited.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                       |                                                             |
| 17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                       |                                                             |
| 18. SUPPLEMENTARY NOTES<br>Item 7: Boris Pasche, Martha J. Giombetti, Stephen E. Benoit, Louis J. Rodino, Marc R. Barnard, and Alan D. Michelson<br>Item 9: Departments of Pediatrics and Surgery, University of Massachusetts Medical School, Worcester, MA                                                                                                                                                                                                                                                                                                                                                                      |                       |                                                             |
| 19. KEY WORDS (Continue on reverse side if necessary and identify by block number)<br>Platelets                      Bleeding time<br>Blood                            Thromboxane B2<br>Cardiopulmonary bypass      Thrombin<br>Flow cytometry                Heparin                                                                                                                                                                                                                                                                                                                                                            |                       |                                                             |
| 20. ABSTRACT (Continue on reverse side if necessary and identify by block number)<br>The use of cardiopulmonary bypass (CPB) during cardiac surgery is associated with a hemostatic defect, the hallmark of which is a markedly prolonged bleeding time. However, the nature of the putative platelet function defect is controversial. In this study, blood from 16 patients was collected and analyzed at 10 time points before, during and after CPB. We utilized a whole blood flow cytometric assay to study platelet surface glycoproteins in a) peripheral blood, b) peripheral blood activated in vitro by either phorbol |                       |                                                             |

DD FORM 1 JAN 73 1473

EDITION OF 1 NOV 65 IS OBSOLETE  
S/N 0102-LF-014-6601

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

myristate acetate, the thromboxane (TX) A2 analogue U46619, or a combination of adenosine diphosphate and epinephrine, and c) the blood emerging from a bleeding time wound (shed blood). Activation-dependent changes were detected by monoclonal antibodies directed against GPIb, the GPIIb-IIIa complex, and P-selectin. In addition, we measured plasma glyocalicin (a proteolytic fragment of GPIb) and shed blood TXB2 (a stable breakdown product of TXA2). In shed blood emerging from a bleeding time wound, the usual time-dependent increase in platelet surface P-selectin was absent during CPB, but returned to normal within 2 hours. This abnormality paralleled both the CPB-induced prolongation of the bleeding time and a CPB-induced marked reduction in shed blood TXB2 generation. In contrast, there was no loss of platelet reactivity to in vitro agonists during or after CPB. In peripheral blood, platelet surface P-selectin was negligible at every time point, demonstrating that CPB resulted in virtually no circulating degranulated platelets. CPB did not change the platelet surface expression of GPIb in peripheral blood, as determined by the platelet binding of a panel of monoclonal antibodies, ristocetin-induced binding of von Willebrand factor, and a lack of increase in plasma glyocalicin. CPB did not change the platelet surface expression of the GPIIb-IIIa complex in peripheral blood, as determined by the platelet binding of fibrinogen and a panel of monoclonal antibodies. In summary, CPB resulted in: 1) no loss of the platelet surface GPIb-IX complex, 2) no loss of the platelet surface GPIIb-IIIa complex, 3) a minimal number of circulating degranulated platelets, 4) normal platelet reactivity in vitro, and 5) markedly deficient platelet reactivity in vivo. These data suggest that the "platelet function defect" of CPB is not a defect intrinsic to the platelet but is an extrinsic defect such as an in vivo lack of availability of platelet agonists. The near universal use of heparin during CPB is likely to contribute substantially to this defect via its inhibition of thrombin, the preeminent platelet activator.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

## ABSTRACT

The use of cardiopulmonary bypass (CPB) during cardiac surgery is associated with a hemostatic defect, the hallmark of which is a markedly prolonged bleeding time. However, the nature of the putative platelet function defect is controversial. In this study, blood from 16 patients was collected and analyzed at 10 time points before, during and after CPB. We utilized a whole blood flow cytometric assay to study platelet surface glycoproteins in a) peripheral blood, b) peripheral blood activated *in vitro* by either phorbol myristate acetate, the thromboxane (TX)  $A_2$  analogue U46619, or a combination of adenosine diphosphate and epinephrine, and c) the blood emerging from a bleeding time wound (shed blood). Activation-dependent changes were detected by monoclonal antibodies directed against GPIb, the GPIIb-IIIa complex, and P-selectin. In addition, we measured plasma glycocalicin (a proteolytic fragment of GPIb) and shed blood  $TXB_2$  (a stable breakdown product of  $TXA_2$ ). In shed blood emerging from a bleeding time wound, the usual time-dependent increase in platelet surface P-selectin was absent during CPB, but returned to normal within 2 hours. This abnormality paralleled both the CPB-induced prolongation of the bleeding time and a CPB-induced marked reduction in shed blood  $TXB_2$  generation. In contrast, there was no loss of platelet reactivity to *in vitro* agonists during or after CPB. In peripheral blood, platelet surface P-selectin was negligible at every time point, demonstrating that CPB resulted in virtually no circulating degranulated platelets. CPB did not change the platelet surface expression of GPIb in peripheral blood, as determined by the platelet binding of a panel of monoclonal antibodies, ristocetin-induced binding of von Willebrand factor, and a lack of increase in plasma glycocalicin. CPB did not change the platelet surface expression of the GPIIb-IIIa complex in peripheral blood, as determined by the platelet binding of fibrinogen and a panel of monoclonal antibodies. In

summary, CPB resulted in: 1) no loss of the platelet surface GPIb-IX complex, 2) no loss of the platelet surface GPIIb-IIIa complex, 3) a minimal number of circulating degranulated platelets, 4) normal platelet reactivity *in vitro*, and 5) markedly deficient platelet reactivity *in vivo*. These data suggest that the "platelet function defect" of CPB is not a defect intrinsic to the platelet but is an extrinsic defect such as an *in vivo* lack of availability of platelet agonists. The near universal use of heparin during CPB is likely to contribute substantially to this defect via its inhibition of thrombin, the preeminent platelet activator.

## INTRODUCTION

The use of cardiopulmonary bypass (CPB) during cardiac surgery is associated with a generalized hemorrhagic defect.<sup>1</sup> Although thrombocytopenia of mild degree and alterations in the fibrinolytic and coagulation systems occur during CPB,<sup>1</sup> a platelet function defect is generally considered to be the primary CPB-induced hemostatic deficit.<sup>1-3</sup> CPB consistently results in a reversible marked prolongation of the bleeding time<sup>1-4</sup> and many,<sup>4-11</sup> but not all,<sup>12,13</sup> studies have reported defects in *in vitro* platelet aggregation. However, the nature of the putative platelet function defect associated with CPB remains controversial. Three types of intrinsic platelet defects have been reported in association with CPB. First, some,<sup>4,7,14</sup> but not other,<sup>15-18</sup> studies have reported that CPB results in partial platelet degranulation. Second, some,<sup>7,17,19</sup> but not all,<sup>20</sup> studies have reported CPB-induced defects in the platelet surface glycoprotein (GP) Ib-IX complex. Third, some,<sup>7,13,16,17</sup> but not other,<sup>18,19</sup> studies have reported defects in the platelet surface GPIIb-IIIa complex. In the present study, whole blood flow cytometry was utilized to circumvent many of the potential methodologic problems of other assays. Evidence is presented that the CPB-induced platelet dysfunction is the result of a defect extrinsic to the platelet.

## METHODS

### *Study Population*

The study was approved by the Human Subjects Committee of the Brockton\West Roxbury Veterans Administration Medical Center. Twenty patients with angiographically documented coronary artery disease referred to the Brockton\West Roxbury Veterans Administration Medical Center for coronary artery revascularization were entered into the study after written informed consent was obtained. All patients were undergoing their first open heart procedure. All patients required coronary artery bypass grafting, while one required concomitant valvular replacement. No patient had a history suggestive of an underlying hemostatic disorder. The age of the subjects in the study was  $62 \pm 2.3$  years (mean  $\pm$  S.E.M.,  $n = 20$ ). Nineteen of the 20 subjects were male. All patients underwent a standard CPB procedure using a membrane oxygenator. The mean aortic cross-clamp time was  $50.4 \pm 4.4$  minutes and the mean duration of CPB was  $109.9 \pm 7.2$  minutes. Patients received intravenous heparin at an initial dose of 4 mg/kg, followed by additional doses as necessary to maintain the activated clotting time (ACT) greater than or equal to 999 seconds. At the completion of CPB, heparin was reversed with protamine sulfate. Heparin reversal was verified by confirming that the ACT had returned to the preoperative value. Maximal hypothermia during CPB was a core temperature of  $26.8 \pm 1.0$  degrees Celsius, as determined by a bladder thermometer (Bard, Boston, MA). Seven patients required no exogenous blood components intraoperatively, but most patients received autologous blood harvested intraoperatively using a cell saver (Haemonetics Corp., Braintree, MA). None of the patients had excessive perioperative bleeding, as defined by transfusion of greater than 10 units of blood in the perioperative period.<sup>1</sup> The number of units of packed red blood cells transfused in the perioperative period was  $1.8 \pm 0.4$  (mean  $\pm$  S.E.M.). The

maximum number of units of packed red blood cells transfused into any one patient was 6 and this patient was found to have surgical bleeding. Data from this patient did not differ statistically from that of the other subjects and was therefore included in the analysis. No patient received a platelet transfusion before or during CPB.

### ***Blood Sampling Time Points***

Peripheral venous blood and the shed blood emerging from a standardized bleeding time wound (Simplate II, Organon Teknika, Durham NC) were collected before, during, and after CPB at the time points listed in Table 1. Not every patient had all studies performed at all time points.

### ***Murine Monoclonal Antibodies***

S12 (provided by Dr. Rodger P. McEver, University of Oklahoma) is a monoclonal antibody directed against P-selectin.<sup>21,22</sup> P-selectin, also referred to as GMP-140,<sup>21</sup> platelet activation-dependent granule-external membrane (PADGEM) protein,<sup>23</sup> and CD62,<sup>24</sup> is a component of the  $\alpha$  granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet secretion.<sup>25</sup>

A panel of platelet GPIb-IX-specific monoclonal antibodies was utilized. 6D1 (provided by Dr. Barry S. Coller, SUNY, Stony Brook) is directed against the von Willebrand factor binding site on the glycosialicin portion of the  $\alpha$ -chain of GPIb.<sup>26,27</sup> TM60 (provided by Dr. Naomasa Yamamoto, Tokyo Metropolitan Institute of Medical Science) is directed against the thrombin binding site on the amino terminal domain of the  $\alpha$  chain of GPIb.<sup>28-30</sup> AK1 (provided by Dr. Michael C. Berndt, Baker Institute, Melbourne, Australia) is directed against the GPIb-IX complex.<sup>31</sup> AK1 only



binds to the intact GPIb-IX complex, not to uncomplexed GPIb or GPIX.<sup>31</sup> FMC25 (provided by Dr. Berndt) is directed against GPIX.<sup>32,33</sup>

A panel of platelet GPIIb-IIIa-specific monoclonal antibodies was utilized. Y2/51 (DAKO, Carpinteria, CA) is directed against platelet membrane GPIIIa.<sup>34</sup> 7E3 (provided by Dr. Collier), 10E5 (provided by Dr. Collier), and M148 (Cymbus Bioscience, Southampton, U.K.) are directed against different epitopes near the fibrinogen binding site on the GPIIb-IIIa complex.<sup>35-38</sup> PAC1 (provided by Dr. Sanford J. Shattil, University of Pennsylvania, Philadelphia) is directed against the fibrinogen binding site exposed on the GPIIb-IIIa complex of activated platelets.<sup>39</sup> Unlike Y2/51, M148, 7E3, and 10E5,<sup>35-37</sup> PAC1 does not bind to resting platelets.<sup>39</sup>

OKM5 (provided by Dr. Patricia Rao, Ortho Diagnostic Systems, Raritan, NJ) is directed against platelet membrane GPIV.<sup>40</sup>

#### *Flow Cytometric Analysis of Platelet Surface Glycoproteins in Peripheral Blood*

A whole blood flow cytometric method was utilized. The method has previously been described in detail<sup>41</sup> and includes no centrifugation, gel filtration, vortexing, or stirring steps that could artefactually activate platelets. Briefly, the method was as follows. The first 2 mL of blood drawn were discarded and then blood was drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ). Within 15 minutes, the anticoagulated blood was: a) diluted in modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM MgCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose, pH 7.4), and b) incubated at 22°C with an agonist (either phorbol myristate acetate [PMA] [Sigma, St. Louis, MO], the stable thromboxane A<sub>2</sub> analogue U46619 [Cayman Chemical, Ann Arbor, MI], purified human  $\alpha$ -thrombin [provided by Dr. John Fenton II, New York Department of Health, Albany] together with 2.5 mM glycyl-L-

prolyl-L-arginyl-L-proline [Calbiochem, San Diego, CA] [an inhibitor of fibrin polymerization<sup>41</sup>], or a combination of adenosine diphosphate [ADP] [Bio/Data Corp., Hatboro, PA] and epinephrine [Sigma]) or buffer only. In kinetic studies, the samples were then fixed with 1% formaldehyde at various time points after the addition of the agonist. In all other studies, fixation was performed 15 minutes after the addition of the agonist, as previously described.<sup>41</sup> After fixation, all samples were diluted and incubated at 22°C for 15 minutes with a) a saturating concentration of a biotinylated monoclonal antibody (directed against either P-selectin, the GPIIb-IIIa complex, or the GPIb-IX complex) and b) a saturating concentration of either fluorescein isothiocyanate (FITC)-conjugated GPIV-specific monoclonal antibody OKM5 or FITC-conjugated GPIIIa-specific monoclonal antibody Y2/51. The samples were then incubated at 22°C for 15 minutes with phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA). (When monoclonal antibody M148 was used, the incubation step with phycoerythrin-streptavidin was unnecessary because the antibody was purchased directly conjugated with R-phycoerythrin.) Within 24 hours of antibody tagging, the samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL). After identification of platelets by gating on both FITC positivity and their characteristic light scatter, binding of the biotinylated monoclonal antibody was determined by analyzing 5,000 individual platelets for phycoerythrin fluorescence. In addition to platelets, OKM5 binds to monocytes<sup>40</sup> but these were completely gated out by size (forward light scatter). We<sup>41</sup> and others<sup>42</sup> have shown that this method results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody tagging. In addition to our standard method of determination of binding by fluorescence intensity relative to a maximally degranulated platelet,<sup>41</sup> some samples were analyzed, as indicated in Results, by the percentage of P-selectin-positive platelets. The percentage of P-selectin-positive platelets at different time points during CPB was

defined as the percentage of platelets that had an S12 fluorescence greater than 98% of the platelets in samples from the preoperative time point (without an added exogenous platelet agonist).

In a separate subgroup of patients, the response of washed platelets to thrombin was studied. Peripheral blood was collected as described above and the platelets separated and washed in modified Tyrode's buffer as previously described.<sup>43</sup> The platelets ( $75,000/\mu\text{L}$ ) were incubated with 0.05, 0.1, or 1 U/mL of purified human  $\alpha$ -thrombin or buffer only, and then analyzed by flow cytometry for the binding of a biotinylated monoclonal antibody (either S12, PAC1, 7E3, or 6D1) as previously described.<sup>44</sup>

#### *Flow Cytometric Analysis of Platelet Surface P-selectin in Shed Blood*

The platelet surface expression of P-selectin in blood emerging from a standardized bleeding time wound was analyzed by the whole blood flow cytometric method. The method has been previously described in detail.<sup>41</sup> Duplicate standardized bleeding times were performed on the forearm of patients with a Simplate II device (General Diagnostics, Durham, NC). The blood emerging from the bleeding time wound (shed blood) was collected with a micropipet at 2 minute intervals until the bleeding stopped. After each pipetting, any residual blood at the bleeding time wound site was removed with filter paper. Immediately after collection at each time point, the pipetted blood was added to a microfuge tube containing sodium citrate, fixed for 30 minutes at  $22^{\circ}\text{C}$  with an equal volume of 2% formaldehyde, and diluted 1:10 by volume in modified Tyrode's buffer. As described above, the fixed diluted whole blood samples were then labeled with the FITC-conjugated GPIV-specific monoclonal antibody OKM5 and the biotinylated P-selectin-specific monoclonal antibody S12, and the individual platelets analyzed in an EPICS Profile flow cytometer to assess S12 binding.

### *Plasma Glycocalicin Assay*

Plasma glycocalicin was determined by a modified version of a previously described competitive inhibition assay.<sup>45,46</sup> A subsaturating concentration of FITC-conjugated monoclonal antibody 6D1 (1.2  $\mu\text{g/mL}$ ) was incubated at 22°C for 20 minutes with either: a) test plasma that had been filtered through a 0.22  $\mu\text{m}$  Acrodisc (Gelman, Ann Arbor, MI) and the pH buffered to 7.4; or b) various concentrations of purified glycocalicin (prepared as previously described<sup>27</sup>). Samples were then incubated at 22°C for 20 minutes with fixed, washed platelets (final concentration 100,000/ $\mu\text{L}$ ) and diluted 20-fold in modified Tyrode's buffer, pH 7.4 before the platelet binding of 6D1 was analyzed by flow cytometry. Linear regression analysis was used to generate a standard curve from 0 to 70 nM from the purified glycocalicin samples. The plasma glycocalicin concentration of unknown plasma samples was derived from this standard curve.

### *Ristocetin-induced Binding of von Willebrand Factor to Platelets*

The ristocetin-induced binding of normal von Willebrand factor to washed platelets from the patient was determined by the following method. The patient's platelets were washed as previously described,<sup>43</sup> fixed in 1% formaldehyde, and resuspended at a concentration of 75,000/ $\mu\text{L}$  in modified Tyrode's buffer, pH 7.4. Twenty  $\mu\text{L}$  of the platelet suspension was incubated at 22°C for 15 minutes with 20  $\mu\text{L}$  of pooled platelet-poor plasma from normal donors (as a source of von Willebrand factor) and 5  $\mu\text{L}$  of ristocetin (BioData, Horsham, PA) (final concentration 1.4 mg/mL). The mixture was then incubated at 22°C for 15 minutes with 0.028 mg/mL of either polyclonal FITC-conjugated anti-von Willebrand factor goat IgG antibody (Atlantic Antibodies, Stillwater, MN) or FITC-conjugated non-specific goat IgG (Atlantic Antibodies). The sample was then diluted 16-fold in modified Tyrode's buffer, pH 7.4 and analyzed for green fluorescence by flow cytometry.

The fluorescence of the sample incubated with the non-specific goat IgG was subtracted from the sample incubated with the anti-von Willebrand factor antibody.

#### ***von Willebrand Factor Assay***

The plasma concentration of von Willebrand factor was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Asserachrom vWF, Diagnostica Stago, Asnieres-Sur-Seine, France).

#### ***Fibrinogen Binding to Platelets***

Fibrinogen binding to ADP-stimulated washed platelets was determined by a direct binding assay using radioiodinated fibrinogen, as previously described.<sup>47</sup>

#### ***Radioimmunoassay of Shed Blood Thromboxane B<sub>2</sub>***

The method has been previously described.<sup>48</sup> A standardized bleeding time wound was performed, as described above. The shed blood emerging from the wound was aspirated through a blunt needle into a tuberculin syringe coated with heparin (1,000 U/mL) and containing 20  $\mu$ L of ibuprofen for each 1 mL of blood (1.9 mg/mL). Samples were collected every 30 seconds until a 600  $\mu$ L aliquot of blood was obtained. The thromboxane B<sub>2</sub> concentration of the aliquot was determined with an RIA kit (New England Nuclear, Boston, MA).

#### ***Hematocrit and Platelet Count***

Hematocrit and platelet counts were measured using a Coulter J.T. Electronic Particle Counter (Coulter Electronics, Hialeah, FL).

*Statistical Analysis*

All statistical analyses were performed using Systat (Systat Inc., Evanston, IL) version 5.02. The reported p values were generated using either paired Student's t-test analysis or ANOVA for repeated measures, as indicated. Unless otherwise stated, all p values were generated by comparing measurements of a given parameter in blood obtained during bypass to measurements of the same parameter before and after bypass. Statistical significance was assumed for p values less than or equal to 0.05.

## RESULTS

### *Effect of CPB on the Bleeding Time and Platelet Count*

The bleeding time was modestly prolonged by heparin administration ( $10.4 \pm 0.5$  minutes) (mean  $\pm$  S.E.M.,  $n = 16$ ), markedly prolonged during CPB ( $33.6 \pm 3.7$  minutes), and again only modestly prolonged immediately after the completion of CPB (Fig 1, panel A).

A platelet function defect appeared to be the basis for this marked prolongation of the bleeding time during CPB, because a) the platelet count declined to  $166.1 \pm 12.7 \times 10^3/\mu\text{L}$  after the start of CPB and remained at only marginally thrombocytopenic levels at all time points up to and including 24 hours after the completion of CPB (Fig 1, panel B), and b) von Willebrand factor levels remained within or above the normal range at all time points (Fig 1, panel C).

### *Effect of CPB on the Platelet Surface GPIb-IX Complex*

The platelet surface expression of the GPIb-IX complex can be markedly reduced by platelet activation<sup>41,43,49</sup> and washing.<sup>50</sup> In order to avoid these potential artifacts, we utilized a whole blood flow cytometric method that did not require any centrifugation, gel filtration, vortexing, or stirring steps.<sup>41</sup> As determined by this method, CPB did not result in significant change in the platelet surface expression of the GPIb-IX complex, irrespective of whether the monoclonal antibody utilized was directed against the von Willebrand factor binding site on GPIb (Fig 2, panel A), the thrombin binding site on GPIb (Fig 2, panel B), GPIX (Fig 2, panel C), or the GPIb-IX complex (Fig 2, panel D).

In addition, we examined the ristocetin-induced binding of von Willebrand factor to platelets. This binding, which reflects the availability of GPIb as a platelet surface receptor for von Willebrand factor,<sup>51,52</sup> was unchanged during and after CPB (Fig 2, panel E).

Furthermore, we assayed the patients' plasma for glycocalicin, a proteolytic product of GPIb.<sup>53,54</sup> Plasma glycocalicin did not rise above the preoperative concentration at any time point during or after CPB (Fig 3). The observed reduction in plasma glycocalicin during CPB was probably dilutional because the reduction paralleled the reductions in hematocrit (Fig 3) and serum albumin (data not shown).

In summary, these data demonstrate that CPB did not result in a loss of the platelet surface GPIb-IX complex, as determined by a) a panel of GPIb-IX-specific monoclonal antibodies in a whole blood assay, b) ristocetin-induced binding of von Willebrand factor, and c) plasma glycocalicin.

#### ***Effect of CPB on the Platelet Surface GPIIb-IIIa Complex***

The platelet surface GPIIb-IIIa complex was analyzed by whole blood flow cytometry. CPB did not result in any significant change in the platelet surface expression of the GPIIb-IIIa complex, as determined by monoclonal antibodies (7E3, 10E5, and M148) directed against different epitopes near the fibrinogen binding site on the GPIIb-IIIa complex and a monoclonal antibody (Y2/51) directed against GPIIIa (Fig 4, panels A, B, C, and D).

Direct measurement of the binding of exogenous fibrinogen to platelets activated *in vitro* with ADP (reflecting exposure of the fibrinogen binding site on the GPIIb-IIIa complex<sup>55</sup>) demonstrated an increase in the  $K_D$  from 0.43 to 0.54  $\mu\text{M}$  and no loss of binding sites during CPB (Fig 4, panel E).



### *Effect of CPB on Platelet Degranulation*

The presence of activated platelets circulating in peripheral blood was investigated by the platelet binding of S12, a monoclonal antibody directed against P-selectin. Again, in order to avoid artefactual platelet activation during the separation of platelets, a whole blood flow cytometric assay was utilized.<sup>41</sup> CPB did not result in any significant overall increase in P-selectin expression on the surface of circulating platelets (Fig 5, panel A, closed circles). Analysis of the same data in terms of the percentage of circulating platelets that were P-selectin-positive resulted in a minimal (but statistically significant) increase during CPB. Thus, the percentage of circulating platelets that were P-selectin-positive were:  $2.0 \pm 0.0\%$  (mean  $\pm$  S.E.M.,  $n = 14$ ) preoperatively,  $3.1 \pm 0.3\%$  at 45 minutes after the start of CPB ( $p = 0.0054$  compared to preoperatively by paired Student t-test), and  $2.9 \pm 0.3\%$  at the completion of CPB, immediately following the administration of protamine ( $p = 0.0078$  compared to preoperatively).

### *Effect of CPB on Platelet Reactivity in Vitro*

In the whole blood flow cytometric assay, CPB did not significantly affect the up-regulation of platelet surface of P-selectin induced *in vitro* by either PMA (Fig 5, panel A), the stable thromboxane  $A_2$  analogue U46619 (Fig 6, panel A), or a combination of ADP and epinephrine (Fig 6, panel A). Similarly, CPB did not significantly affect the down-regulation of the platelet surface GPIb-IX complex or the up-regulation of the platelet surface GPIIb-IIIa complex induced *in vitro* by either PMA, U46619, or ADP/epinephrine (Fig 5, panel B and Fig 6, panels B and C). CPB did not alter the kinetics of up-regulation of P-selectin (Fig 7, panel A), down-regulation of GPIb-IX complex (Fig 7, panel B), or up-regulation of the GPIIb-IIIa complex (Fig 7, panel C).

Interestingly, administration of heparin appeared to augment both the PMA-induced up-regulation of P-selectin (Fig 5, panel A), and down-regulation of the GPIb-IX complex (Fig 5, panel B). Protamine sulfate administration appeared to reverse this augmentation (Fig 5, panels A and B).

In order to assess the reactivity of platelets to thrombin, the patients' platelets were washed free of heparin. CPB did not inhibit the thrombin-induced up-regulation of platelet surface P-selectin, up-regulation of the GPIIb-IIIa complex, or down-regulation of the GPIb-IX complex (Fig 8, panels A, B, C, and D). However, as expected, when the thrombin reactivity of platelets in whole blood containing the therapeutically administered heparin was measured, platelets were completely unreactive to thrombin at the following time points: 5 minutes after heparin administration, but before the start of CPB; after the start of CPB (normothermic conditions); beginning of maximal hypothermia on CPB; and 45 minutes after the start of CPB (hypothermic conditions) (data not shown).

Monoclonal antibody PAC1 does not bind to resting platelets, only to activated platelet that have exposed their fibrinogen binding site on the GPIIb-IIIa complex.<sup>39</sup> Therefore, the studies with PAC1 (Fig 8, panel D) demonstrate that a) CPB did not result in significant exposure of the fibrinogen binding site on the GPIIb-IIIa complex and b) CPB did not inhibit the ability of platelets to expose their fibrinogen receptors.

### ***Effect of CPB on Platelet Reactivity in Vivo***

In order to determine platelet reactivity *in vivo*, the time-dependent up-regulation of platelet surface P-selectin was determined by whole blood flow cytometry in the blood emerging from a standardized bleeding time wound (Fig 9). The previously reported<sup>18,41</sup> time-dependent up-regulation of platelet surface P-selectin was observed in samples obtained from bleeding time incisions

performed preoperatively ("PRE OP" time point in Fig 9) and after the start of anesthesia and surgery but before heparin and CPB ("PRE HEP" time point in Fig 9). However, this *in vivo* up-regulation of P-selectin was abolished 5 minutes after heparin administration but before the start of CPB ("HEP" time point in Fig 9) and 45 minutes after the start of CPB ("CPB 45" time point in Fig 9). The *in vivo* up-regulation of P-selectin returned to near normal within 2 hours after the completion of CPB ("POST 2" time point in Fig 9) and was completely normal within 24 hours after the completion of CPB ("POST 24" time point in Fig 9). The maximum up-regulation of P-selectin in shed blood collected during bypass was significantly decreased as compared to the mean values in shed blood collected before and after bypass (F statistic = 33.97, df = 2,28,  $p < 0.001$ , ANOVA for repeated measures).

These data demonstrate that *in vivo* platelet reactivity during CPB is markedly deficient (Fig 9), whereas *in vitro* platelet reactivity during CPB is normal (Figs 5, 6, 7, and 8). These contrasting *in vivo* and *in vitro* results were obtained on samples obtained from the same set of patients at the same time points using the same assays run in parallel with the same reagents.

#### ***Effect of CPB on Platelet Generation of Thromboxane $A_2$ In Vivo***

To provide further evidence that platelet reactivity *in vivo* is deficient during CPB, thromboxane  $B_2$  (a stable metabolite of thromboxane  $A_2$ ) was assayed in the shed blood emerging from the standardized bleeding time wound. Generation of thromboxane  $B_2$  in shed blood was reduced 5 minutes after heparin administration but before the start of CPB ("HEP" time point in Fig 10) and decreased further 45 minutes after the start of CPB ("CPB 45" time point in Fig 10). Generation of thromboxane  $B_2$  in shed blood returned towards normal after the completion of CPB ("CPB END", "POST 2", and "POST 24" time points in Fig 10).

## DISCUSSION

The most important factor contributing to the hemostatic defect associated with CPB is generally considered to be a platelet function defect.<sup>1-4,56</sup> However, the precise nature of the putative CPB-induced platelet function defect is controversial.<sup>1,3</sup>

### *Effect of CPB on the Platelet Surface GPIb-IX Complex*

Some,<sup>7,17,19</sup> but not all,<sup>20</sup> previous studies have concluded that a CPB-induced decrease in platelet surface GPIb plays a role in the pathogenesis of the CPB-induced platelet dysfunction. GPIb is a receptor for von Willebrand factor and is important in the process of platelet adhesion to damaged blood vessel walls.<sup>52</sup> The study by George et al.<sup>17</sup> has been widely interpreted as evidence for a CPB-induced reduction in platelet surface GPIb. However, in George's study<sup>17</sup> all values for platelet surface GPIb during CPB were within or close to the normal range. van Oeveren et al.<sup>19</sup> reported a 25% reduction in GPIb during CPB, but, unlike the study by George et al.<sup>17</sup> and the present study, these investigators centrifuged and gel filtered the platelets prior to assay, thereby introducing the possibility of an artefactual *in vitro* decrease in platelet surface GPIb as a result of proteolysis<sup>50</sup> or activation.<sup>49</sup> Rinder et al.<sup>7</sup> reported a maximal reduction of platelet surface GPIb of 28%, but the only significant reduction in GPIb was at the completion of and after CPB. Thus, Rinder et al.<sup>7</sup> did not observe a significant reduction in platelet surface GPIb during CPB, when the bleeding time is most prolonged. Furthermore, carriers of Bernard-Soulier syndrome, an autosomal recessive inherited deficiency of platelet glycoproteins Ib, IX, and V, have a normal bleeding time and no hemorrhagic diathesis, despite an approximately 50% reduction in platelet surface GPIb.<sup>51,57,58</sup> Thus, the modest reductions in GPIb found by some investigators<sup>7,19</sup> do not provide a satisfactory

explanation either for the hemorrhagic diathesis or for the prolonged bleeding time associated with CPB.

We have recently developed a flow cytometric method that allows us to study the platelet surface expression of GPIb in whole blood, thereby avoiding potential artefactual reductions in platelet surface GPIb.<sup>41</sup> In the present study, as demonstrated by this whole blood assay, CPB did not result in a decrease in the platelet surface expression of the GPIb-IX complex (Fig 2). The possibility of a CPB-induced conformational change in the GPIb-IX complex, or a CPB-induced proteolysis of a fragment of the GPIb-IX complex, was excluded by the lack of change in the platelet binding of a panel of monoclonal antibodies (6D1, TM60, AK1, and FMC25) known to be directed against different epitopes on the GPIb-IX complex (Fig 2). Furthermore, CPB neither reduced the ristocetin-induced binding of von Willebrand factor to GPIb (Fig 2) nor increased the plasma concentration of glycalicin, a proteolytic fragment of GPIb (Fig 3). Thus, by a combination of methods, the present study demonstrates that CPB is not associated with a loss of platelet surface GPIb.

#### *Effect of CPB on the Platelet Surface GPIIb-IIIa Complex*

Some,<sup>7,13,16,17</sup> but not other,<sup>18,19</sup> previous studies have concluded that a CPB-induced decrease in the platelet surface GPIIb-IIIa complex plays a role in the pathogenesis of the CPB-induced platelet dysfunction. The GPIIb-IIIa complex is a receptor for fibrinogen and is important in the process of platelet aggregation.<sup>55</sup> Using washed platelet methods, Wenger et al.<sup>13</sup> reported a decrease of 25% in the whole platelet GPIIIa content during CPB and a decrease of 41% in the platelet binding of exogenous fibrinogen. Dechavanne et al.<sup>16</sup> also used a washed platelet method and reported a 32% decrease in the platelet surface GPIIb-IIIa complex during CPB. Utilizing a whole blood method,

George et al.<sup>17</sup> reported a slight decrease in platelet surface GPIIb during CPB. However, in this study<sup>17</sup> all values for platelet surface GPIIb during CPB were within or close to the normal range. Rinder et al.,<sup>7</sup> using a whole blood method, reported a 21% (but statistically insignificant) decrease in the platelet surface GPIIb-IIIa complex after CPB. Furthermore, carriers of Glanzmann's thrombasthenia, an autosomal recessive inherited deficiency of the platelet GPIIb-IIIa complex, have a normal bleeding time and no hemorrhagic diathesis, despite an approximately 50% reduction in the platelet surface GPIIb-IIIa complex.<sup>57,59</sup> Therefore, the quantitatively more modest reductions in the platelet surface GPIIb-IIIa complex found during CPB by some investigators<sup>7,13,16,17</sup> do not provide a satisfactory explanation for the prolonged bleeding time and hemorrhagic diathesis associated with CPB. Moreover, van Oeveren et al.<sup>19</sup> detected a modest increase in both platelet surface GPIIb-IIIa and ADP-induced fibrinogen binding during CPB. Finally, utilizing a whole blood flow cytometric method, Abrams et al.<sup>18</sup> reported that fibrinogen binding to the platelet GPIIb-IIIa complex was slightly increased during CPB, as determined by the binding of a monoclonal antibody (PAC1) directed against the fibrinogen binding site on the GPIIb-IIIa complex and by the binding of a monoclonal antibody (9F9) directed against platelet-bound fibrinogen.

In the present study, as demonstrated by a whole blood flow cytometric assay, CPB did not result in a decrease in the platelet surface expression of the GPIIb-IIIa complex (Fig 4). The possibility of a CPB-induced conformational change in the GPIIb-IIIa complex, or a CPB-induced proteolysis of a fragment of the GPIIb-IIIa complex, was excluded by the lack of change in the platelet binding of a panel of monoclonal antibodies (7E3, 10E5, M148, and Y2/51) known to be directed against different epitopes on the GPIIb-IIIa complex (Fig 4). In addition, we directly studied the binding of exogenous fibrinogen to the GPIIb-IIIa complex and found no CPB-induced reduction (Fig 4). Finally, we demonstrated that irrespective of whether peripheral blood samples were drawn

before, during, or after CPB, maximal *in vitro* stimulation of washed platelets by thrombin resulted in the same exposure of the fibrinogen binding site on the GPIIb-IIIa complex, as determined by monoclonal antibody PAC1 (Fig 8, panel D). Thus, by a combination of methods, the present study demonstrates that CPB is not associated with a loss of the platelet surface GPIIb-IIIa complex.

### ***Effect of CPB on Platelet Degranulation***

Plasma assays of  $\beta$ -thromboglobulin ( $\beta$ -TG) and platelet factor 4 (PF4) have been widely used as markers of platelet degranulation and secretion.<sup>60</sup> Most studies have reported increased plasma concentrations of  $\beta$ -TG and PF4 during and after CPB.<sup>4,6,15,61-63</sup> Some,<sup>62</sup> but not other,<sup>64</sup> studies have found that platelet levels of  $\beta$ -TG were reduced by CPB and concluded that degranulation had occurred during CPB. However, there are two major problems with the use of plasma assays of  $\beta$ -TG and PF4 to monitor platelet degranulation and secretion. First, separation of plasma from platelets prior to the performance of these assays frequently results in artefactual *in vitro* degranulation and secretion.<sup>60</sup> Second, elevated plasma concentrations of plasma  $\beta$ -TG and PF4 reflect not only the number of circulating degranulated platelets but also lysed platelets and non-circulating degranulated platelets adherent to synthetic surfaces or vessel walls.<sup>3,15</sup>

Electron microscopic studies of platelet degranulation during CPB are also fraught with the possibility of artefactual *in vitro* platelet activation. Using electron microscopy, Harker et al.<sup>4</sup> found that platelets circulating during CPB had partially released their  $\alpha$  granules but not their dense granules, whereas neither Zilla et al.<sup>15</sup> nor Dechavanne et al.<sup>16</sup> found CPB-induced degranulation of circulating platelets.

More recently, a number of investigators have studied the effect of CPB on the binding of monoclonal antibodies directed against granule antigens that are only present on the platelet surface

after degranulation.<sup>7,14,16-18</sup> Utilizing washed platelet systems, Nieuwenhuis et al.<sup>14</sup> found a modest increase during CPB of the platelet binding of a monoclonal antibody directed against a 53 kDa lysosomal antigen, whereas Dechavanne et al.<sup>16</sup> found that CPB did not result in an increase in the platelet surface expression of the  $\alpha$  granule membrane protein P-selectin (also known as GMP-140 and PADGEM protein). Utilizing whole blood methods that are much less likely than washed platelet methods to result in artefactual *in vitro* platelet degranulation,<sup>65</sup> both George et al.<sup>17</sup> and Abrams et al.<sup>18</sup> demonstrated that CPB resulted in no increase in the platelet surface expression of P-selectin. Although Rinder et al.<sup>7</sup> used a whole blood flow cytometric assay and found a 29% increase in P-selectin-positive platelets at the end of CPB, the amount of increase in P-selectin on the P-selectin-positive platelets was modest.

Consistent with these previous studies, the present whole blood flow cytometric study of peripheral blood samples during CPB revealed minimal P-selectin expression on the platelet surface (Fig 5, panel A, closed circles). This virtual absence of circulating degranulated platelets suggests that the previously reported CPB-induced increase in plasma concentrations of  $\beta$ -TG and PF4<sup>4,6,15,61-63</sup> originates from either a) degranulated platelets that are very rapidly cleared from the circulation<sup>66</sup> (possibly by circulating monocytes and neutrophils<sup>67,68</sup>), b) non-circulating degranulated platelets adherent to synthetic surfaces or vessel walls, and/or c) platelet lysis *in vivo*<sup>15</sup> or *in vitro*.

### ***Effect of CPB on Platelet Reactivity in Vitro***

The results of studies of the effect of CPB on platelet aggregation, as determined by standard nephelometric methods, are inconsistent.<sup>4-12,15,69</sup> Although many of these studies describe CPB-induced defects in platelet aggregation, the reported responses to known platelet agonists are widely variable. Some of the reported defects are modest<sup>15,69</sup> and some investigators did not detect CPB-



induced defects in platelet aggregation.<sup>12,13</sup> These inconsistencies probably result in part from the fact that platelet aggregation, especially in a complex clinical setting, is semiquantitative and subject to standardization problems.<sup>70,71</sup> Furthermore, most of the reported platelet aggregometry studies during CPB were performed in platelet-rich plasma without normalizing the platelet count. The CPB-induced "platelet aggregation defect" may therefore simply reflect the CPB-induced thrombocytopenia.

In this study, in contrast to all previous studies, platelet reactivity *in vitro* was analyzed not by aggregometry but by whole blood flow cytometric assay of peripheral blood samples obtained before, during, and after CPB. CPB did not result in a defect in platelet reactivity *in vitro*, as determined by agonist-induced up-regulation of the platelet surface P-selectin (monitored by monoclonal antibody S12), up-regulation of the fibrinogen binding site on the GPIIb-IIIa complex (monitored by monoclonal antibodies PAC1 and 7E3), and down-regulation of the GPIb-IX complex (monitored by monoclonal antibody 6D1) (Figs 5, 7, and 8). The results were the same irrespective of whether the *in vitro* platelet agonist was PMA, the stable thromboxane A<sub>2</sub> agonist U46619, a combination of ADP and epinephrine, or, in a washed platelet system, thrombin (Figs 5, 6, 7, and 8), thereby excluding the possibility of a CPB-induced signal transduction defect. One could speculate that the heparin-induced augmentation of platelet reactivity *in vitro* (Fig 5), which was reversed by protamine sulfate administration (Fig 5), might provide a specific mechanism through which platelets may be particularly prone to artefactual *in vitro* activation during the separation of platelet-rich plasma and therefore appear to be less reactive to exogenous agonists in platelet aggregation studies.

### ***Effect of CPB on Platelet Reactivity in Vivo***

No previous study of CPB has attempted to directly assess the effect of platelet reactivity *in vivo*. This was accomplished in the present study by two assays of the shed blood emerging from a standardized *in vivo* bleeding time wound.<sup>18,41</sup>

First, as determined by whole blood flow cytometry, the previously described<sup>18,41</sup> time-dependent up-regulation of the platelet surface expression of P-selectin was observed in shed blood samples obtained from bleeding time incisions performed preoperatively (Fig 9). However, this *in vivo* up-regulation of P-selectin was abolished a) 5 minutes after heparin administration but before the start of CPB and b) 45 minutes after the start of CPB (Fig 9). The *in vivo* up-regulation of P-selectin returned to near normal within 2 hours of the completion of CPB and completely normal within 24 hours after the completion of CPB (Fig 9).

Second, as determined by radioimmunoassay, generation of thromboxane B<sub>2</sub> (a stable metabolite of thromboxane A<sub>2</sub> and an important marker of platelet activation<sup>72</sup>) was reduced in shed blood samples during CPB (Fig 10).

Thus, by a combination of methods, the present study demonstrates that, in striking contrast to the normal *in vitro* platelet reactivity observed during CPB (see above), *in vivo* platelet reactivity during CPB is markedly deficient.

### ***The Platelet Function Defect of CPB***

CPB is associated with a hemostatic defect, the hallmark of which is a prolonged bleeding time.<sup>1-4</sup> A platelet function defect appears to be the basis for this prolongation of the bleeding time during CPB, because: i) the modest decrease in the platelet count during CPB is insufficient to account for the marked prolongation of the bleeding time during CPB (Fig 1, panel B, and ref. 73);

ii) the relationship between bleeding time and degree of thrombocytopenia is discordant at the completion of CPB and after CPB (Fig 1, panels A and B, and ref. 4); iii) von Willebrand factor levels remain within or above the normal range during and after CPB (Fig 1, panel C and refs. 69,74,75); iv) up-regulation of platelet surface P-selectin *in vivo* is abolished during CPB (Fig 9); and v) *in vivo* generation of thromboxane A<sub>2</sub>, an important marker of platelet activation,<sup>72</sup> is deficient during CPB (Fig 10).

However, the hypothesis that CPB results in an intrinsic platelet function defect is not supported by the present findings during and after CPB: a) normal antigenic and functional expression of the platelet surface GPIb-IX and GPIIb-IIIa complexes, b) absence of circulating degranulated platelets, and c) normal platelet reactivity *in vitro*. Taken together with these findings, the demonstration of a CPB-induced deficiency of platelet reactivity *in vivo* suggests that the "platelet function defect" of CPB is the result of a defect extrinsic to the platelet, such as an *in vivo* lack of availability of platelet agonists. Thrombin, the preeminent platelet agonist *in vivo*,<sup>76-78</sup> is functionally deficient during CPB because of the presence of high circulating concentrations of heparin. Although thrombin bound to fibrin clots is relatively protected from inhibition by heparin-antithrombin III,<sup>79,80</sup> the high concentrations of heparin used during CPB (4.5 U/mL, with an ACT greater than or equal to 999 seconds, in this study) are sufficient to completely overcome this protection.<sup>79</sup> The importance of heparin inhibition of thrombin in the extrinsic platelet function defect of CPB is suggested by the finding that 5 minutes after heparin administration but before the start of CPB: a) the *in vivo* up-regulation of platelet surface P-selectin was abolished ("HEP" time point in Fig 9), b) the *in vivo* generation of thromboxane A<sub>2</sub> was almost maximally inhibited ("HEP" time point in Fig 10), c) the bleeding time was prolonged ("HEP" time point in Fig 1) and d) platelets in whole blood were completely unreactive to thrombin *in vitro* (data not shown).

Heparin is nearly universally used during CPB. The present study provides evidence for two distinct effects of heparin on platelet function during CPB. First, heparin augments platelet activation in whole blood exposed to an *exogenous* platelet agonist *in vitro* (figure 5). Second, as discussed above, heparin suppresses platelet activation *in vivo* via inhibition of *endogenous* thrombin. These two apparently opposite effects of heparin on platelets are neither inconsistent nor contradictory. Thus, although heparin augments the activatability of platelets, the platelets are not in fact activatable *in vivo* because thrombin, the preeminent agonist,<sup>76-78</sup> is unavailable.

Neither the presence of fibrin degradation products nor the use of hypothermia can account for the differences between platelet function *in vitro* and *in vivo* observed in the present study. Inhibition of platelet function by circulating fibrin(ogen) degradation products<sup>81</sup> does not account for our findings because platelet activation *in vitro* was normal. Although hypothermia is likely to play a role in the extrinsic platelet function defect associated with CPB,<sup>82-84</sup> hypothermia does not account for the differences between platelet activatability *in vitro* and *in vivo*, because our *in vitro* studies were performed at 22°C.

In summary, CPB resulted in: 1) no loss of the platelet surface GPIb-IX complex, 2) no loss of the platelet surface GPIIb-IIIa complex, 3) a minimal number of circulating degranulated platelets, 4) normal platelet reactivity *in vitro*, and 5) markedly deficient platelet reactivity *in vivo*. These data suggest that the "platelet function defect" of CPB is not a defect intrinsic to the platelet but is an extrinsic defect such as an *in vivo* lack of availability of platelet agonists. The near universal use of heparin during CPB is likely to contribute substantially to this defect via its inhibition of thrombin.

### ACKNOWLEDGEMENTS

The authors thank Drs. Michael C. Berndt, Barry S. Coller, John W. Fenton II, Rodger P. McEver, Patricia Rao, Sanford J. Shattil and Naomasa Yamamoto for generously providing reagents, Dr. Peter H. Levine and James J. Hoogasian for their support, and Nancy Healy, Mheir Doursounian, and Stephanie Francis for their essential contributions. Dr. Kestin dedicates this paper to Dr. Joseph Kestin.

## REFERENCES

1. Woodman RC, Harker LA: Bleeding complications associated with cardiopulmonary bypass. *Blood* 76:1680, 1990
2. Harker LA: Bleeding after cardiopulmonary bypass. *N Engl J Med* 314:1446, 1986
3. Michelson AD: Pathomechanism of defective haemostasis during and after extracorporeal circulation: The role of platelets, in Hetzer R (ed): *Blood use in cardiac surgery*, Darmstadt, Steinkopff, 1990,
4. Harker LA, Malpass TW, Branson HE, Hessel EA, Slichter SJ: Mechanism of abnormal bleeding in patients undergoing cardiopulmonary bypass: Acquired transient platelet dysfunction associated with selective  $\alpha$ -granule release. *Blood* 56:824, 1980
5. Bick RL: Hemostasis defects associated with cardiac surgery, prosthetic devices, and other extracorporeal circuits. *Semin Thromb Hemost* 11:249, 1985
6. Mammen EF, Koets MH, Washington BC, Wolk LW, Brown JM, Burdick M, Selik NR, Wilson RF: Hemostasis changes during cardiopulmonary bypass surgery. *Semin Thromb Hemost* 11:281, 1985
7. Rinder CS, Mathew JP, Rinder HM, Bonan J, Ault KA, Smith BR: Modulation of platelet surface adhesion receptors during cardiopulmonary bypass. *Anesthesiology* 75:563, 1991
8. Rinder CS, Bohnert J, Rinder HM, Mitchell J, Ault K, Hillman R: Platelet activation and aggregation during cardiopulmonary bypass. *Anesthesiology* 75:388, 1991
9. Edmunds LH, Ellison N, Colman RW, et al.: Platelet function during open heart surgery: Comparison to the membrane and bubble oxygenators. *J Thorac Cardiovasc Surg* 83:805, 1982

10. Mammen EF, Koets MH, Washington BC, Wolk LW, Brown JM, Burdick M, Selik NR, Wilson RF: Hemostasis changes during cardiopulmonary bypass surgery. *Semin Thromb Hemost* 281:292, 1985
11. Wachtfogel YT, Musial J, Jenkin B, Niewiarowski S, Edmunds LH, Colman RW: Loss of platelet  $\alpha_2$ -adrenergic receptors during simulated extracorporeal circulation: Prevention with prostaglandin E1. *J Lab Clin Med* 105:601, 1985
12. Holloway DS, Summaria L, Sandesara J, Vagher JP, Alexander JC, Caprini JA: Decreased platelet number and function and increased fibrinolysis contribute to postoperative bleeding in cardiopulmonary bypass patients. *Thromb Haemost* 59:62, 1988
13. Wenger RK, Lukasiewicz H, Mikuta BS, Niewiarowski S, Edmunds LH: Loss of platelet fibrinogen receptors during clinical cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 97:235, 1989
14. Nieuwenhuis HK, van Oosterhout JJG, Rozemuller E, van Iwaarden F, Sixma JJ: Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* 70:838, 1987
15. Zilla P, Fasol R, Groscurth P, Klepetko W, Reichenspurner H, Wolner E: Blood platelets in cardiopulmonary bypass. Recovery occurs after initial stimulation, rather than continual activation. *J Thorac Cardiovasc Surg* 97:379, 1989
16. Dechavanne M, Ffrench M, Pages J, Ffrench P, Boukerche H, Bryon PA, McGregor JL: Significant reduction in the binding of a monoclonal antibody (LYP 18) directed against the IIb-IIIa glycoprotein complex to platelets of patients having undergone extracorporeal circulation. *Thromb Haemost* 57:106, 1987

17. George JN, Pickett EB, Saucerman S, McEver RP, Kunicki TJ, Kieffer N, Newman PJ: Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 78:340, 1986
18. Abrams CS, Ellison N, Budzynski AZ, Shattil SJ: Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 75:128, 1990
19. van Oeveren W, Harder MP, Roozendaal KJ, Eijssman L, Wildevuur CRH: Aprotinin protects platelets against the initial effect of cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 99:788, 1990
20. Vandenveld C, Fondu P, Dubois-Primo J: Low-dose aprotinin for reduction of blood loss after cardiopulmonary bypass. *Lancet* 337:1157, 1991
21. Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF: A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 101:880, 1985
22. Bevilacqua M, Butcher E, Furie B, Gallatin M, Gimbrone M, Harlan J, Kishimoto K, Lasky L, McEver R, Paulson J, Rosen S, Seed B, Siegelman M, Springer T, Stoolman L, Tedder T, Varki A, Wagner D, Weissman I, Zimmerman G: Selectins: a family of adhesion receptors [letter]. *Cell* 67:233, 1991
23. Hsu-Lin S-C, Berman CL, Furie BC, August D, Furie B: A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. *J Biol Chem* 259:9121, 1984
24. Knapp W, Dorken B, Rieber P, Schmidt RE, Stein H, von dem Borne AEGKr: CD antigens 1989. *Blood* 74:1448, 1989



25. McEver RP: Properties of GMP-140, an inducible granule membrane protein of platelets and endothelium. *Blood Cells* 16:73, 1990
26. Collier BS, Peerschke EI, Scudder LE, Sullivan CA: Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. *Blood* 61:99, 1983
27. Michelson AD, Loscalzo J, Melnick B, Collier BS, Handin RI: Partial characterization of a binding site for von Willebrand factor on glyocalicin. *Blood* 67:19, 1986
28. Yamamoto N, Kitagawa H, Tanoue K, Yamazaki H: Monoclonal antibody to glycoprotein Ib inhibits both thrombin- and ristocetin-induced platelet aggregations. *Thromb Res* 39:751, 1985
29. Yamamoto K, Yamamoto N, Kitagawa H, Tanoue K, Kosaki G, Yamazaki H: Localization of a thrombin-binding site on human platelet glycoprotein Ib determined by a monoclonal antibody. *Thromb Haemost* 55:162, 1986
30. Katagiri Y, Hayashi Y, Yamamoto K, Tanoue K, Kosaki G, Yamazaki H: Localization of von Willebrand factor and thrombin-interactive domains on human platelet glycoprotein Ib. *Thromb Haemost* 63:122, 1990
31. Du X, Beutler L, Ruan C, Castaldi PA, Berndt MC: Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. *Blood* 69:1524, 1987
32. Berndt MC, Gregory C, Kabral A, Zola H, Fournier D, Castaldi PA: Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. *Eur J Biochem* 151:637, 1985
33. Berndt MC, Chong BH, Bull HA, Zola H, Castaldi PA: Molecular characterization of quinine/quinidine drug-dependent antibody platelet interaction using monoclonal antibodies. *Blood* 66:1292, 1985

34. Gatter KC, Cordell JL, Turley H, Heryet A, Kieffer N, Anstee DJ, Mason DY: The immunohistological detection of platelets, megakaryocytes and thrombi in routinely processed specimens. *Histopathology* 13:257, 1988
35. Jones D, Fritschy J, Garson J, Nokes TJC, Kemshead JT, Hardisty RM: A monoclonal antibody binding to human medulloblastoma cells and to the platelet glycoprotein IIb-IIIa complex. *Br J Haematol* 57:621, 1984
36. Collier BS: A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. *J Clin Invest* 76:101, 1985
37. Collier BS, Peerschke EI, Scudder LE, Sullivan CA: A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J Clin Invest* 72:325, 1983
38. Collier BS: Activation-specific platelet antigens, in Kunicki TJ, George JN (eds): *Platelet Immunobiology: Molecular and Clinical Aspects*, Philadelphia, J.B.Lippincott, 1989, p 166
39. Shattil SJ, Hoxie JA, Cunningham M, Brass LF: Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J Biol Chem* 260:11107, 1985
40. Asch AS, Barnwell J, Silverstein RL, Nachman RL: Isolation of the thrombospondin membrane receptor. *J Clin Invest* 79:1054, 1987
41. Michelson AD, Ellis PA, Barnard MR, Matic GB, Viles AF, Kestin AS: Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, ADP or an in vivo wound. *Blood* 77:770, 1991
42. Shattil SJ, Cunningham M, Hoxie JA: Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* 70:307, 1987

43. Michelson AD, Barnard MR: Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex. *Blood* 70:1673, 1987
44. Yamamoto N, Greco NJ, Barnard MR, Tanoue K, Yamazaki H, Jamieson GA, Michelson AD: Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. *Blood* 77:1740, 1991
45. Michelson AD, Adelman B, Barnard MR, Carroll E, Handin RI: Platelet storage results in a redistribution of glycoprotein Ib molecules. Evidence for a large intraplatelet pool of glycoprotein Ib. *J Clin Invest* 81:1734, 1988
46. Michelson AD, Barnard MR: Plasmin-induced redistribution of platelet glycoprotein Ib. *Blood* 76:2005, 1990
47. Mendelsohn ME, O'Neill S, George D, Loscalzo J: Inhibition of fibrinogen binding to human platelets by S-nitroso-N-acetylcysteine. *J Biol Chem* 265:19028, 1990
48. Valeri CR, Cassidy G, Khuri S, Feingold H, Ragno G, Altschule MD: Hypothermia-induced reversible platelet dysfunction. *Ann Surg* 205:175, 1987
49. Michelson AD: Thrombin-induced down-regulation of the platelet membrane glycoprotein Ib-IX complex. *Semin Thromb Hemost* 18:18, 1992
50. George JN, Thoi LL, Morgan RK: Quantitative analysis of platelet membrane glycoproteins: Effect of platelet washing procedures and isolation of platelet density subpopulations. *Thromb Res* 23:69, 1981
51. George JN, Nurden AT, Phillips DR: Molecular defects in interactions of platelets with the vessel wall. *N Engl J Med* 311:1084, 1984
52. Ruggeri ZM: The platelet glycoprotein Ib-IX complex. *Prog Hemost Thromb* 10:35, 1991

53. Okumura T, Lombart C, Jamieson GA: Platelet glycoprotein IIb/IIIa. II. Purification and characterization. *J Biol Chem* 251:5950, 1976
54. Collier BS, Kalomiris E, Steinberg M, Scudder LE: Evidence that glycoprotein IIb/IIIa circulates in normal plasma. *J Clin Invest* 73:794, 1984
55. Phillips DR, Charo IF, Parise LV, Fitzgerald LA: The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 71:831, 1988
56. Edmunds LH, Addonizio VP: Extracorporeal circulation, in Colman RW, Hirsh J, Marder VJ, Salzman EW (eds): Hemostasis and thrombosis. Basic principles and clinical practice, Philadelphia, Lippincott, 1987, p 901
57. Michelson AD: Flow cytometric analysis of platelet surface glycoproteins: Phenotypically distinct subpopulations of platelets in children with chronic myeloid leukemia. *J Lab Clin Med* 110:346, 1987
58. George JN, Reimann TA, Moake JL, Morgan RK, Cimo PL, Sears DA: Bernard-Soulier disease: a study of four patients and their parents. *Br J Haematol* 48:459, 1981
59. George JN, Caen JP, Nurden AT: Glanzmann's Thrombasthenia: the spectrum of clinical disease. *Blood* 75:1383, 1990
60. Levine SP: Secreted platelet proteins as markers for pathological disorders, in Phillips DR, Shuman MA (eds): Biochemistry of platelets, Orlando, Academic Press, 1986, p 378
61. Aren C, Feddersen K, Radegran K: Effects of prostacyclin infusion on platelet activation and postoperative blood loss in coronary bypass. *Ann Thorac Surg* 36:49, 1983
62. Mezzano D, Aranda E, Urzua J, Lema G, Habash J, Irarrazabal MJ, Pereira J: Changes in platelet B-thromboglobulin, fibrinogen, albumen, 5-hydroxytryptamine, ATP, and ADP during and after surgery with extracorporeal circulation in man. *Am J Hemat* 22:133, 1986

63. Colman RW: Platelet and neutrophil activation in cardiopulmonary bypass. *Ann Thorac Surg* 49:32, 1990
64. Pumphey CW, Dawes J: Platelet alpha granule depletion: findings in patients with prosthetic heart valves and following cardiopulmonary bypass surgery. *Thromb Res* 30:257, 1983
65. Abrams CS, Shattil SJ: Immunological detection of activated platelets in clinical disorders. *Thromb Haemost* 65:467, 1991
66. Rinder HM, Murphy M, Mitchell JG, Stocks J, Ault KA, Hillman RS: Progressive platelet activation with storage: evidence for shortened survival of activated platelets after transfusion. *Transfusion* 31:409, 1991
67. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B: PADGEM protein: A receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 59:305, 1989
68. Hamburger SA, McEver RP: GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 75:550, 1990
69. Mohr R, Golan M, Martinowitz U, Rosner E, Goor DA, Ramot B: Effect of cardiac operation on platelets. *J Thorac Cardiovasc Surg* 92:434, 1986
70. Kinlough-Rathbone RL, Packham MA, Mustard JF: Platelet aggregation, in Harker LA, Zimmerman TS (eds): *Measurements of platelet function*, New York, Churchill Livingstone, 1983, p 64
71. George JN, Shattil SJ: The clinical importance of acquired abnormalities of platelet function. *N Engl J Med* 324:27, 1991

72. Marcus AJ: Platelet eicosanoid metabolism, in Colman RW, Hirsh J, Marder VJ, Salzman EW (eds): Hemostasis and thrombosis. Basic principles and clinical practice, Philadelphia, Lippincott, 1987, p 676
73. Harker LA, Slichter SJ: The bleeding time as a screening test for the evaluation of platelet function. *N Engl J Med* 287:155, 1972
74. Jones DK, Luddington R, Higenbottam TW, Scott J, Cavarocchi N, Reardon D, Calvin J, Wallwork J: Changes in factor VIII proteins after cardiopulmonary bypass in man suggest endothelial damage. *Thromb Haemost* 60:199, 1988
75. Hackmann T, Gascoyne RD, Naiman SC, Growe GH, Burchill LD, Jamieson WRE, Sheps SB, Schechter MT, Townsend GE: A trial of desmopressin (1-desamino-8-D-arginine vasopressin) to reduce blood loss in uncomplicated cardiac surgery. *N Engl J Med* 321:1437, 1989
76. Hansen SR, Harker LA: Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin PPACK. *Proc Natl Acad Sci USA* 85:3184, 1988
77. Eidt JF, Allison P, Nobel S, Ashton J, Golino P, McNatt J, Buja LM, Willerson J: Thrombin is an important mediator of platelet aggregation in stenosed coronary arteries. *J Clin Invest* 84:18, 1989
78. Kelly AB, Marzec UM, Krupski W, Bass A, Cadroy Y, Hanson SR, Harker LA: Hirudin interruption of heparin-resistant arterial thrombus formation in baboons. *Blood* 77:1006, 1991
79. Weitz JI, Huduba M, Massel D, Maragnore J, Hirsh J: Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III independent inhibitors. *J Clin Invest* 86:385, 1990

80. Badimon L, Badimon JJ, Lassila R, Heras M, Chesebro JH, Fuster V: Thrombin regulation of platelet interaction with damaged vessel wall and isolated collagen type I at arterial flow conditions in a porcine model: effects of hirudins, heparin, and calcium chelation. *Blood* 78:423, 1991
81. Kowalski E, Kopec M, Wegrzynowicz Z: Influence of fibrinogen degradation products (FDP) on platelet aggregation, adhesiveness, and viscous metamorphosis. *Thromb Diath Haemorrh* 10:406, 1963
82. Khuri SF, Wolfe JA, Josa M, Axford TC, Szymanski I, Assousa S, Ragno G, Patel M, Silverman A, Park M, Valeri CR: Hematologic changes during and after cardiopulmonary bypass and their relationship to the bleeding time and non-surgical blood loss. *J Thorac Cardiovasc Surg* 104:94, 1992
83. Valeri CR, Kabbaz K, Khuri S, Marquardt C, Ragno G, Feingold H, Gray A, Axford T: Effect of skin temperature on platelet function in extracorporeal bypass patients. *J Thorac Cardiovasc Surg* 104:108, 1992
84. Michelson AD, MacGregor H, Kestin AS, Barnard MR, Rohrer MJ, Valeri CR: Hypothermia-induced reversible inhibition of human platelet activation in vitro and in vivo. *Blood* 78:389a, 1991

# FIGURE LEGENDS

**Fig 1.** Effect of CPB on the bleeding time (panel A), platelet count (panel B), and plasma von Willebrand factor concentration (panel C). The abbreviations listed on the horizontal axis refer to the blood sampling time points before, during, and after CPB, as defined in Table 1. Data are mean  $\pm$  S.E.M.,  $n = 16$  separate patients.

**Fig 2.** Effect of CPB on the platelet surface expression of the GPIb-IX complex. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Panels A through D: the platelet binding of a panel of GPIb-IX-specific monoclonal antibodies, as determined by whole blood flow cytometry. The antibodies are directed against the von Willebrand factor binding site on GPIb (6D1), the thrombin binding site on GPIb (TM60), GPIX (FMC25), and the GPIb-IX complex (AK1). Panel E: ristocetin-induced binding of exogenous von Willebrand factor (vWf) to washed platelets, as determined by flow cytometry with a polyclonal anti-vWf antibody. In each panel, antibody binding at the preoperative ("PRE OP") time point was assigned a value of 100 units of fluorescence. Data are mean  $\pm$  S.E.M.  $n = 4$ .

**Fig 3.** Effect of CPB on the plasma concentration of glyocalicin (a proteolytic fragment of GPIb). The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Plasma glyocalicin (solid line) was measured by a competitive inhibition assay. In order to assess the effect of hemodilution during the bypass procedure, hematocrit (dotted line) was also measured. Data are mean  $\pm$  S.E.M.  $n = 16$ .



**Fig 4.** Effect of CPB on the platelet surface expression of the GPIIb-IIIa complex. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Panels A through D: the platelet binding of a panel of GPIIb-IIIa-specific monoclonal antibodies, as determined by whole blood flow cytometry. Antibodies 7E3, 10E5, and M148 are directed against different epitopes near the fibrinogen binding site on the GPIIb-IIIa complex. Y2/51 is directed against the GPIIIa subunit. In each panel, antibody binding at the preoperative ("PRE OP") time point was assigned a value of 100 units of fluorescence. Panel E: binding of exogenous radioiodinated fibrinogen to ADP-stimulated washed platelets. Fibrinogen binding is expressed as molecules per platelet. Data are mean  $\pm$  S.E.M.,  $n = 4$ .

**Fig 5.** Effect of CPB on platelet reactivity to PMA, as determined by whole blood flow cytometry. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Blood samples were incubated with PMA 0 (solid circles), 0.25 (solid triangles) or 10  $\mu$ M (solid squares). Panel A: platelet surface expression of platelet-selectin, as determined by monoclonal antibody S12. The fluorescence intensity of platelets incubated with PMA 10  $\mu$ M at the preoperative ("PRE OP") time point was assigned a value of 100 units. Panel B: platelet surface GPIb, as determined by monoclonal antibody 6D1. The fluorescence intensity of platelets incubated without PMA at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean  $\pm$  S.E.M.  $n = 16$ .

**Fig 6.** The effect of CPB on platelet reactivity to the stable thromboxane A<sub>2</sub> analogue U46619 and to a combination of ADP and epinephrine (Epi), as determined by whole blood flow cytometry. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Blood samples were incubated with the indicated agonist, and the platelet binding measured of monoclonal antibodies S12 (P-selectin-specific) (panel A), 6D1 (GPIIb-specific) (panel B), and 7E3 (GPIIb-IIIa complex-specific) (panel C). For assays with S12 and 7E3, the fluorescence intensity of platelets incubated with 1 U/mL thrombin at the preoperative ("PRE OP") time point was assigned a value of 100 units. For assays with 6D1, the fluorescence intensity of platelets without added exogenous agonist at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean  $\pm$  S.E.M.,  $n = 4$ .

**Fig 7.** The effect of CPB on the kinetics of the up-regulation of P-selectin and the GPIIb-IIIa complex and the down-regulation of GPIIb. Blood samples were drawn at the perioperative time points listed in the small box and fully defined in Table 1. The samples were incubated with U46619 at 22°C for various lengths of time, immediately fixed with 1% formaldehyde, and analyzed by whole blood flow cytometry. For assays with S12 (panel A) and 7E3 (panel C), the fluorescence intensity of platelets incubated for 10 minutes with 1 U/mL thrombin at the preoperative ("PRE OP") time point was assigned a value of 100 units. For assays with 6D1 (panel B), the fluorescence intensity of platelets without added exogenous agonist at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean  $\pm$  S.E.M.  $n = 4$ .

Fig 8. The effect of CPB on the thrombin reactivity of platelets. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. The platelets were washed and incubated with the indicated thrombin concentrations. Thrombin-induced up-regulation of the platelet surface expression of P-selectin (panel A) and the GPIIb-IIIa complex (panels C and D) and down-regulation of GPIb (panel B) was measured by flow cytometry. For assays with S12 (panel A), 7E3 (panel C), and PAC1 (panel D), the fluorescence intensity of platelets incubated with thrombin 1 U/mL at the preoperative ("PRE OP") time point was assigned a value of 100 units. For assays with 6D1 (panel B), the fluorescence intensity of platelets without added exogenous thrombin at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean  $\pm$  S.E.M.  $n = 4$ .

Fig 9. The effect of CPB on activation-induced up-regulation of the platelet surface expression of P-selectin in whole blood *in vivo*. Blood samples were drawn at the perioperative time points listed to the right of each panel and fully defined in Table 1. A standardized bleeding time was performed and, without touching the wound, the shed blood was collected with a micropipet directly from the wound site at 2 minute intervals (as shown on the horizontal axis) until the cessation of bleeding. The sample was immediately fixed and then analyzed by whole blood flow cytometry with monoclonal antibody S12. The maximal binding of S12 to platelets at the preoperative ("PRE OP") time point was assigned a value of 100 units of fluorescence. This experiment is representative of 9 separate experiments.

Fig 10. The effect of CPB on thromboxane B<sub>2</sub> generation in response to a standardized vascular injury *in vivo*. The abbreviations listed on the horizontal axis refer to the perioperative time points, as defined in Table 1. At each of these perioperative time points, a standardized bleeding time wound was performed and the shed blood collected every 30 seconds until a 600  $\mu$ L aliquot of blood was obtained. The thromboxane B<sub>2</sub> concentration of the aliquot was determined by radioimmunoassay. Data are mean  $\pm$  S.E.M. n = 16.

**Table 1. Blood sampling time points before, during, and after CPB.**

|                |                                                                        |
|----------------|------------------------------------------------------------------------|
| <b>PRE OP</b>  | Preoperative.                                                          |
| <b>PRE HEP</b> | After the start of anesthesia and surgery, but before heparin and CPB. |
| <b>HEP</b>     | 5 minutes after heparin administration, but before the start of CPB.   |
| <b>CPB NT</b>  | After the start of CPB (normothermic conditions).                      |
| <b>CPB HT</b>  | Beginning of maximal hypothermia on CPB.                               |
| <b>CPB 45</b>  | 45 minutes after the start of CPB (hypothermic conditions).            |
| <b>CPB END</b> | Completion of CPB, immediately following administration of protamine.  |
| <b>POST 2</b>  | 2 hours after completion of CPB.                                       |
| <b>POST 24</b> | 24 hours after completion of CPB.                                      |
| <b>POST 48</b> | 48 hours after completion of CPB                                       |

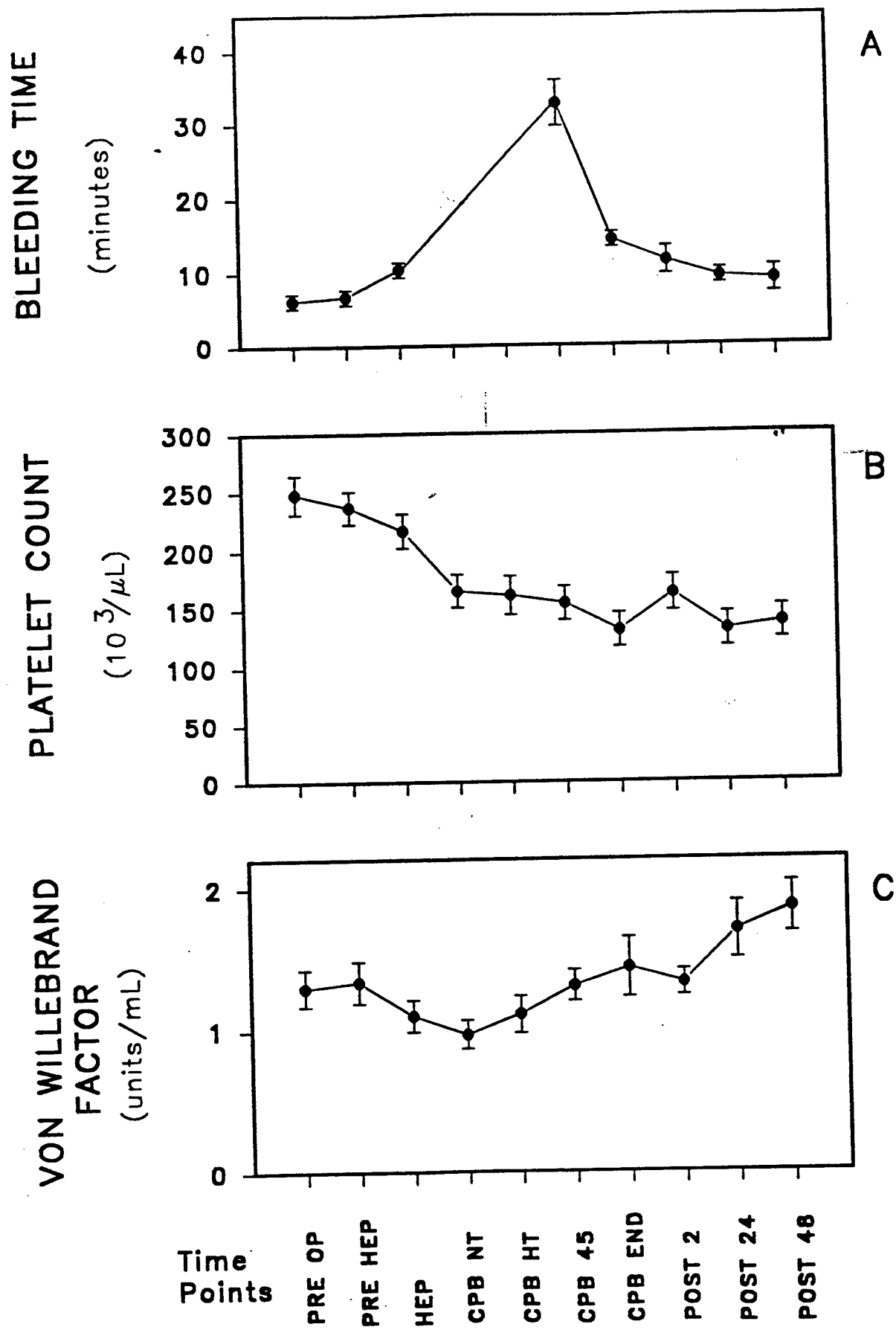


Fig. 1

# GPIb/IX

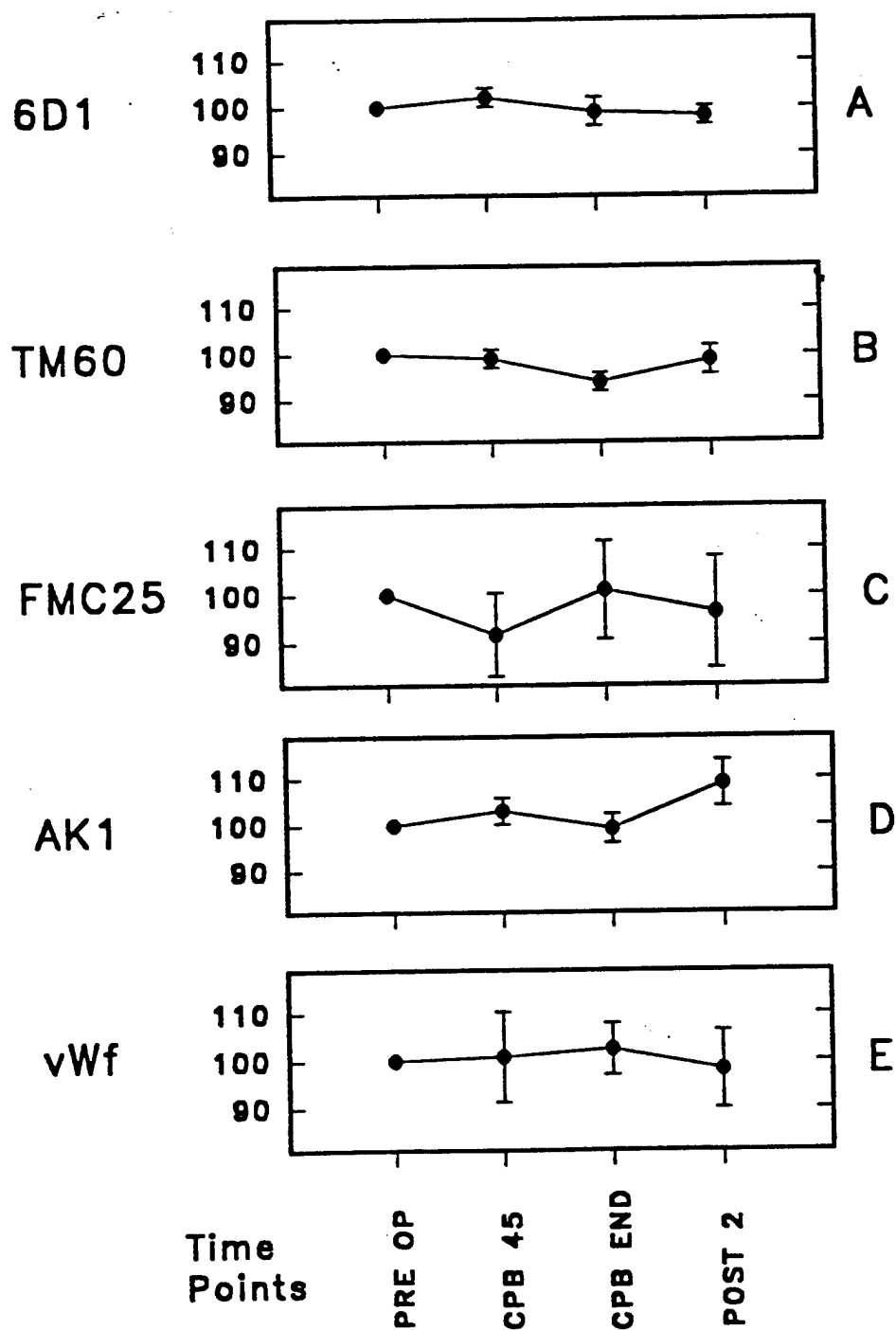


Fig. 2

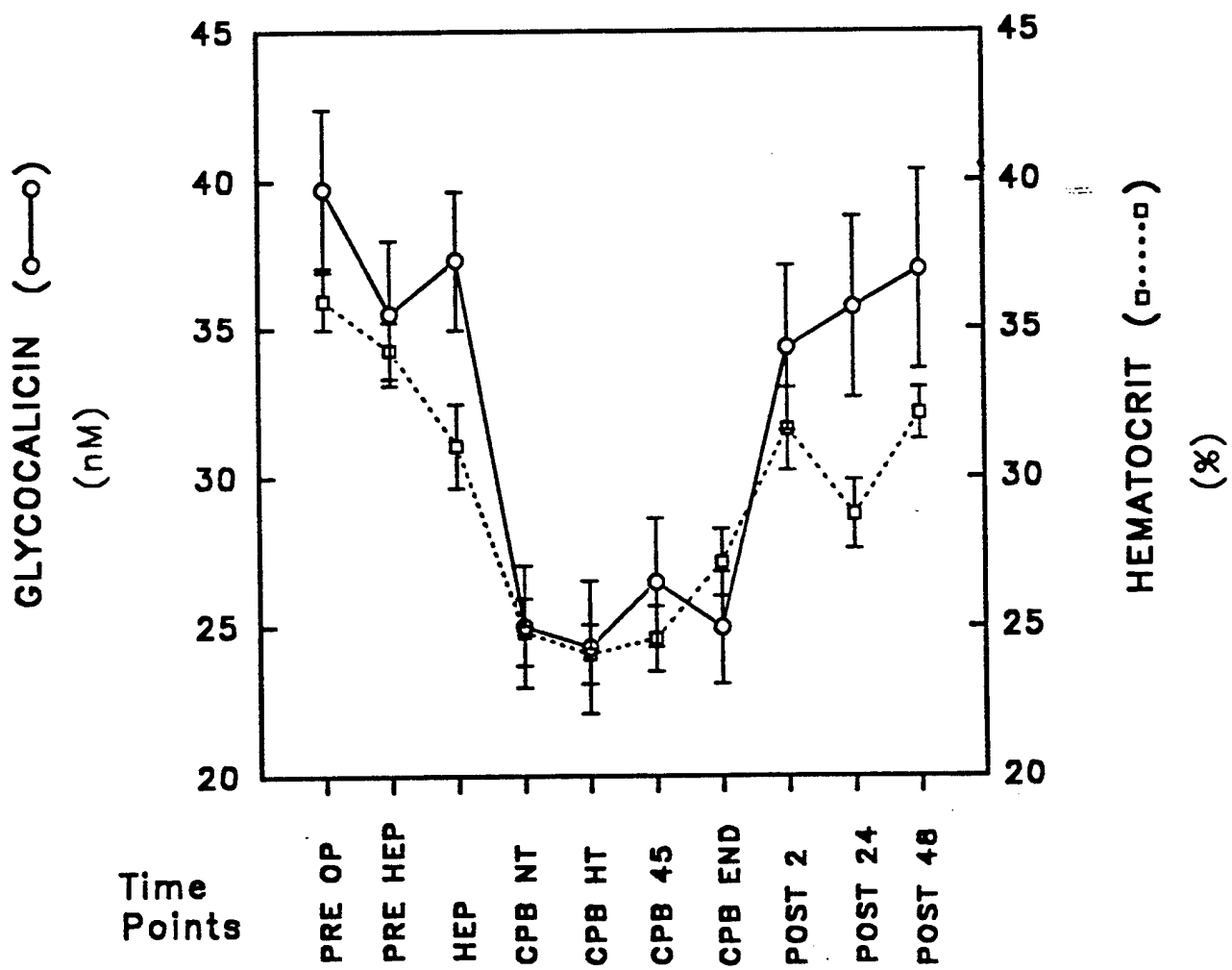
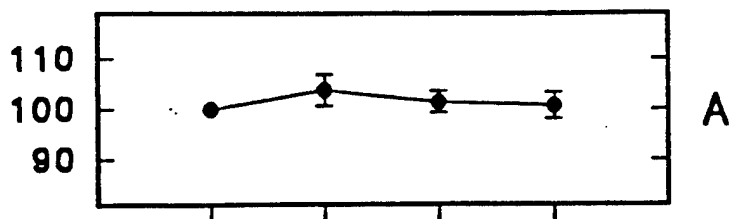


Fig.3

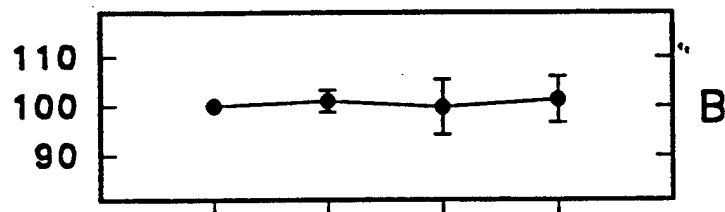


# GPIIb/IIIa

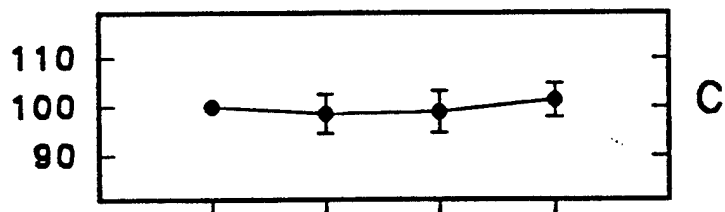
7E3



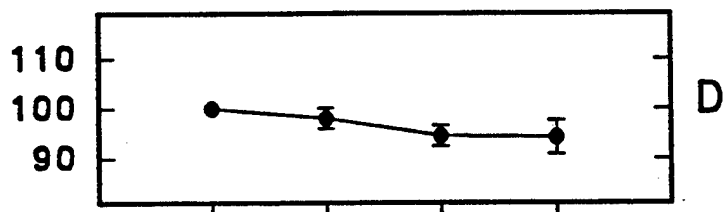
10E5



M148



Y2/51



FIBRINOGEN

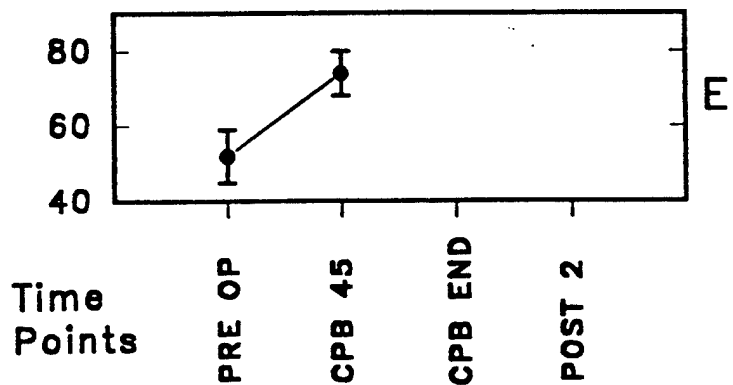


Fig. 4

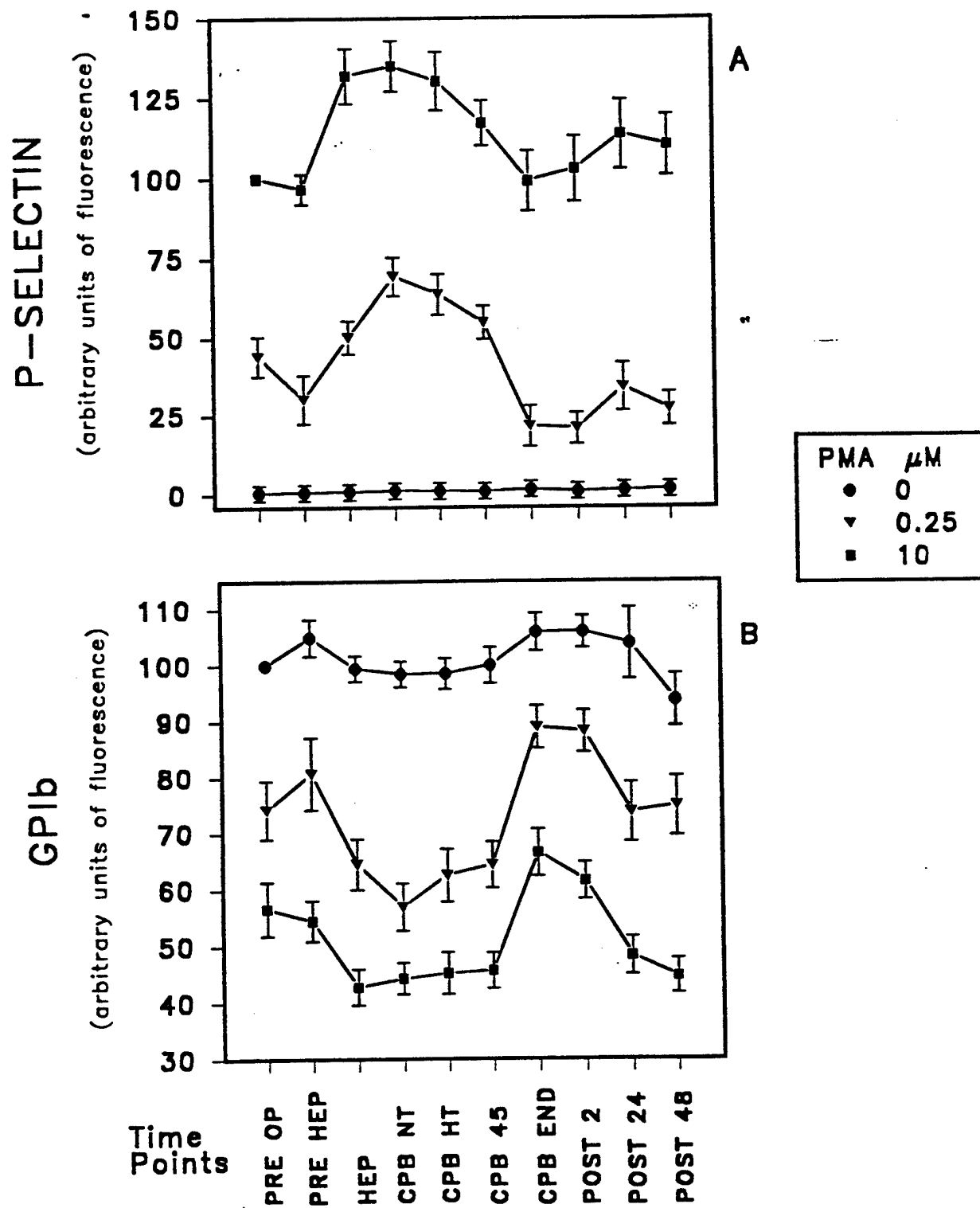


Fig. 5

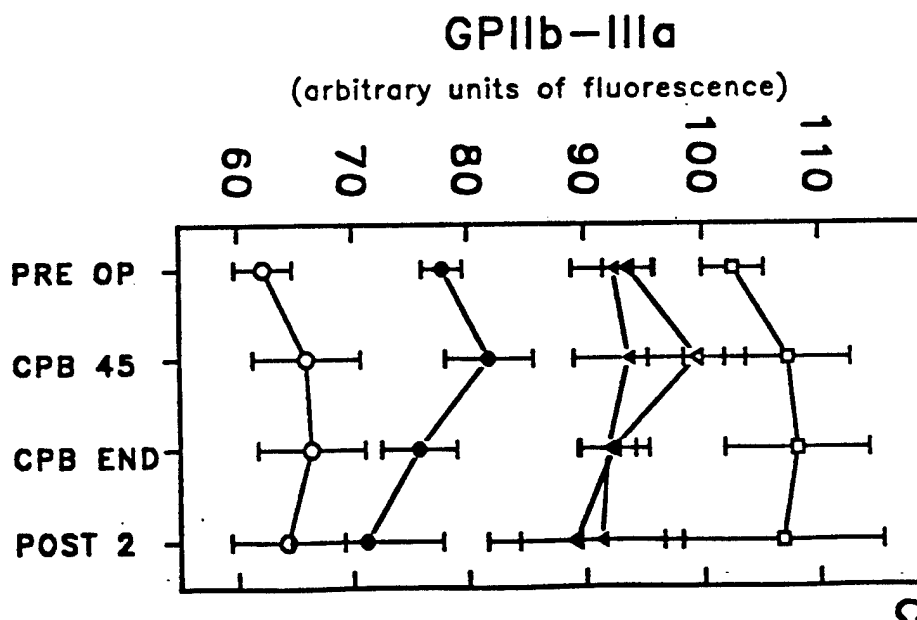
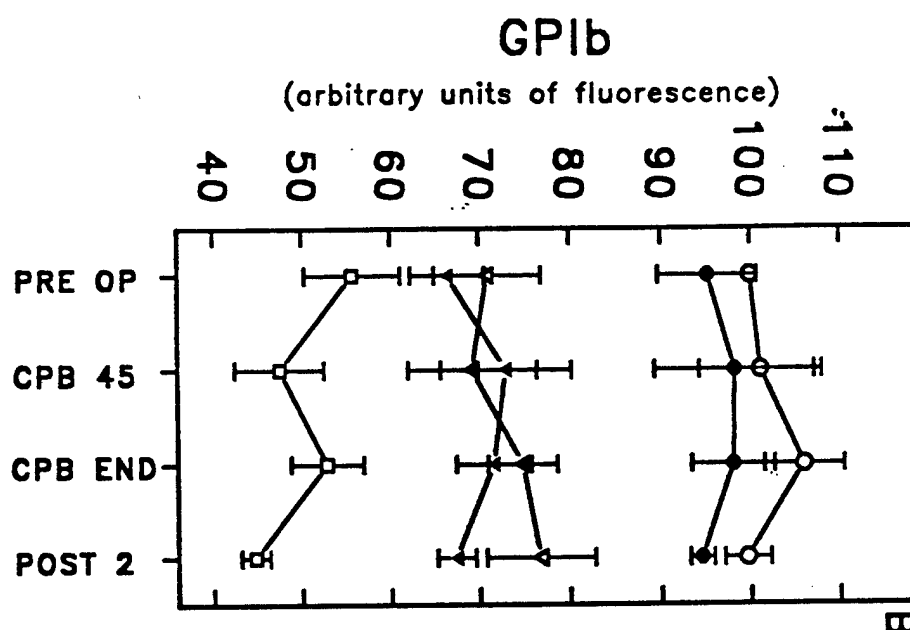
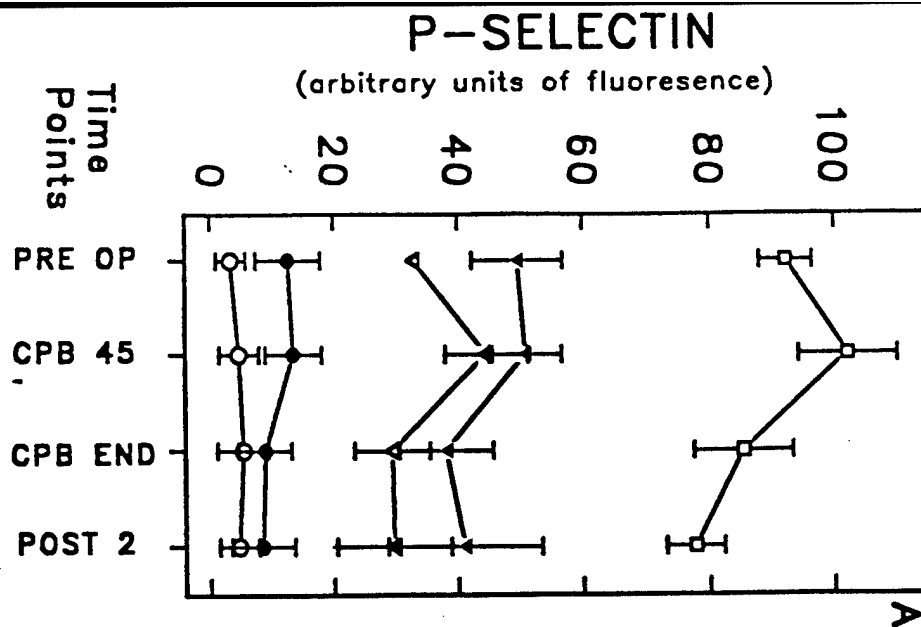
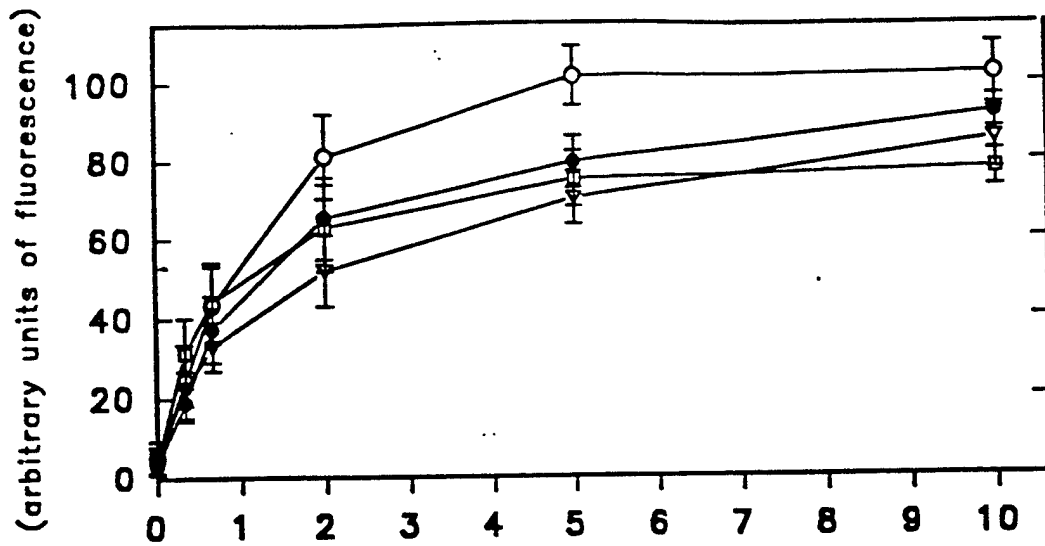


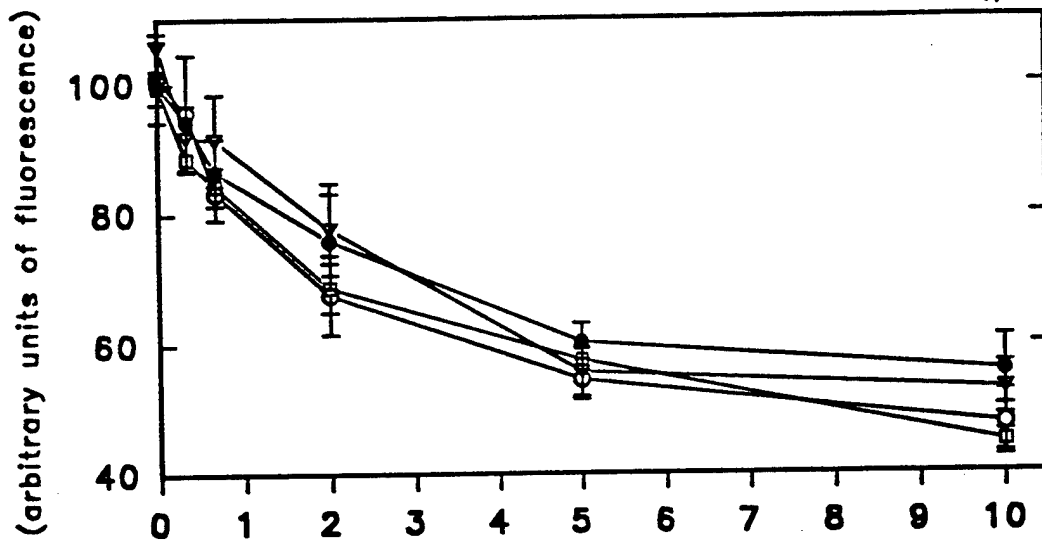
Fig. 6

P-SELECTIN



A

GPIIb

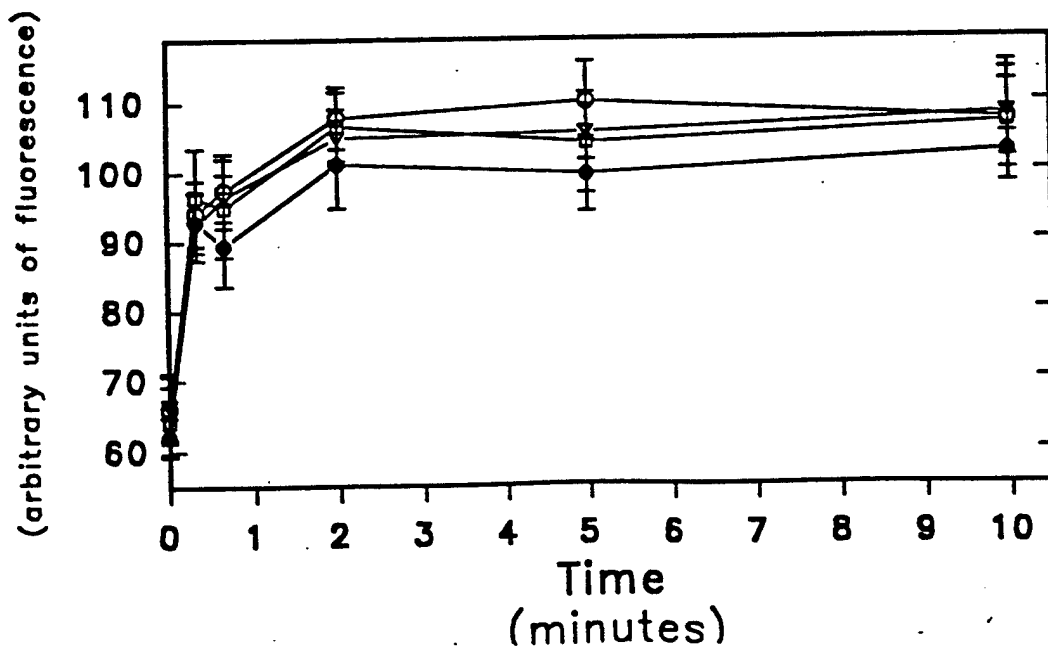


B

Time Points

- PRE OP
- CPB 45
- ▽ CPB END
- POST 2

GPIIb-IIIa

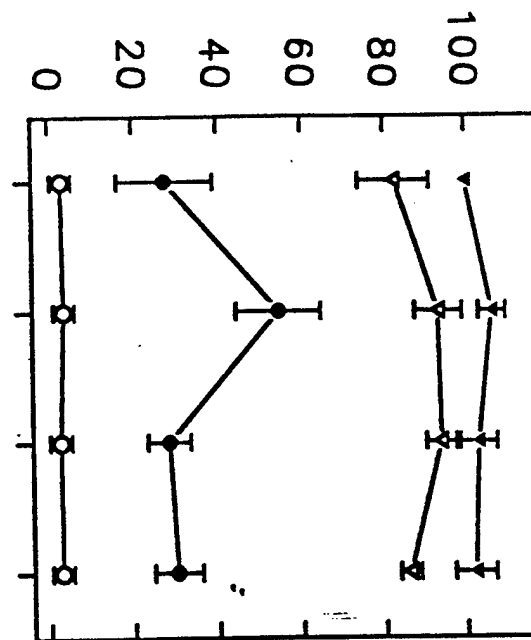


C

Fig. 7

## P-SELECTIN

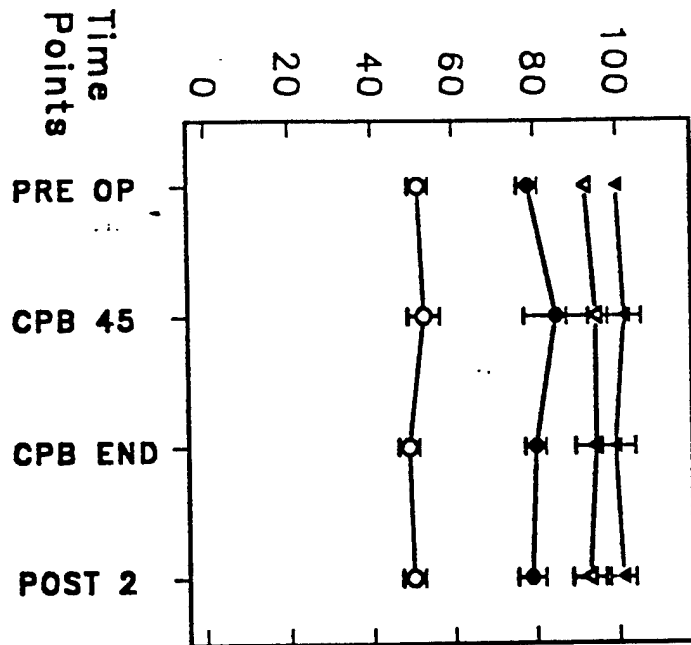
(arbitrary units of fluorescence)



A

## GPIIb-IIIa

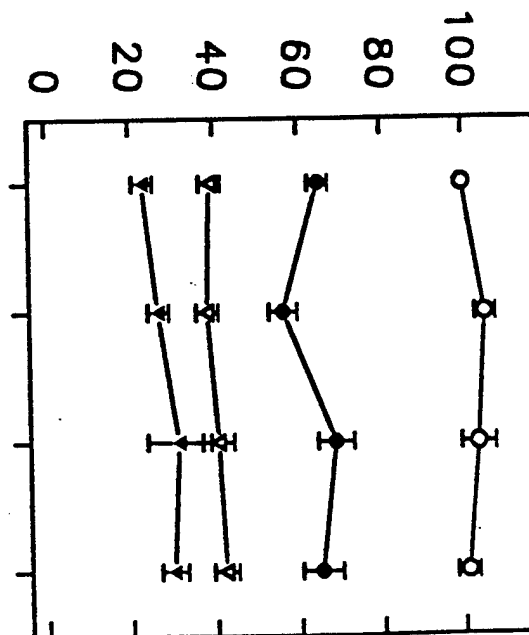
(arbitrary units of fluorescence)



C

## GPIb

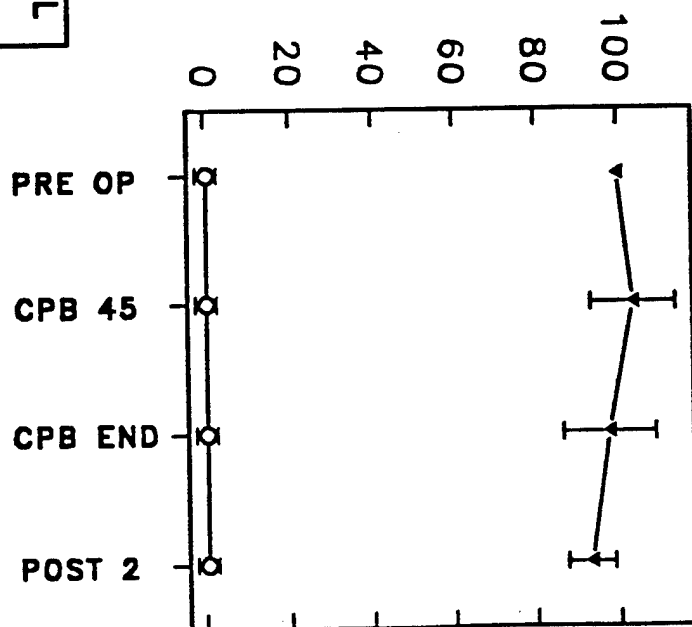
(arbitrary units of fluorescence)



B

## GPIIb-IIIa

(arbitrary units of fluorescence)



D

THROMBIN U/mL

○ 0

● 0.05

▼ 0.1

Fig. 8

# P-SELECTIN

(arbitrary units of fluorescence)

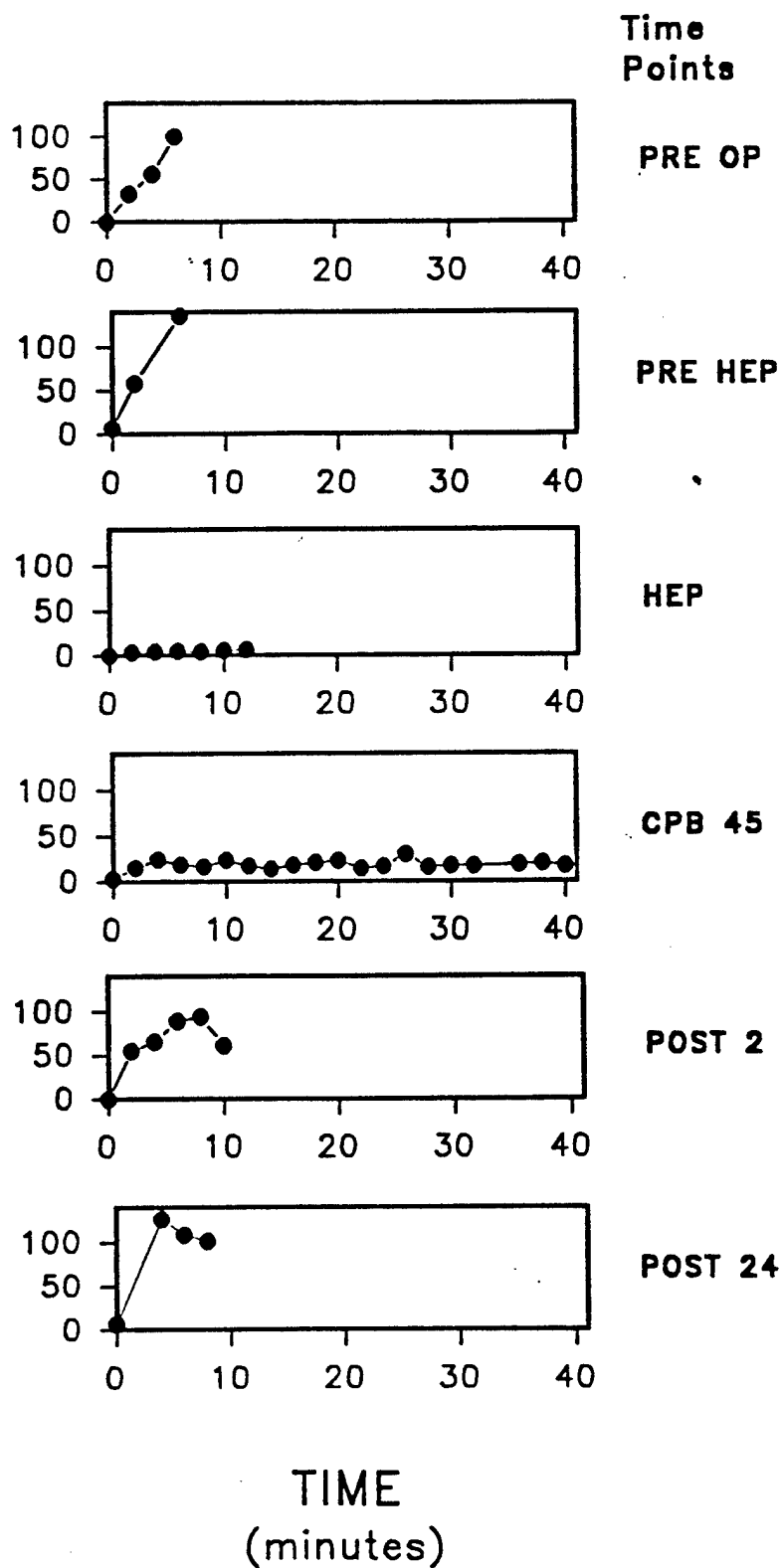


Fig. 9

SHED BLOOD THROMBOXANE B<sub>2</sub>  
(pg/0.1 mL)

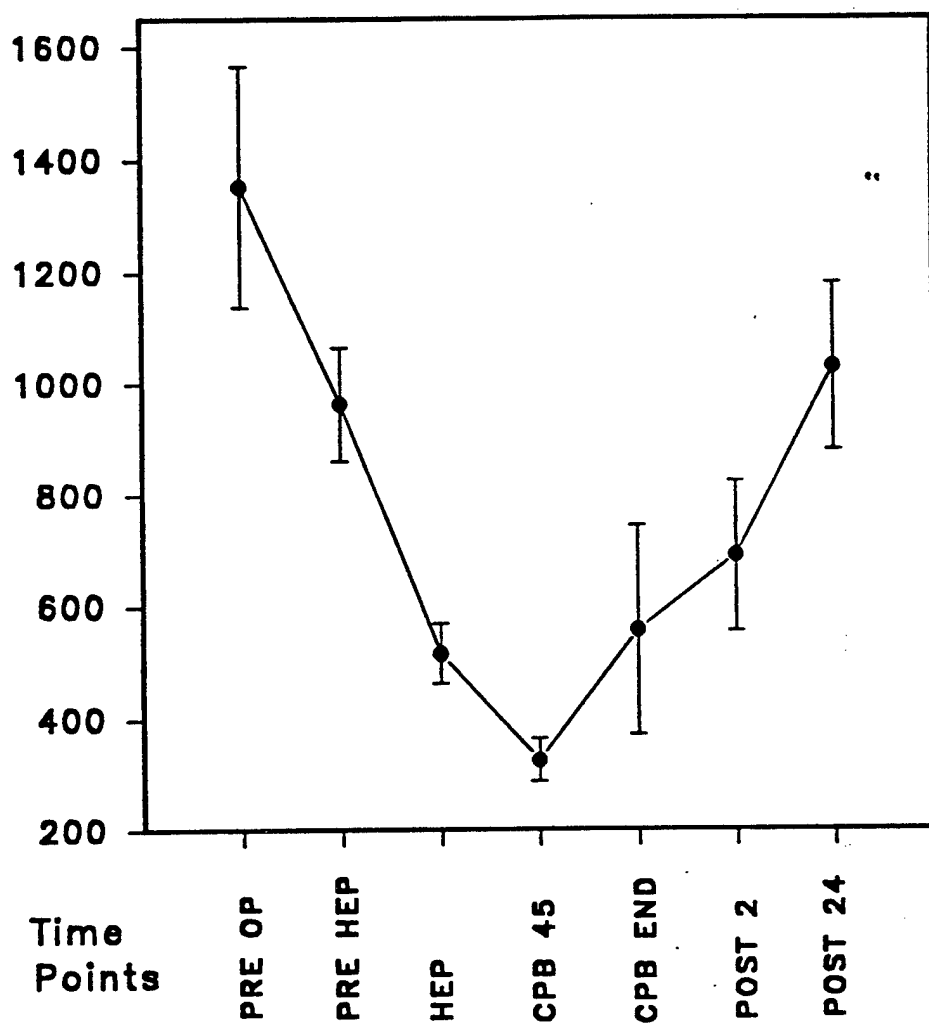


Fig. 10