PROGRESS REPORT

August 1, 1976 to October 31, 1977
ERDA - Contract #(11-1)-2783
"Physiopathology of Blood Platelets
and Development
of Platelets Substitutes"
Presented by Mario G. Baldini, M.D.
on July 31, 1977

DISCLAIMER

INTRODUCTION

This progress report covers the period August 1, 1976 to July 31, 1977 although the contract itself will continue until October 31, 1977. This report is submitted by Mario G. Baldini, M.D., Principal Investigator.

During the past year, work was concentrated on two main subjects: 1) Pathogenesis and prevention of thromboembolic phenomena, and 2) Platelet alloimmunization and the effect of antibodies on studies of platelet kinetics.

Results on these subjects were interesting and developed while work was going on, so that less activity was devoted to other subjects which were also included in our project proposal, i.e. studies on platelet nucleotides (which are presently being carried out more intensively), and those on the effect of ionizing radiation on platelets which were left aside.

Several of our publications appeared during the past year, others were published in abstract form and are presently being completed for publication. Several papers were reported at national and international conferences. All these are listed in the following section. Reprints of papers published are being submitted simultaneously. Copy of the abstracts are here included in one of the following sections.

All the investigators who were listed in our project proposal worked actively on these projects during the past year. These included:

1. Mario G. Baldini, M.D.-Principal Investigator (20% of time).
2. Manfred Steiner, M.D., Ph.D.-Co-Principal Investigator (15% of time).
3. Byung K. Kim, M.D.-Senior Investigator (50% of time).
4. Toshiro Nagasawa, M.D.-Co-Investigator (100% of time).
5. Hiroshi Nagasawa, M.D.-Co-Investigator (100% of time).

The non-professional personnel who collaborated in these investigations were:

1. Elaine Therrien, Research Technician (80% of time).
2. Joyce Merchant, Research Technician (80% of time).
3. Donna Field, Senior Research Technician (20% of time).

PUBLICATIONS WHICH APPEARED IN PRINT SINCE LAST PROGRESS REPORT ONE YEAR AGO.

1. Castellan, R.M. and Steiner, M. "Effect of Platelet Age on Adhesiveness to Collagen and Platelet Surface Charge". Thrombosis and Haemostasis 36:392-400, 1976.

2. Agarwal, K.C. and Steiner, M. "Effect of Serotonin on Cyclic Nucleotides of Human Platelets". Biochem. and Biophys. Res. Communications. 69:962-969, 1976.

3. Kim, B.K., Tanoue, K. and Baldini, M.G. "Storage of Human Platelets by Freezing". Vox Sanguinis 30:401-411, 1976.

4. Kim, B.K. and Baldini, M.G. "Platelet Reversal Reaction: Its Mechanism and Significance" Chapter 13 in "Blood Bank Technology" 2nd Ed. Williams and Wilkins, Baltimore, 1976, pages 126-130.

5. Baldini, M.D. and Ikeda, Y. "Purpura Due to Capillary and Platelet Disorders" Chapter in "Current Diagnosis", ed. by H.F. Conn and R.B. Conn Jr., W.B. Saunders and Co., Philadelphia, Pa., 1976, pages 533-550.

PAPERS PUBLISHED IN ABSTRACT FORM

1. Kim, B.K., Tanoue, K. and Baldini, M.G. "Adenine Nucleotide Pools and Aggregability of Human Platelets during Preservation at 22°C." Proc. XVI Intern. Congress of Hematology, Sept. 1976, Kyoto, Japan. p. 289

2. Baldini, M.G. "Kinetics of ^{51}Cr-labeled Platelets." Proc. XVI Intern. Congress of Hematology, Sept. 1976, Kyoto, Japan. p. 40

3. Baldini, M.G., Nagasawa, T. and Kim, B.K. "Arrest or Prevention of Hemorrhage in Severely Alloimmunized Thrombocytopenic Patients." Proc. XVI Intern. Congress of Hematology, Sept. 1976, Kyoto, Japan. p. 136.

4. Kim, B.K. and Baldini, M.G. "Early Platelet Activation During Perfusion of a Hollow Fiber Artificial Kidney." Proc.VI Intern. Congress on Thrombosis and Hemostasis, Philadelphia, June 1977, p. 204

5. Nagasawa, T., Kim, B.K. and Baldini, M.G. "Platelet Alterations Caused by Antiplatelet Antibodies in the Circulation." Proc. VI Intern. Congress on Thrombosis and Hemostasis, Philadelphia, June 1977, p. 142

6. Nagasawa, H., Steiner, M. and Baldini, M.G. "Modification of Thrombin-Induced Platelet Aggregation by Estrogen." Proc. VI Intern. Congress on Thrombosis and Hemostasis, Philadelphia, June 1977, p. 186

7. Nagasawa, T., Kim, B.K. and Baldini, M.G. "In vivo Elution of ^{51}Cr From Labeled Platelets Induced by Antibody." Federation Proc., Chicago, April 1977, p. 380

Work performed during the past year will be summarized under the following headings:

A) THE EFFECT OF ESTROGENS ON PLATELET AGGREGABILITY AND THROMBUS FORMATION

A growing number of studies show that a state of hypercoagulability does exist in users of oral contraceptives. There seems to be general agreement that factors II, VII, X and fibrinogen are increased and antithrombin III decreased. Reduction in the level of this inhibitor has been repeatedly confirmed both by coagulant and immunological methods. As the actual role of this protein in the regulation of blood coagulation is being more fully appreciated, understanding the mechanism by which oral contraceptives decrease its plasma levels becomes of increasing importance. The studies performed by us address themselves to this problem.

Almost all of our studies have been carried out with materials derived from human sources. Platelets were isolated by conventional methods from citrated fresh blood which was obtained from donors who had been off any medication known to influence platelet aggregation for at least seven days. The platelets were washed and suspended in Ca^{2+}-free Tyrode buffer. Antithrombin III was isolated by the method of Abildgaard with inclusion of a final purification step on an isoelectric focusing column containing carrier ampholines in the pH range of 4-6. Antithrombin III was focused at a pH of 5.3. The fractions making up the peak were combined and dialyzed against 0.1M Tris buffer, pH 7.4 containing 0.15M NaCl. The purity of the isolated inhibitor was determined by electrophoresis on polyacrylamide gels with and without SDS and by immunoelectrophoresis using a commercially available antiserum against whole human serum. The specific activity of the final protein preparation was twice the normal plasma antithrombin activity per mg protein.

Bovine thrombin used for most of our aggregation studies was obtained from a commercial source, and was purified by the method of Lundblad. Human thrombin was a commercial product with a specific activity of 1800 NIH U/mg protein. The purity of both proteases was tested by SDS gel electrophoresis and is shown in the next slide.

All steroids were of highest available purity. They were initially dissolved in hot ethanol and were then diluted with Ca^{2+}-free Tyrode buffer to the desired concentration. The final concentration of ethanol in the incubation mixture was 0.0025%. This concentration of ethanol did not produce any change in platelet aggregation.

In previous studies, one of us (Dr. H. Nagasawa) had shown that estrogen in concentrations of 3-24 mM can inhibit in vitro in a dose-dependent manner the thrombin-neutralizing activity of antithrombin III measured immunologically or by thrombin-fibrinogen time. Searching for a more sensitive method to detect the effect of estrogens on antithrombin III, we have investigated during the past year the platelet aggregating ability of thrombin (Fig. 1). To 0.4 ml of platelet suspension were added in the indicated sequence, buffer, antithrombin III and thrombin, each in a volume of 10µl. 0.015U of thrombin was the minimum concentration to produce complete aggregation in the absence of antithrombin III. Increasing concentration of antithrombin III added to the system resulted in a progressive reduction of thrombin-induced aggregation. 0.15U antithrombin III was the smallest concentration able to inhibit platelet aggregation induced by 0.025U

thrombin (Fig. 2). These concentrations of protease and inhibitor were used in subsequent experiments in which the effect of estrogens and other steroid hormones was investigated. The addition of estrogen in the indicated final concentrations progressively returned thrombin-induced aggregation, restoring it to its initial level at 1.25×10^{-7}. In order to rule out a possible influence of estrogen on thrombin or platelets, the following experiment was performed.

The minimal amount of thrombin producing only very slight aggregation was established and the effect of addition of estrogen studied. Concentration of the steroid hormone up to ten times that which were found to be effective in overcoming the antithrombin III related neutralization of thrombin, did not enhance thrombin-induced aggregation of platelets. This result indicates that estrogens exert their effect through interaction with antithrombin III and not through thrombin or platelets.

In further experiments we investigated the specificity of the steroid effect on antithrombin III. The major classes of steroids which were examined and their effectiveness in this system are shown in Fig. 3 where equimolar concentrations are compared. They range in decreasing order of effectiveness from ethinyl estradiol, to progesterone, testosterone, cortisone and cholesterol (Fig. 3)

To obtain direct evidence that estrogen is bound to antithrombin III we carried out a number of different binding studies. The results of one study in which antithrombin III was incubated for 60 minutes at 4°C with different concentrations of estrogen are shown in Fig. 4. The reactions were terminated by addition of a measured amount of DEAE Sephadex sufficient to bind >95% of the antithrombin III in the mixture. The result, corrected for absorption of estrogen onto the walls of the tube, were plotted according to Scatchard. From the intercept on the abscissa we estimated approximately one binding site per molecule of antithrombin III (Fig. 4).

The next question which arises is whether the binding of estrogen changes the structure of antithrombin III in such a way that it is incapable of forming a complex with thrombin. That the biological function of the inhibitor is altered is clearly suggested by the results described before. A structural change in the protein molecule induced by estrogen was looked for by SDS polyacrylamide gel electrophoresis in the presence of sulfhydroreducing agent. There was no evidence that estrogens prevented the complex formation between antithrombin III and thrombin. Omission of the detergents, however, revealed a striking difference between control and estrogen-treated complexes. It should be stress also that heating of the misture containing estrogen-treated antithrombin III-thrombin complex abolished the migration differences between control and estrogen-treated samples.

In conclusion, our studies have shown that:

1) Antithrombin III can bind estrogen in a specific manner,
2) Estrogen-treated antithrombin III exhibits impaired thrombin-neutralization,
3) Estrogen does not affect the activity of thrombin nor does it cause any apparent alteration of platelet function,
4) Steroid hormones other than estrogen and cholesterol also

decrease antithrombin III activity,

5) Steroids in nanomolar concentrations alter antithrombin III activity as measured by our platelet aggregation system,

6) Complex formation between estrogen-treated antithrombin III and thrombin is not blocked but the antithrombin III molecule seems to have undergone a structural change resulting in an alteration of the binding site of thrombin such that the serine protease bound to the inhibitor remains active,

7) Finally, our studies strongly indicate that steroids exert a very important role in hemostasis by modulating the activity of antithrombin III.

MODIFICATION BY ANTITHROMBIN III OF THROMBIN INDUCED PLATELET AGGREGATION

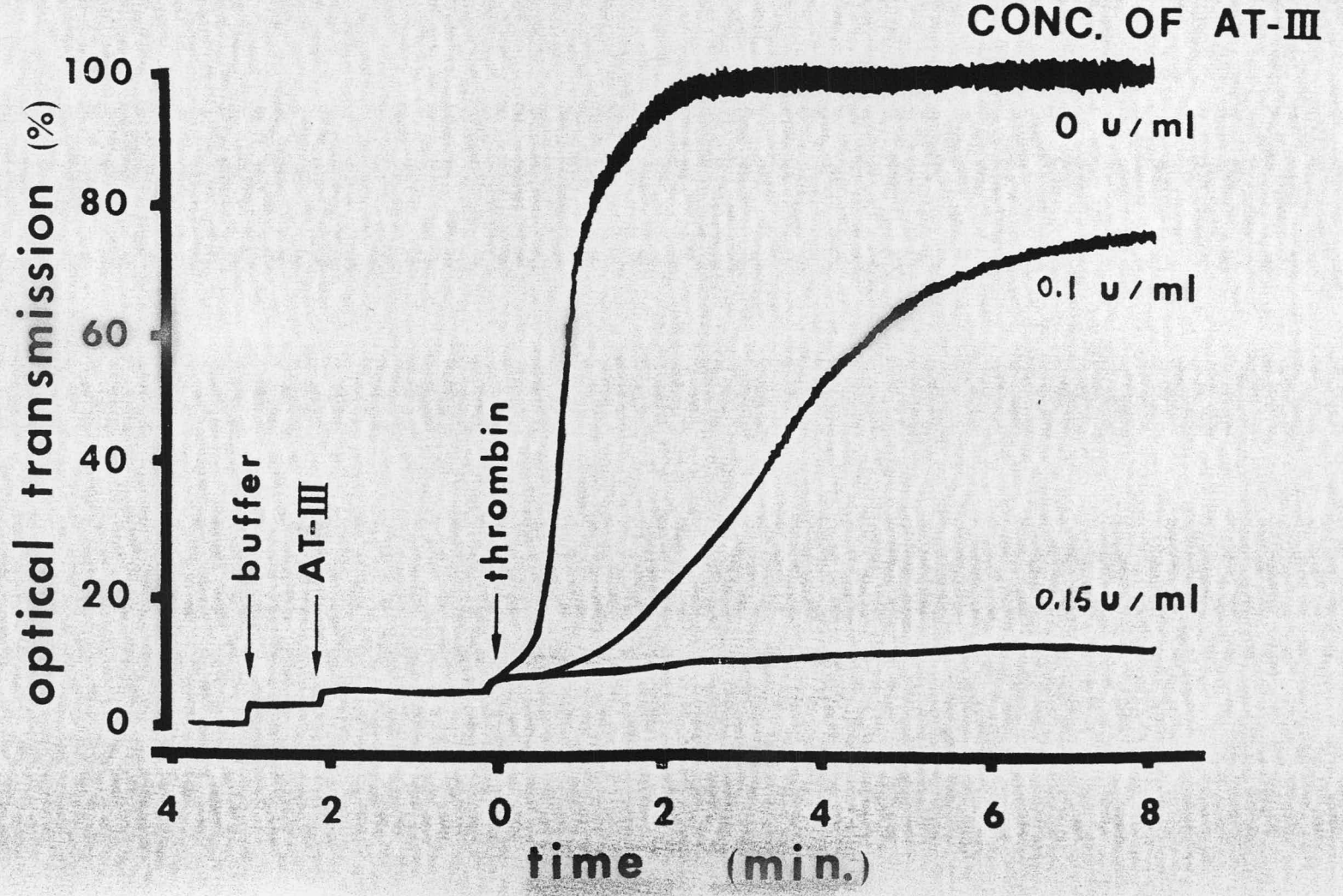


Fig. 1

EFFECT OF ESTROGEN ON AT-III. MODIFICATION OF THROMBIN INDUCED PLATELET AGGREGATION

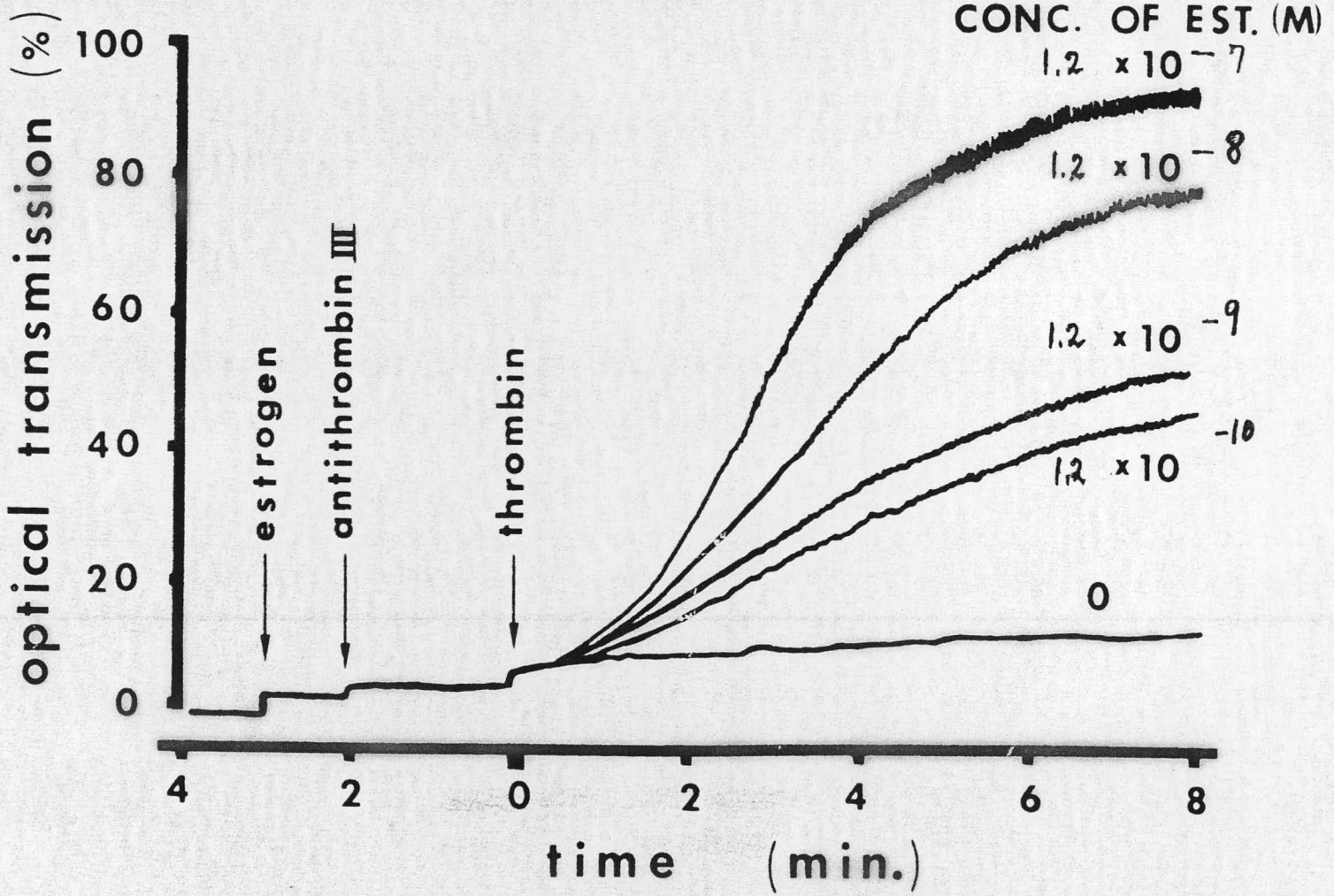


Fig. 2

EFFECT OF STEROIDS ON AT-III, MODIFICATION OF PLATELET AGGREGATION BY THROMBIN

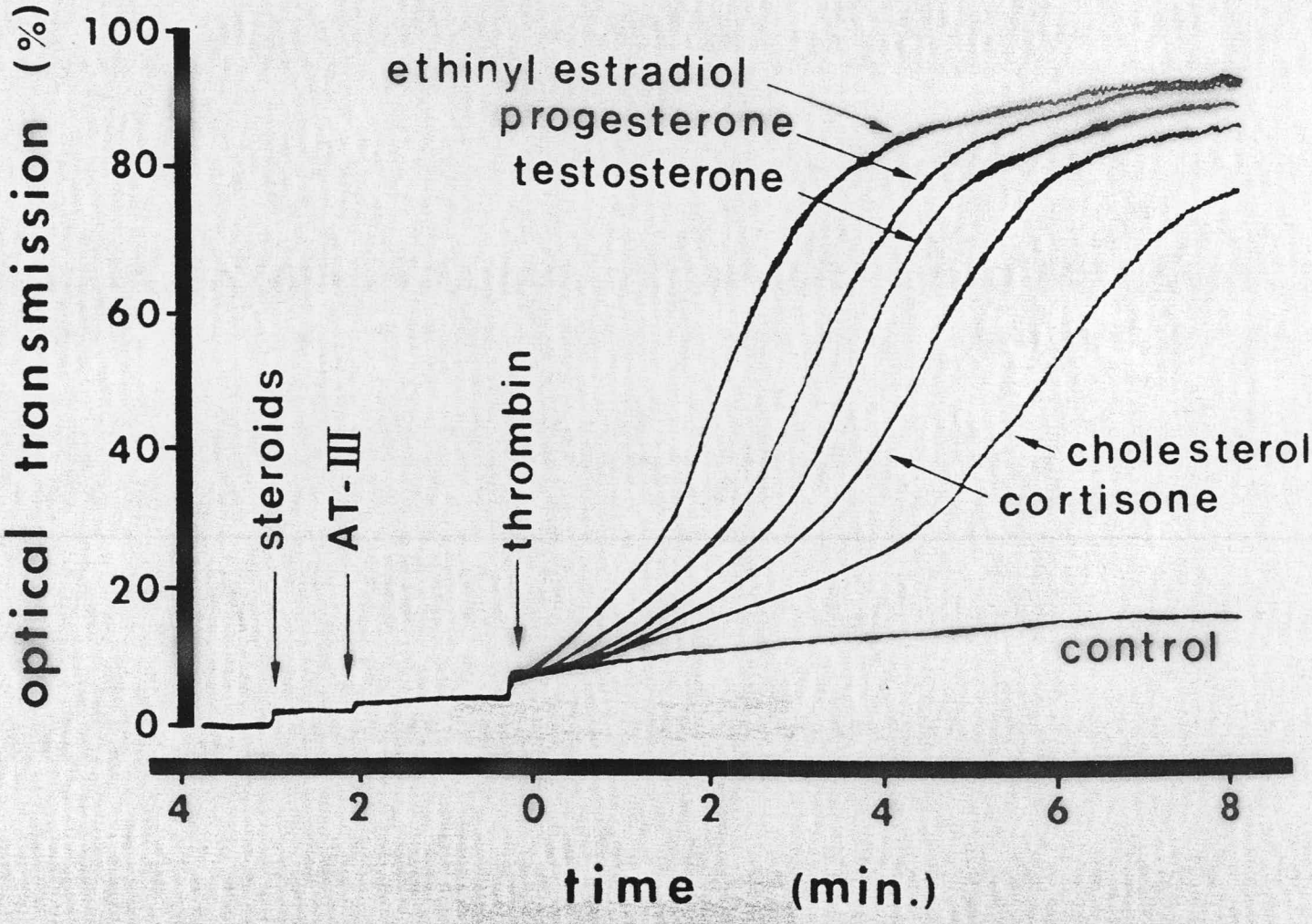
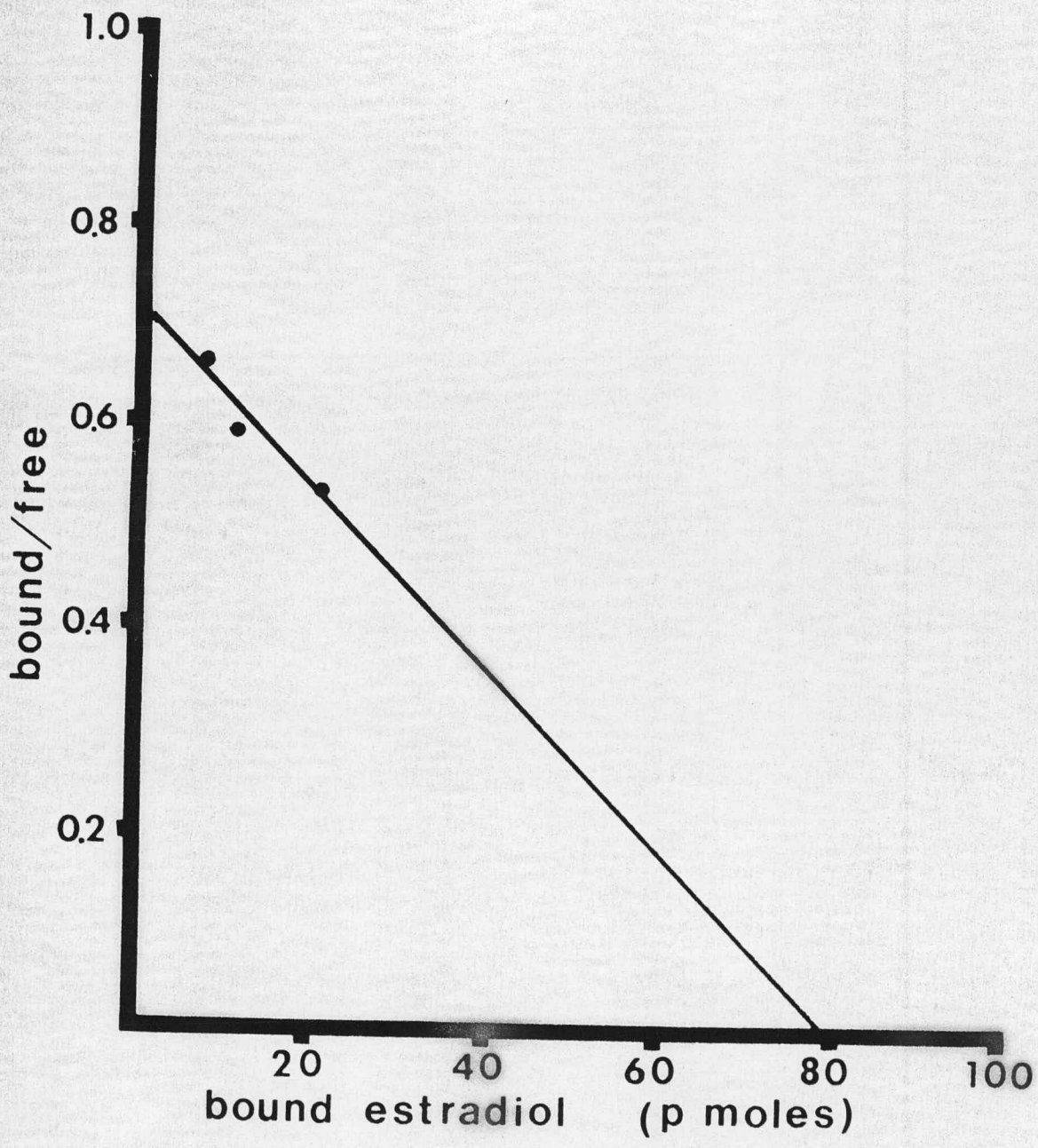


Fig. 3

Fig. 4

SCATCHARD ANALYSIS OF BINDING BETWEEN AT-III & 17β-ESTRADIOL



B) STUDY ON THE ANTITHROMBOTIC EFFECT OF PLATELET INHIBITING AGENTS IN A BENCH MODEL OF ARTIFICIAL KIDNEY.

A small bench model of a hollow fiber artificial kidney has been developed in our laboratory during the past few years and has been used to study and measure the effect of antithrombotic drugs. This device containing 64 hollow silicone rubber fibers (Cordis-Dow hollow cellulose semipermeable fibers) is enclosed in a polyurethane casing six inches in length. It is inserted in a flow-circuit of silicone rubber tubing in which freshly collected human blood from a reservoir bag circulates at constant speed by the aid of a roller-pump. Pressure of the blood in the tubing between the pump and perfusion device is monitored by the use of a sensitive pressure transducer (Model 377, Harvard Apparatus, Millis, Mass.) connected with a recorder (Fig. 1). Increase of pressure in the circuit indicates blocking of the hollow fibers by platelet clumps adhering to their inlet and thrombus formation.

Our preliminary observations during the development phase of this system in the past few years were obtained by the use of ACD-anticoagulated blood and suggested that our bench model of perfusion organ is a reliable tool to study the effect of antithrombotic drugs. During the past year our experiments were conducted with heparinized blood since heparin is the anticoagulant used in the actual operation of most artificial perfusion organs. Thirty ml of normal human blood anticoagulated with 150 units of heparin were perfused at a rate of 6 ml per min. Each experiment was done with blood from a different donor. The mean value of pressure increase observed in 45 experiments using 6 equal perfusion devices was 63.9 mmHg after 15 min and 99.8 mmHg after 30 min of perfusion (Table I). The values of pressure increment during perfusion for 30 min varied depending on the individual device used (Table I).

To minimize variations, experiments were paired, one done with pure blood (control) and the other with blood to which an antithrombotic agent had been added, and both experiments were done using a single perfusion unit and blood from one single donor. The whole circuit including the perfusion device was cleaned immediately after each experiment by washing in 5% biodegradable detergent (FL-70, Fisher Scientific Corp., Fairlawn, N.J.) for two hours. Two different concentrations of platelet inhibiting agents were used as indicated in Table 2. Pressure increments in the tubing during 15 min and 30 min perfusion were expressed in percent of the respective control value. The agents studied exhibited a dose dependent antithrombotic effect although variations in results were wide (Table 2). The most potent inhibiting effect was obtained with dipyridamole and the least potent with Phenylbutazone. More doses are, however, being studied. Chelating calcium ion with ACD-anticoagulant solution (acid-citrate-dextrose, "Formula A") also suppressed the pressure increment, indicating its antithrombotic effect.

Combinations of two different agents to examine their synergistic effect are reported in Table 3. In this study, a lower than usual concentration of each drug was used. Aspirin appeared to enhance the antithrombotic effect of dipyridamole (Table 3). The combination of dipyridamole and sulfinpyrazone, sulfinpyrazone and phenylbutazone or sulfinpyrazone and aspirin, however, did not show any increased effect when compared to the results obtained with the simple drugs (Table 3).

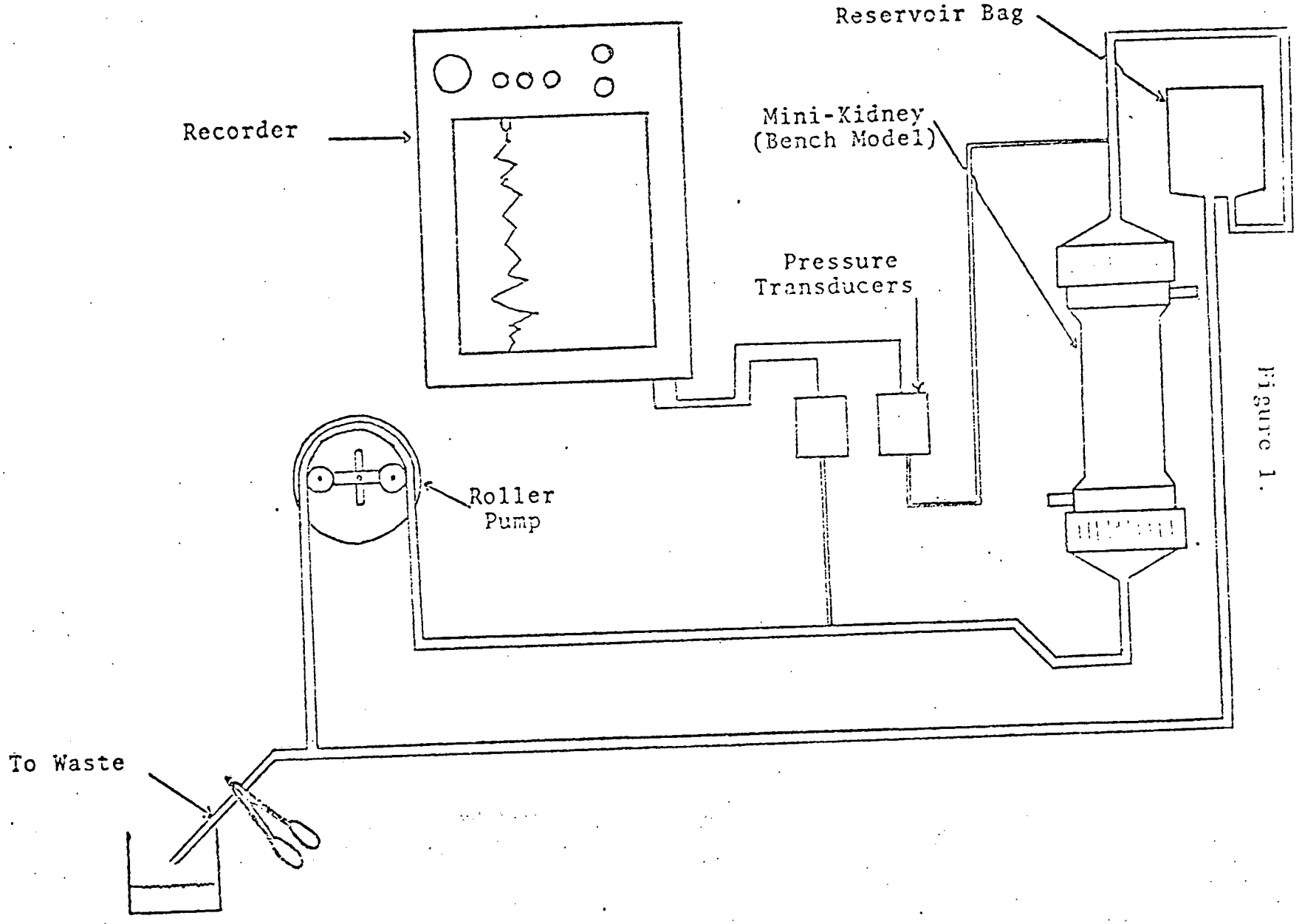

Reservoir Bag
Recorder
Mini-Kidney
(Bench Model)
Pressure
Transducers
Roller
Pump
To Waste

Figure 1.

Perfusion device, number	No. of Expts.	Period of Perfusion 15 min	30 min
		mmHg	mmHg
1	10	83.0 ± 26.5	133.8 ± 30.4
2	10	61.2 ± 20.3	92.2 ± 25.0
3	10	67.1 ± 19.8	92.4 ± 28.5
4	5	66.0 ± 23.1	119.9 ± 33.1
5	5	36.1 ± 11.4	68.0 ± 18.2
6	5	50.6 ± 14.1	73.7 ± 24.6
TOTAL	45	63.9 ± 16.2	99.8 ± 25.7

TABLE I: Pressure changes in the tubing after 15 min and 30 min perfusion in our bench model of artificial organ. Heparinized blood (5 units per ml) was circulated at a rate of 6 ml/min. Mean pressure increments ± 1 SD are reported. These were obtained with one or with multiple artificial units as indicated in the left column. One different blood donor was used for each experiment.

Platelet Inhibiting Agents	Conc. of Agent	No. of Expts.	Period of Perfusion 15 min	30 min
Dipyridamole	10^{-5}M	6	31.5 ± 14.2%	42.6 ± 17.1%
	10^{-4}M	6	15.1 ± 10.9	13.2 ± 11.4
Sulfinpyrazone	$6x10^{-5}$M	5	30.5 ± 11.9	54.3 ± 23.7
	$2.4x10^{-4}$M	5	29.6 ± 15.6	38.2 ± 21.3
Phenylbutazone	10^{-4}M	4	80.8 ± 26.2	107.5 ± 19.6
	$5x10^{-4}$M	4	88.7 ± 21.8	88.1 ± 17.0
Aspirin	$2.5x10^{-5}$M	4	86.5 ± 19.2	87.3 ± 25.3
	$5x10^{-5}$M	4	69.2 ± 12.7	69.5 ± 20.9
Motrin	$5x10^{-5}$M	5	110.4 ± 27.6	85.5 ± 29.4
	10^{-4}M	5	121.0 ± 32.7	72.9 ± 19.1
ACD Solution	10%	5	80.1 ± 23.4	83.5 ± 30.3
	15%	5	49.0 ± 28.3	51.9 ± 27.8

TABLE II: Antithrombotic effect of various platelet inhibiting agents; pressure changes in the circuit tubing observed after 15 min and 30 min perfusion were espressed in % of respective control values.

Inhibitory Agents	Concentration of Agent	No. of Expts.	Period of Perfusion 15 min	30 min
1. Dipyridamole	$1x10^{-5}M$	6	31.5 ± 14.2%	42.6 ± 17.1%
2. Combined with Sulfinpyrazone	$6x10^{-5}M$	6	33.9 ± 10.3	42.2 ± 13.5
3. Combined with Aspirin	$2.5x10^{-5}M$	6	25.0 ± 11.0	29.1 ± 10.2
1. Sulfinpyrazone alone	$6x10^{-5}M$	5	30.5 ± 11.9	54.3 ± 23.7
2. Combined with Aspirin	$2.5x10^{-5}M$	5	46.5 ± 10.7	52.7 ± 15.0
3. Combined with Phenylbutazone	$1x10^{-5}M$	5	34.2 ± 10.4	49.0 ± 14.6

TABLE III: Antithrombotic effect of combinations of two different drugs. Pressure changes in the circuit tubing were monitored after 15 and 30 minutes perfusion and expressed in % of respective control values.

C) PLATELET ACTIVATION DURING PERFUSION THROUGH A HOLLOW FIBER ARTIFICIAL KIDNEY.

The aim of this project was to study early platelet changes caused by contact with artificial surfaces. It is well known that adhesiveness to the artificial surface and aggregation of blood platelets play a central role in thrombus formation in artificial blood perfusion organs. It has been speculated that during the early phase of blood perfusion in an artificial organ, the platelets would be "activated" and become progressively more sticky until adhesion, then, aggregation occur blocking the blood flow in the perfusion system. We believe that recognition of platelet activation and its measurement may offer new ways of predicting platelet adhesion and aggregation, of understanding these phenomena better and, finally, of suggesting new ways of preventing them. To study the early activation caused by contact with a foreign surface, our bench model of hollow fiber artificial kidney described in the preceding section of this report (Antithrombotic Effect of Platelet Inhibiting Agents) was used.

Thirty ml of platelet rich plasma (PRP; 3.5 to 6.0 x 10^8 cells per ml plasma) or platelet concentrate (2 x 10^9 platelets per ml plasma) prepared from ACD-anticoagulated blood were circulated at a rate of 6 ml per min in the artificial perfusion device (Fig 1, previous section). Sampling was done before and after 5, 15, 30 and 60 min of perfusion. There was no decrease in platelet count and no visible thrombus formation in the fibers during the first 60 min of perfusion. A number of parameters were used to measure platelet activation : 1) platelet aggregation induced with graded doses of ADP and collagen, 2) platelet coagulant activities, 3) ^{14}C-serotonin release, 4) release of platelet enzymes, e.g., β-glucuronidase (a lysosomal enzyme) and purine nucleoside phosphorylase (a cytoplasmic enzyme), 5) synthesis of prostaglandin E2 and $F2_{\alpha}$.

Platelet aggregation was determined by measurement of the maximal change in light transmission using the chronolog aggregometer (Model 300, Chronolog Co. Broomall, PA) and was expressed in percent of light transmission difference between platelet rich plasma and platelet poor plasma. Five to 50 μM ADP and 25 to 100 μg collagen were used as the aggregating agents. In each experiment, the proper concentration for each agent capable of producing a moderate degree of aggregation (40 to 70% increase of light transmission) was determined in a first series of experiments. The control value of ADP-induced platelet aggregation before perfusion in 11 experiments was 55.6 ± 5.8% (mean ± MSE). It became significantly enhanced already after 5 min perfusion and reached the maximum value of 79.6 ± 8.5% after 15 to 30 min perfusion (Fig. 1). When perfusion was further continued, aggregability gradually reduced. After 60 min of perfusion, it became 70.9 ± 5.6. In two experiments, the perfusion period was extended to 120 min and ADP-induced aggregation became 55.8% with a control value of 66.3% at 15 min, 62.8% at 30 min, 57.0% at 60 min, 52.3% at 90 min and 46.5% after 120 min (Fig. 2). These findings indicate that the platelets became activated during the early phase of perfusion, but that they later became less responsive.

Collagen-induced aggregation in the control samples had an average value of 67.2 ± 5.28% (mean ± MSE). It became increased to 79.8 ± 8.3% after 5 min perfusion and reached a maximum of 81.9 ± 6.3% after 30 to 60 min perfusion (Fig. 3).

Platelet coagulant activities, e.g., platelet factor 3 availability and contact product forming activity, progressively increased during perfusion (Table 1). Platelet factor 3 availability was determined by measuring the kaolin clotting time (Hardisty and Hutton; Brit J. Haemat. 12;764, 1966) and contact product forming activity was determined by measuring the recalcification clotting time following preincubation of platelets with 400 M ADP (Walsh: Brit J. Haemat. 22: 205, 1972). Shortening of clotting time indicated enhancement of platelet coagulant activity. The rate of increment in coagulant activities was greater during the first 15 min of perfusion than during the subsequent 45 min.

To investigate the possible mechanisms of platelet activation caused by contact with the foreign surface of the artificial organ, significant changes in the release of platelet granules and in prostaglandin synthesis during the perfusion were investigated. Release of platelet granules was determined by measuring the release of ^{14}C-serotonin (dense granules) from the previously labeled platelets, and β-glucuronidase (lysosomal enzyme) into the plasma. Release of purine nucleoside phosphorylase (a cytoplasmic enzyme) was also measured as a reference indicator of platelet destruction. Platelet release of ^{14}C-serotonin detected in the plasma was only 1.2% after 5 min perfusion, 2.7% after 30 min and 3.2% after 60 min indicating that significant degrees of release reaction were absent during the early phase of perfusion. The amount of purine nucleoside phosphorylase and β-glucuronidase released into the plasma medium in 60 minutes of perfusion was only 1 to 2% of the respective platelet enzyme content indicating that platelet destruction was practically insignificant.

Platelet prostaglandin synthesis was determined by measuring production of radioactive prostaglandin from ^{14}C-arachidonic acid labeled platelets during perfusion. One unit of platelets (5-7 x 10^{10}) concentrated in 10 ml plasma was incubated with ^{14}C-arachidonic acid (0.4 μCi/2.44μg in 20 μl) at 22°C for 60 min and was, then, diluted to 30 ml plasma. Prostaglandin extraction (Jaffe et al: J. Clin. Invest. 52:398, 1973) from the samples obtained before perfusion and after 15 min and 30 min of perfusion was done. Radioactive prostaglandins E2 and $F2_\alpha$ were determined by scanning of silica gel thin layer chromatography (Flowers and Blackwell; Biochem. Pharmacol. 25: 285, 1976). After 15 min and 30 min perfusion of the platelets in the artificial kidney, the levels of total radioactive prostaglandins E2 and $F2_\alpha$ were 97 ± 11.4% and 106 ± 14.2% of control value (before perfusion), respectively. The results indicated that platelet prostaglandin synthesis was not significantly enhanced during 30 min perfusion in our experimental system.

Presently, further experiments are being carried out to study the possibility of inhibiting platelet activation by various chemical agents.

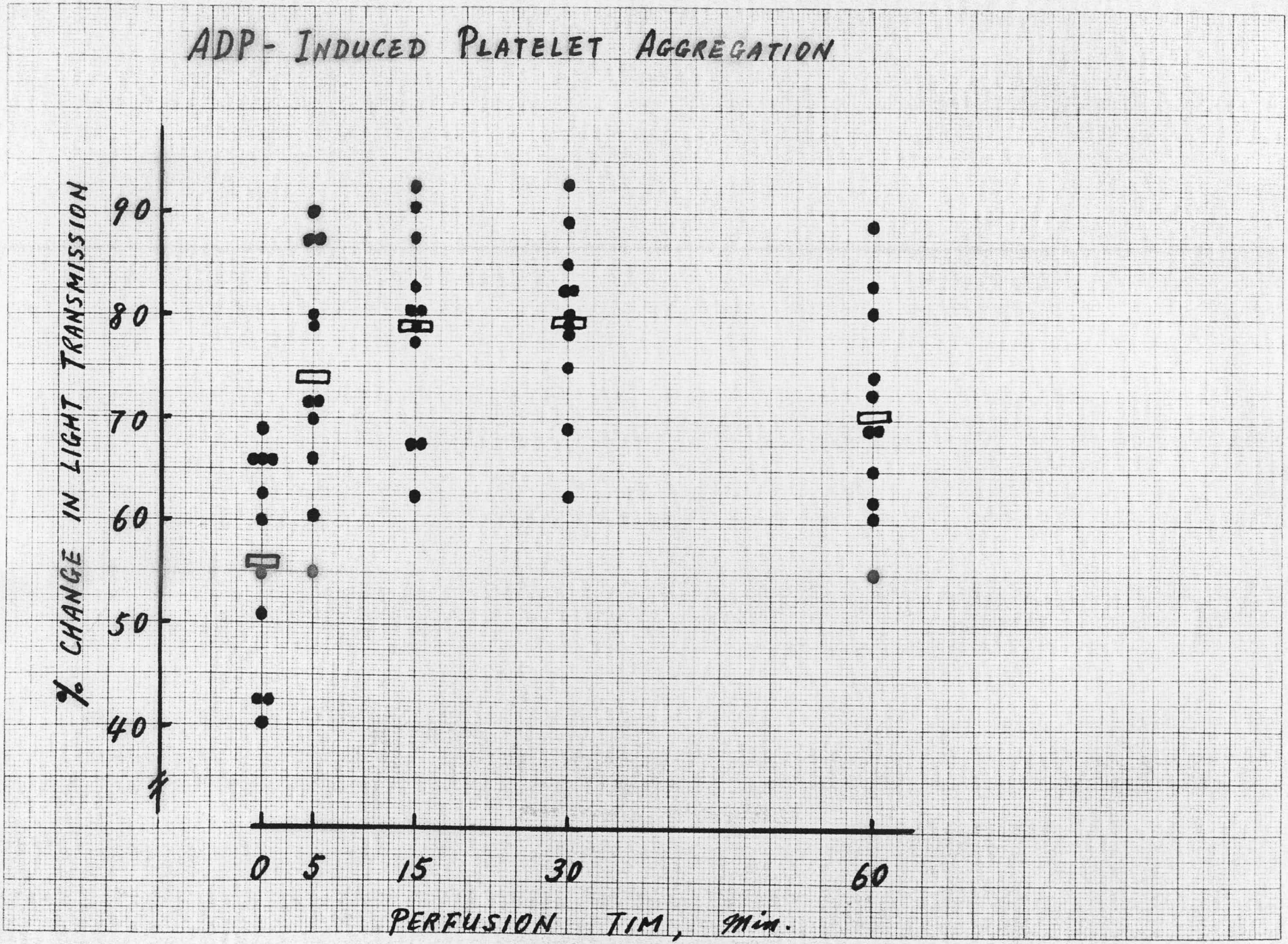

ADP- INDUCED PLATELET AGGREGATION
% CHANGE IN LIGHT TRANSMISSION
90
80
70
60
50
40
0
5
15
30
60
PERFUSION TIM, min.

Figure 1

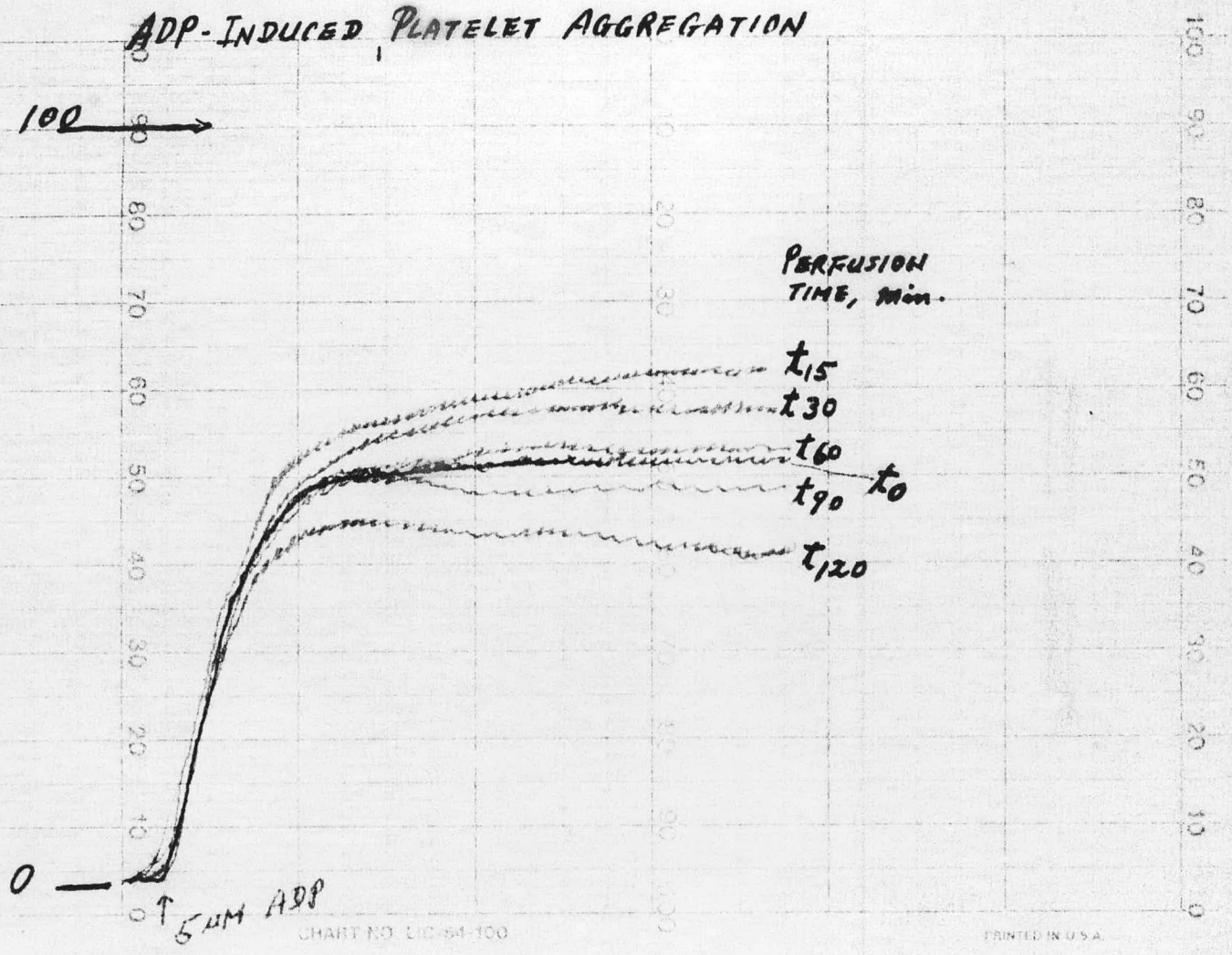

ADP-INDUCED PLATELET AGGREGATION
100
0
5 μM ADP
PERFUSION TIME, Min.
t_{15}
t_{30}
t_{60}
t_0
t_{90}
t_{120}
CHART NO. LIC-64-100
PRINTED IN U.S.A.

Figure 2

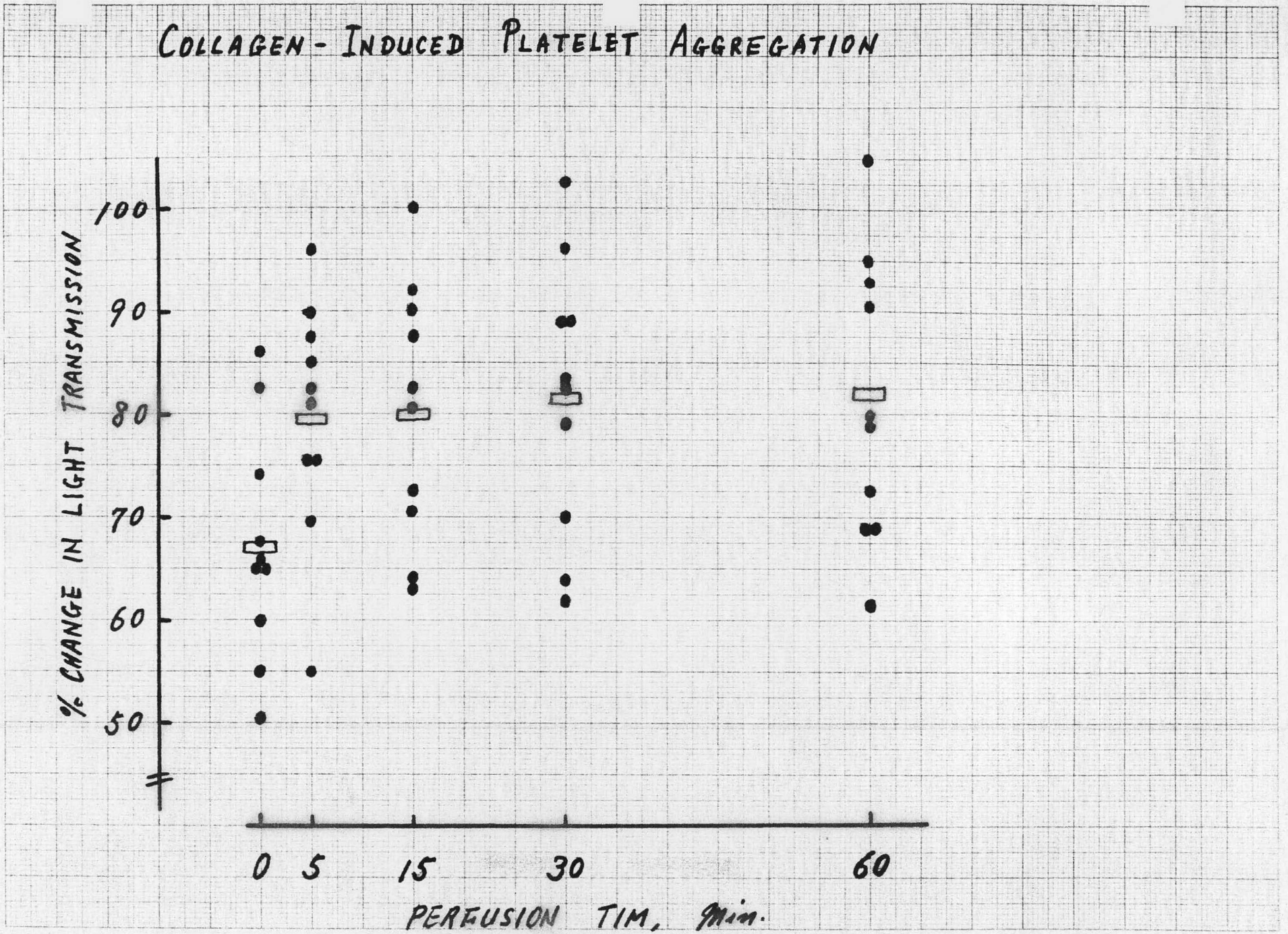


Figure 3

Perfusion Period, min	Platelet coagulant activities	
	Kaolin Clotting Time	Recalcification C T
0	108 ± 6 sec	440 ± 23 sec
5	93 ± 3	390 ± 27
15	84 ± 2	375 ± 33
30	76 ± 3	351 ± 23
60	71 ± 4	351 ± 15

Table 1: Platelet Coagulant Activities: Kaolin clotting time represents platelet factor 3 availability, and recalcification clotting time represents contact product forming activity. Mean value ± MSE observed from 5 experiments is reported.

D) STUDIES ON PLATELET ALLOIMMUNIZATION - THE ARREST OF HEMORRHAGE IN SEVERELY ALLOIMMUNIZED THROMBOCYTOPENIC PATIENTS.

In the progress report of last year, preliminary results were reported of our studies on platelet alloimmunization. During the current year, we concentrated our studies on a particular aspects of platelet alloimmunization, i.e. on the possibility of arresting of hemorrhage in severely alloimmunized thrombocytopenic patients by overwhelming the patient's circulation with an excess of antigen so that the subsequently transfused platelets could survive longer and be effective on arresting hemorrhage. These experiments were first carried out in experimental animals, then in patients.

White rabbits weighing 3 to 4.5 Kg were alloimmunized by weekly intradermal injections of $1x10^9$ allogenic platelets from more than five donor rabbits. Alloimmunization in the recipient animals was documented by measuring the survival of donor platelets labeled with Cr^{51} in the circulation of the recipient animals (1,2).

Severe alloimmunization could be produced in most of the recipient animals. After 15 injections, maximum recovery values of the infused ^{51}Cr donor platelets were less than 20%, and life span less than three hours.

An attempt was, then, made to adsorb the alloantibody by infusing large numbers ($1x10^9$, $1x10^{10}$ and $4x10^{10}$) of viable allogenic platelets. Thirty minutes thereafter, survival of fresh donor platelets leveled with Cr^{51} was, again, measured in the circulation of the sensitized recipients. Values became significantly improved after the infusion, indicating a reduction in the level of alloantibody (Fig. 1). Furthermore, in subsequent experiments, it could be shown that there was a direct relationship between the number of platelets infused to adsorb the alloantibody and survival values of the allogenic Cr^{51}-labeled platelets infused thereafter (Fig. 1). A considerable reduction in the degree of alloimmunization could also be observed when the same number ($4x10^{10}$) of stored (4°C for one week) rather than fresh platelets from donor animals was infused. In other experiments, similar results (although of lesser magnitude) could be obtained with the infusion of lyophilized donor platelets (Fig. 2).

The hypothesis that improvement in survival of allogenic platelets infused in the sensitized animals was obtained because of blockage of the R.E. system by the massive infusion of fresh, stored or lyophilized platelets with consequent inhibition of phagocytosis, was examined by infusing large numbers of red cell ghosts or latex particles rather than platelets (3). Results obtained with $2x10^{10}$, $5x10^{10}$ and $1x10^{11}$ red cell ghosts were almost insignificant. Small , but more significant increases in survival could be obtained with the latex particles 2.0 μm in diameter. With $1x10^{11}$ latex particles, the platelet recovery value doubled, but the survival time was still unchanged and the allogenic platelets disappeared from the circulation within three hours (Fig. 3).

In another series of experiments, alloantibody in the serum of the sensitized animals was measured before and after massive infusions

of fresh donor platelets. The in vitro ^{14}C-serotonin release method was used and it was seen that before infusion, up to 32% serotonin release occurred in 30 min of incubation at 37°C in the presence of the sensitized serum. Thirty min after the infusion, values dropped to <5% and were still within the normal range (4% to 9.5%) after 3 to 24 hours. This gave the direct demonstration of the effectiveness of the platelet infusion in adsorbing the alloantibody.

In subsequent experiments, two patients with aplastic anemia were studied. Both had severe thrombocytopenia (<5,000 platelets per cumm), and prominent bleeding manifestations with epistaxes, gastrointestinal bleeding, hematuria and generalized purpura. They were both severely sensitized to repeated infusions of platelets from random donors and very low values of recovery and survival of allogenic platelets were observed in the patients. After the slow infusion over 30 min of six units ($40x10^9$ to $75x10^9$ platelets per unit) of fresh platelets from randomly selected donors, recovery and survival values of ^{51}Cr-labeled allogenic platelets were clearly improved in both patients (Fig. 4). Furthermore, in patient #2, a nearly normal survival curve of ^{51}Cr-labeled allogenic platelets was obtained after the slow infusion of 20 units of donor platelets stored for ten days at 4°C, that is non-viable platelets. By the use of only four units of freshly prepared platelets from randomly selected donors, signs of hemostatic effectiveness were rather dramatic with disappearance of most of the purpuric manifestations, a more stable hemoglobin level and a marked reduction of the hematuria. These improvements remained persistent for about ten days.

It is concluded that prompt arrest of acute hemorrhage in bleeding thrombocytopenic patients severely alloimmunized towards platelets from all available donors is feasible by the use of a two-step infusion of 1) a large number of platelets (20 to 30 units) to adsorb the antibody, immediately followed by, 2) the infusion of one to four units of freshly collected platelets from random donors. To adsorb the antibody, stored (non-viable) or lyophilized platelets collected as byproducts of daily blood banking can be effectively utilized.

It is presently considered that with repeated platelet infusions over long intervals, platelets from 25% to 50% of phenotypically (HLA) matched donors may lose the capacity to produce an effective post-transfusion response in the respective recipients (4). The clinician, therefore, is often faced with the difficult task of arresting hemorrhage in a bleeding thrombocytopenic patient who has become refractory to platelet transfusions because of the development of alloimmunity towards donor's platelets. In these extreme cases the above method of using an excess of antigen for adsorption of the alloantibody may be lifesaving on a number of occasions.

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Figure 1

SURVIVAL OF ^{51}Cr-LABELED ALLOGENIC PLATELETS AFTER INFUSION OF FRESH PLATELETS IN ALLOIMMUNIZED RABBITS

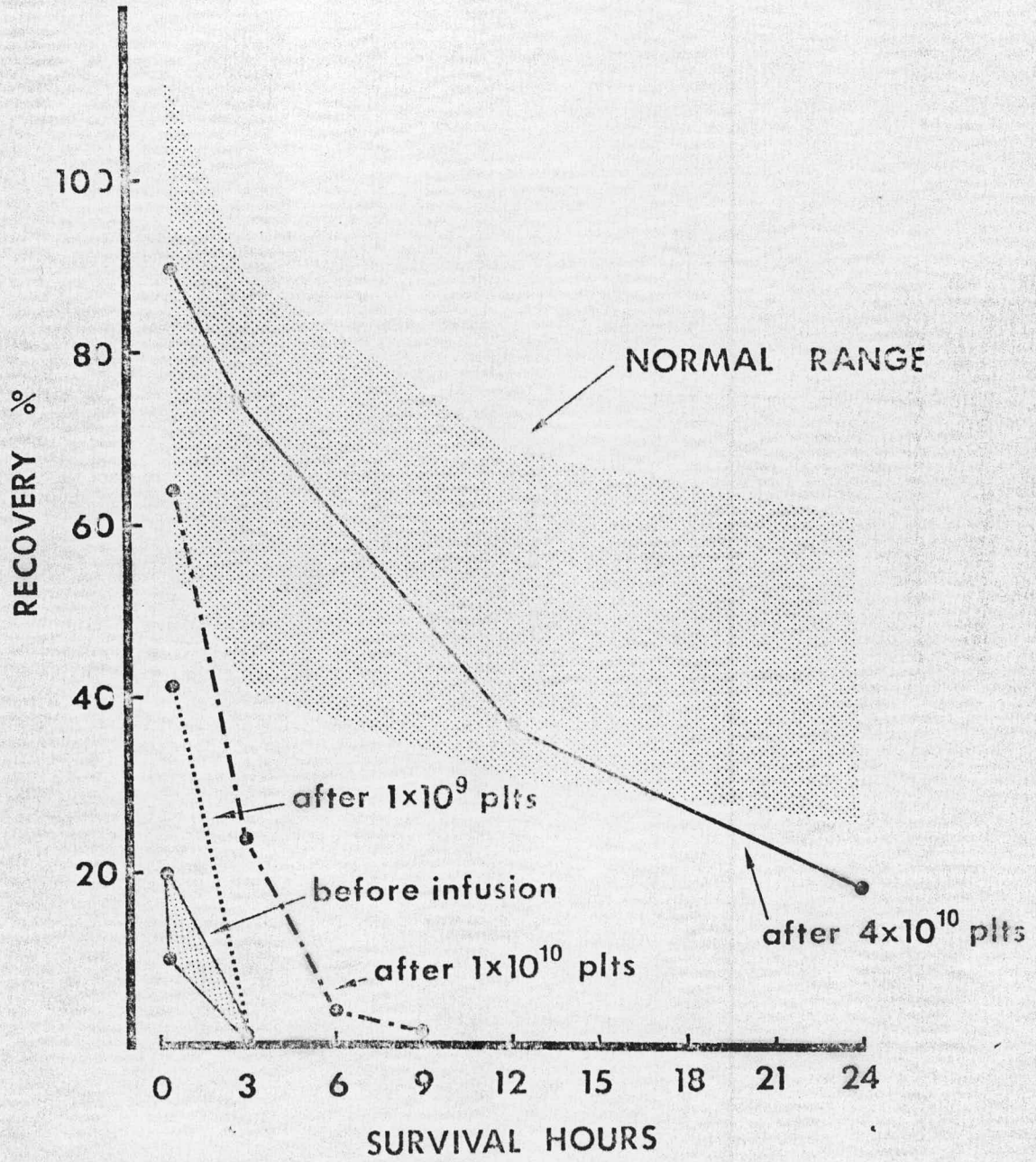
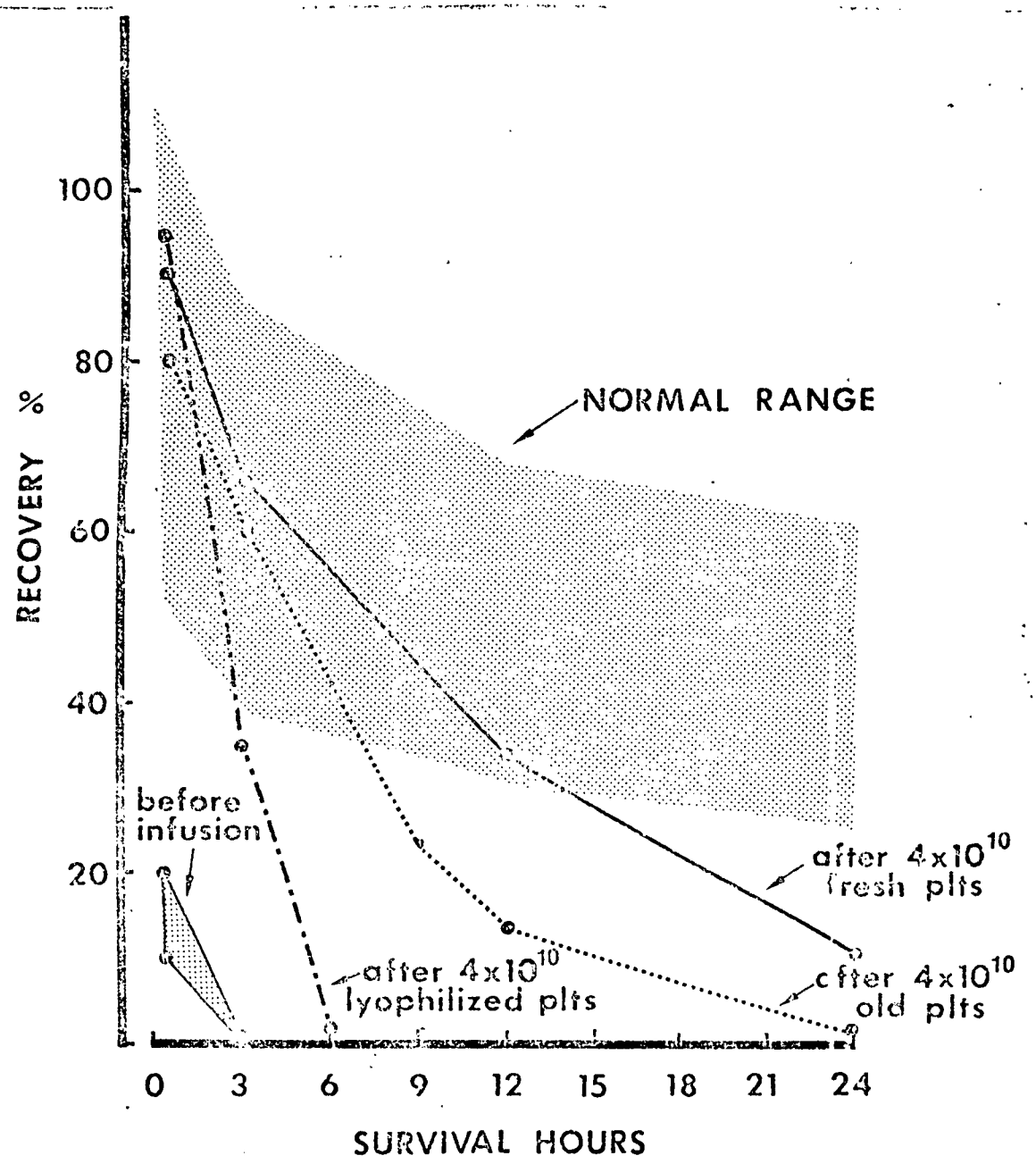
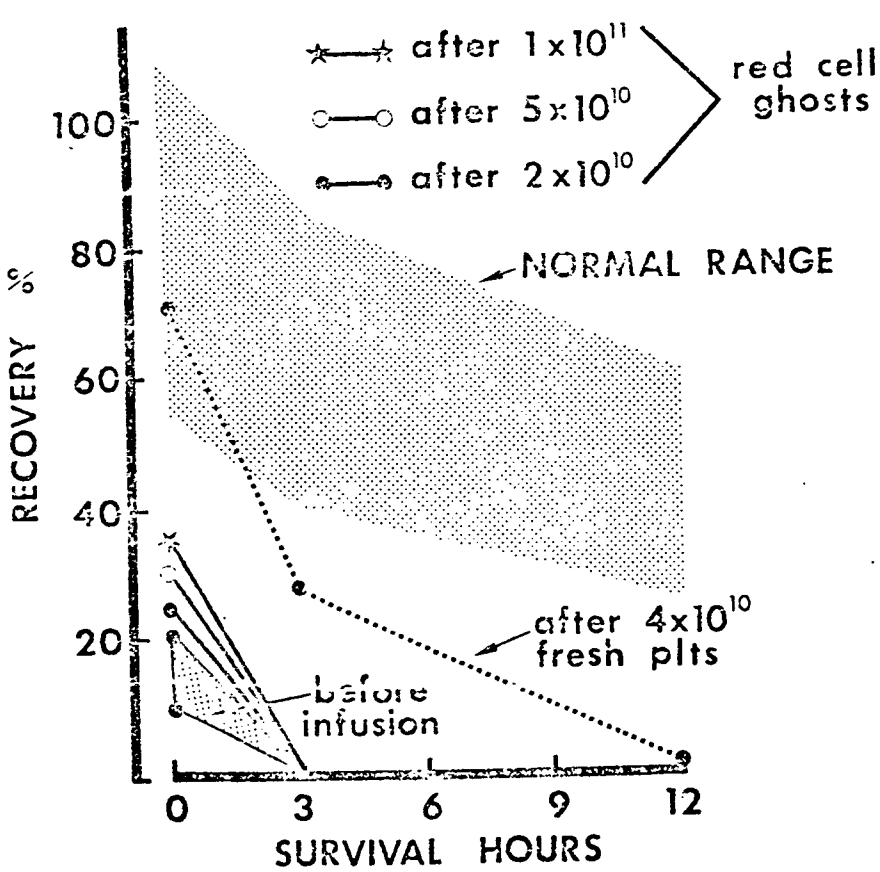


Figure 2

SURVIVAL OF ^{51}Cr-LABELED ALLOGENIC PLATELETS AFTER INFUSION OF STORED OR LYOPHILIZED PLATELETS IN ALLOIMMUNIZED RABBITS



SURVIVAL OF ^{51}Cr-LABELED ALLOGENIC PLATELETS AFTER R.E.S. BLOCKADE



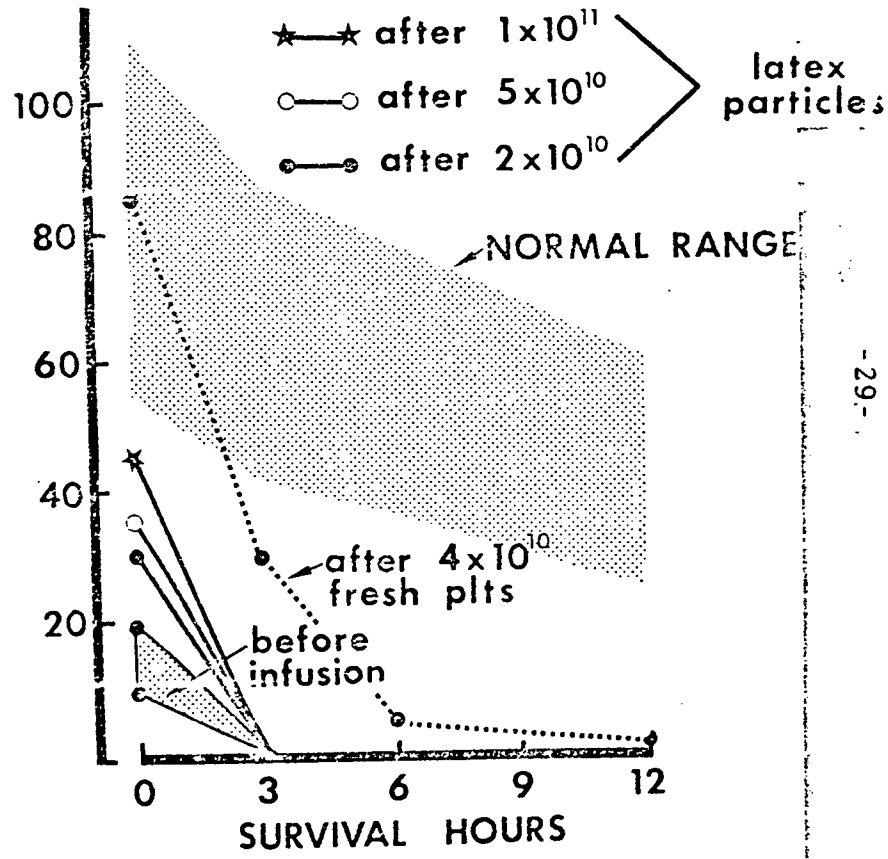
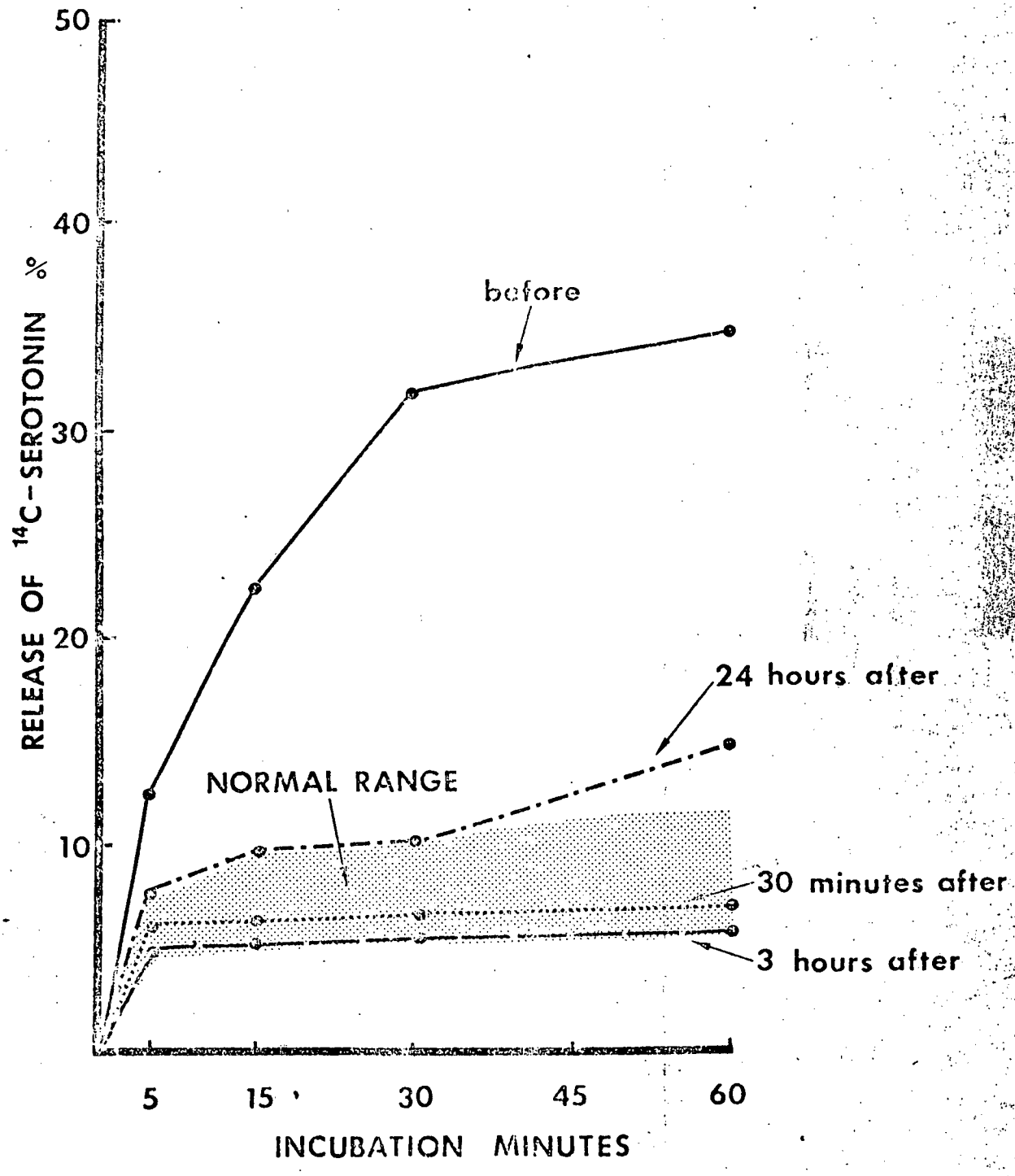


Figure 3

Figure 4

IN VITRO SEROTONIN RELEASE BEFORE AND AFTER ADSORPTION OF CIRCULATING ALLOANTIBODIES IN RABBITS



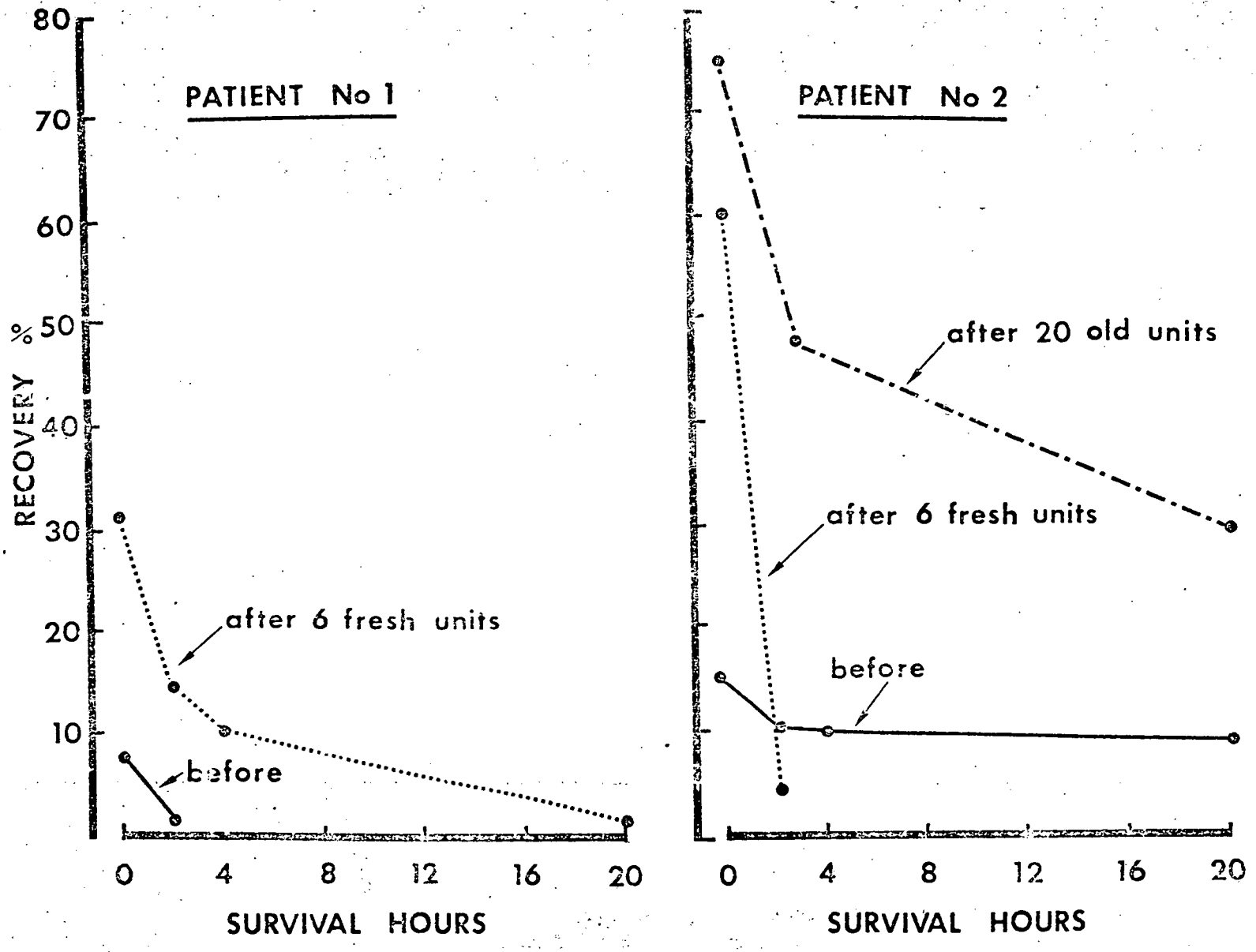

PATIENT No 1
PATIENT No 2
RECOVERY %
80
70
60
50
40
30
20
10
after 6 fresh units
before
after 20 old units
after 6 fresh units
before
0
4
8
12
16
20
SURVIVAL HOURS
SURVIVAL HOURS

Figure 5

E) IN VIVO ELUTION OF ^{51}Cr FROM LABELED PLATELETS INDUCED BY ANTIBODY

Chromium-51 sodium chromate has most widely been used as a radioisotopic label to study platelet survival and kinetics in experimental animals and in human subjects. A major prerequisite for a cell label in kinetic studies in that its binding to the cell be perfectly stable. In previous experiments by Steiner and Baldini (1965), it was demonstrated that antiplatelet heteroantibody in vitro can cause release of ^{51}Cr from labeled platelets. We have postulated that antiplatelet antibodies may cause elution of the ^{51}Cr-label from platelets also in the circulation. This would render inaccurate measurements of platelet survival and kinetics by the ^{51}Cr method in animals or patients with antiplatelet antibodies.

To examine this hypothesis, we have studied the effect of antiplatelet heteroantibody and alloantibody on ^{51}Cr labeled platelets infused in thrombocytopenic rabbits. Two thrombocytopenic patients alloimmunized by repeated platelet transfusions were also studied.

I) Rabbit Experiments

a) Control group

Ten white rabbits were made thrombocytopenic (less than 7,000/cumm) by two injections of ^{32}P sodium phosphate per Kg body weight. $4x10^{10}$ to $5x10^{10}$ ^{51}Cr-labeled platelet collected from donor rabbits were infused in the thrombocytopenic recipients to raise the circulating platelet count from 180,000 to 250,000/cumm. Platelet number, ^{51}Cr recovery and ^{51}Cr platelet specific activity were measured at 30 min, 60 min, 3 hours and 24 hours post infusion. As shown in Fig. 1, the recovery values obtained by enumeration were 13.5% higher than the values of ^{51}Cr-recovery at 10 min after infusion indicating that a significant percentage of radioactivity was lost from the labeled platelets soon after infusion. This difference increased only slightly during their life span, indicating that a small amount of ^{51}Cr label continued to be lost in the circulation.

b) Heteroantibody group

Ten thrombocytopenic rabbits were infused with 4 to $5x10^{10}$ ^{51}Cr labeled platelets as in control group. A small amount (0.2 ml/Kg) of anti-rabbit platelet serum prepared in the guinea pig was, then, intravenously injected. Platelet number, ^{51}Cr recovery, and ^{51}Cr platelet specific activity were followed as in control. As in Fig. 2, the difference between platelet number and ^{51}Cr recovery was larger than that observed in control group, suggesting that elution of ^{51}Cr caused by antiplatelet heteroantibody had occurred. The platelet specific activity values represented by the shaded area, were corrected for the degree of normally occuring elution measured in the control group. These results demonstrated that the hetero-antiplatelet antibody not only caused immediate destruction of platelets, but also caused a significant elution of ^{51}Cr from the platelets remaining in the circulation.

c) Alloantibody group

In these experiments, ten rabbits were first alloimmunized by 15

weekly injections of 10^9 allogenic platelets from donor rabbits. These alloimmunized rabbits were, then, made thrombocytopenic with ^{32}P-sodium phosphate, then infused with 4 to $5x10^{10}$ allogenic platelet labeled with ^{51}Cr. There was a large difference between the platelet number and ^{51}Cr recovery indicating that large amounts of ^{51}Cr-label was lost in the circulation in the presence of antiplatelet alloantibody (Fig. 3). The shaded area reports the value of ^{51}Cr platelet specific activity corrected by the respective control values obtained without antibody. The antiplatelet alloantibody caused a loss of about 50% of the label from the circulating platelets during the first 60 min after infusion.

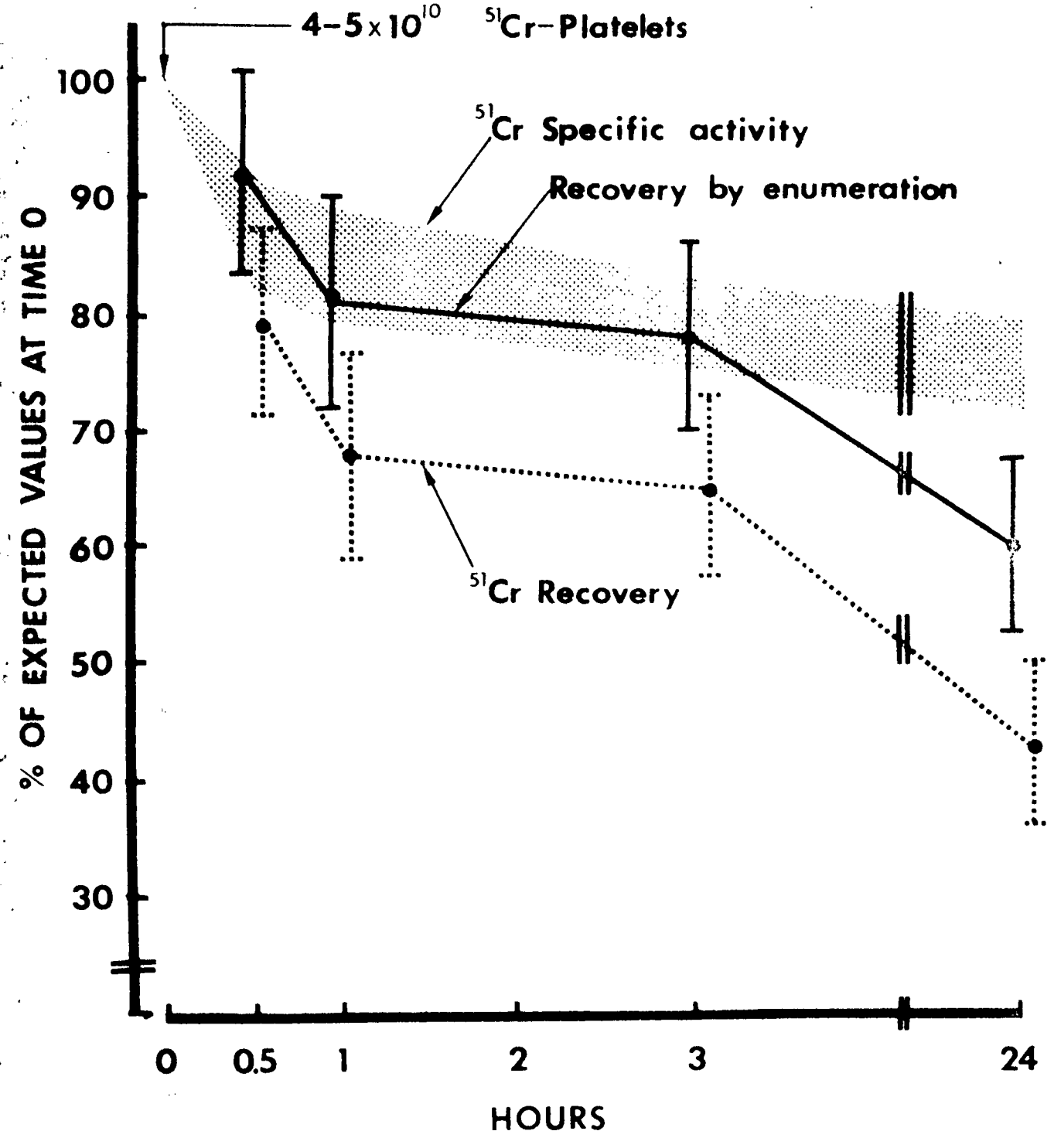
II) Human Studies

Two patients with severe amegakaryocytic thrombocytopenia, alloimmunized by prior transfusions of platelets, were studied for this purpose. Case 1 was a 65 year old female with myelofibrosis and myeloid metaplasia and Case 2 was a 69 year old male with acute myelomonocytic leukemia. Both had received chemotherapy and repeated platelet transfusions. At the time of study, their platelet counts were less than 6,000/cumm. They had both severely alloimmunized to donor platelets with survival times of allogenic platelets of 3 hours and 3½ hours and recovery values of 30% and 31% respectively. Case 1 was infused with $9x10^{11}$ ^{51}Cr-labeled platelets and platelet count rose to 77,000/cumm. The percent difference between recovery values measured by platelet enumeration and those measured by ^{51}Cr was significantly greater than the difference observed in normal subjects (Fig. 4) indicating that also in human subjects the presence of alloantibody in the circulation can cause a significant increase in ^{51}Cr elution from the circulating platelets.

In conclusion, our experiments clearly indicate that hetero- and alloantibodies can cause loss of ^{51}Cr label from the platelets in the circulation. These findings may cast some doubt on the accuracy of platelet survival and kinetics measured by ^{51}Cr in the patients with antiplatelet antibodies.

COMPARATIVE VALUES OF :

- ^{51}Cr PLATELET SPECIFIC ACTIVITY
- ^{51}Cr PLATELET RECOVERY
- RECOVERY BY ENUMERATION



^{51}Cr ELUTION FROM CIRCULATING PLATELETS AFTER INFUSION OF ANTIPLATELET HETEROANTIBODY

Fig. 2

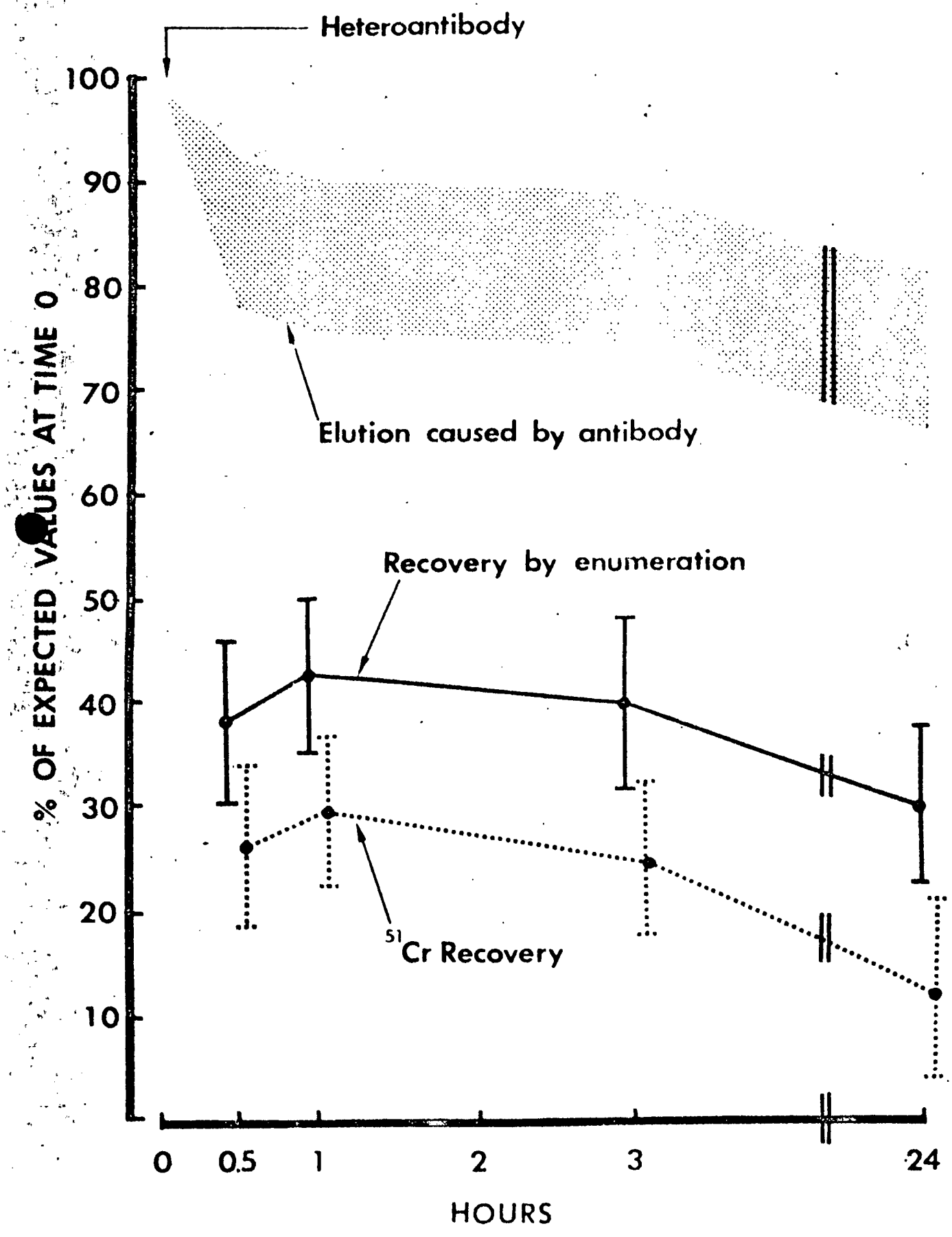
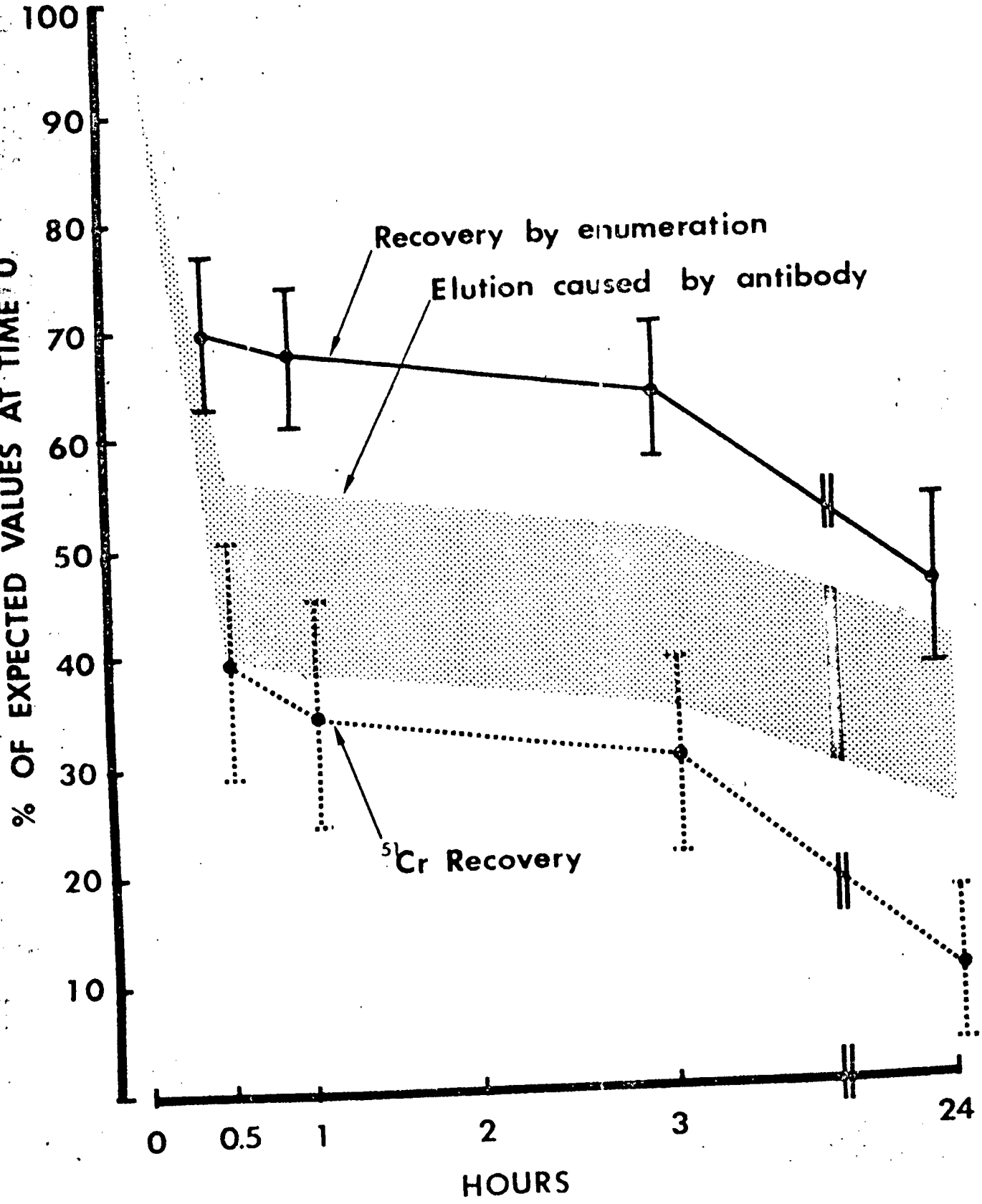


Fig. 3

^{51}Cr ELUTION FROM CIRCULATING PLATELETS IN THE PRESENCE OF ANTIPLATELET ALLOANTIBODY



^{51}Cr-ELUTION FROM CIRCULATING PLATELETS IN HUMAN SUBJECTS