SEQUESTRATION OF RABBIT PLATELETS LABELLED WITH Cr^{51}

by

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INTRODUCTION

It has been demonstrated that a definite decrease in the number of circulating blood platelets occurs in the presence of particulate material. Govaerts[1], in 1921, demonstrated this decrease following the intravenous injection of bacteria. His results were reinforced by studies of Popesco and Combiesco[2] and by Suzuki[3]. Following the intravenous injection of a suspension of quartz particles into rabbits, Tait and Elvidge[4] observed a transitory thrombocytopenia. This decrease in platelet count, which reached its maximum after 3-10 minutes, was followed by a gradual return to the normal value. These same investigators also experimented with other sequestering agents such as India ink and barium sulphate.

A similar transitory thrombocytopenia has been observed by other authors; Lambin and Gerard[5] in 1932 and Reeves and Stuck[6] in 1938 observed this phenomenon following transfusions of thorotrast in rabbits. In 1958, Bloom and Swensson[7] investigated this sequestration phenomenon in the presence of many sequestering agents not employed by Tait and Elvidge. They found that most fine particle suspensions produced the thrombocytopenia but that the phenomenon was not observed with colloidal solutions of an iron preparation or of methylene blue.

It has been assumed that the mechanism of this thrombocytopenia involves the adhesion of platelets to the particles; Bloom, Gustavson, and Swensson[8] have shown that platelets readily adhere to sequestering particles *in vitro*. From this assumption and the observation by Tait and Elvidge that sequestering particles were retained in the reticulo-endothelial system, it was assumed that the platelets which had disappeared from the circulation were likewise retained in the R-E system.

In September 1959, Salvidio and Crosby[9] reported that platelets labelled with P^{32} returned to circulation after being sequestered by India ink. This observation implies that the platelet returns to the circulation after being trapped in the R-E system. It is true that the spleen is capable of removing foreign matter from certain cells without damaging these cells; it can, for example, pick iron granules from siderocytes and return these cells to circulation[10]. However, it seems probable that a platelet which has undergone morphological changes associated with its attachment to a foreign particle would tend to be phagocytized by the spleen. Because of the paradox implied in the reappearance of these platelets, this study was undertaken in order to better elucidate the loci of platelet sequestration in the presence of foreign particles.

MATERIALS AND METHODS

The animals used in these experiments were New Zealand albino rabbits, of average weight 4.0 kg. In order to study the sequestration phenomenon by means of platelets labelled with radioactive chromium ($Na_2Cr^{51}O_4$), it was necessary to develop suitable techniques for tagging the platelets and for accomplishing sequestration. Once the proper sequestering agent had been determined and the tagging technique perfected, the sequestration phenomenon was investigated as follows:

A rabbit was given an autogenous transfusion of platelets labelled with $Na_2Cr^{51}O_4$. After equilibration, a transfusion of sequestering agent was administered. At the time specified fro maximum sequestration to have occurred, the animal was sacrificed and dissected. The radioactivity in aliquots of each organ was then measured and compared to the activity found in a rabbit receiving platelets labelled with $Na_2Cr^{51}O_4$ but no sequestering agent.

The following is a detailed description of the method used to measure the redistribution of platelets in the presence of quartz particles. The amount of radioactivity used, together with the technique employed for radioactive labelling, was determined experimentally. Similarly, the other parameters such as centrifuge speeds, anticoagulant concentration,

and volume of injected substances were determined by preliminary experiments. All glassware in contact with the platelets was sterile and silicone-coated.

The rabbit was placed in a wooden restraining box. Both ears were shaved with a straight razor. One of the ears was then swabbed with xylol which acted as an irritant, causing the artery to stand out. The artery was then entered with a no. 20 gauge needle attached to a 10 cc. syringe. When the flow of blood into the syringe indicated that the artery had been properly entered, the syringe was removed from the hub of the needle and exchanged for a 30 cc. syringe containing 4 cc. of a 3% solution of disodium ethylenediaminetetracetate (EDTA) which was the anticoagulant used throughout the experiment. The rabbit was bled 30 cc. of blood, which was transferred to a tube and centrifuged at 4° C. for 15 minutes at 1000 RPM (180 G) in an International Centrifuge Model PR-2.

This mild centrifugation resulted in the formation of a layer of red cells at the bottom of the tube and a supernatant platelet rich plasma essentially free of red cells. This platelet rich plasma was transferred to a second tube by means of a pipette and rubber bulb. To this was added 150 microcuries of a sterile solution of $Na_2Cr^{51}O_4$, after 0.1 cc. had been drawn into a blood diluting pipette for a platelet count.

In order for proper labelling of platelets to occur, the platelet rich plasma containing the radioactive chromium was allowed to incubate for 20 minutes at room temperature. Following this incubation period, 0.5 cc. of a 5% solution of ascorbic acid was added. This reduced any of the unbound Cr^{+6} to Cr^{+3} which is inactive as a labelling agent. The platelet rich plasma was then poured into a 20 cc. syringe.

The rabbit was again placed in the restraining box. The ear vein was entered with a no. 22 gauge needle attached to a 10 cc. syringe. When it was certain that the vein had been properly entered, the syringe was exchanged for the 20 cc. syringe containing the platelet rich plasma. This was then transfused at the rate of 5 cc. per minute.

Following this platelet transfusion, the rabbit was returned to his cage for a period of time long enough to ensure equilibration of the radioactive platelets in the circulation. It was decided that 24 hours should constitute this equilibration time.

At the end of this time, the rabbit was returned to the restraining box. The ear vein was entered with a no. 22 gauge needle attached to a 10 cc. syringe. When it was certain that the vein had been properly entered, the syringe was exchanged for a 30 cc. syringe containing 25 cc. of quartz suspension. This quartz suspension was transfused at the rate of 5 cc. per minute.

Six minutes following the transfusion of the quartz suspension, the ear artery was entered with a no. 20 needle attached to a 10 cc. syringe. Three cc. of blood was withdrawn, which was transferred to a test tube. Two cc. of this was then transferred by means of a volumetric pipette to a second tube for measurement of radioactivity. The ear vein was then entered with a no. 22 gauge needle attached to a 5 cc. syringe containing a solution of 500 mg. of sodium pentothal (sodium thiopental). The pentothal was injected as rapidly as possible and the rabbit expired instantly.

The rabbit was immediately subjected to dissection. The organs to be studied were each removed carefully, and weighed aliquots were placed in separate test tubes. The aorta was removed and stripped of the adventitia. It was then split longitudinally, and any visible clots were removed. Each tube was then centrifuged for 30 minutes at 3000 RPM (1700 G) to ensure uniform packing of the tissue at the bottom of the tube. Following this centrifugation, the radioactivity was measured in a thallium-activated sodium iodide well-type counter with a three inch shield (Tracerlab Model P-20A Scintillation Counter). The counting error was less than 5%.

To each tube containing an organ specimen, 9cc. of isotonic saline was added. The tubes were inverted several times until the organ specimens were loosened from the bottom and mixed thoroughly with the saline. The tubes were recentrifuged, the supernatant saline discarded, and the activity remeasured.

The results of these measurements were compared with those obtained in identical experiments in which the quartz transfusion was omitted.

Comments on Method

1. Tagging - The tagging procedure was based on studies concerning the labelling of platelets with radioactive chromate which have been reported by Aas and Gardner[11, 12].

2. Preparation of the Quartz Suspension - In an agate ball mill was placed 600 g. of coarse quartz granules. Following the addition of 250 cc. of distilled water, the cover of the mill was firmly attached and the granules were ground for 24 hours. At the end of this period, a sample of the particles was taken for microscopic examination. The particles were ground for a second 24 hours, after which another sample was taken. This procedure was followed for a total of 96 hours.

At the end of the grinding period, the average particle size was of the order of one micron. The resultant slurry was transferred to a porcelain

basin and the mill and balls were rinsed thoroughly in order to achieve maximum recovery. To reduce the volume of liquid, the porcelain basin was placed in an oven at 85° C. for 24 hours.

The quartz, which now had the appearance of a gray paste, was transferred to an 8 liter pyrex battery jar and placed on a gas stove. Six pounds of concentrated hydrochloric acid and 7 lb. of concentrated nitric acid was added, and the mixture was heated nearly to boiling for a period of 9 hours. At this point, a second portion of 6 lb. of hydrochloric acid and 7 lb. of nitric acid was added. The mixture was evaporated to 1/3 of its volume and a third portion of acid was added. The mixture was heated to 70° for 13 hours.

Following this final heating period, the quartz lost its tendency to agglutinate and the solution assumed a cream color. The contents of the battery jar were transferred to four 1 liter polyethylene bottles. These bottles were then placed in an International Model no. 2 Reinforced Centrifuge and were spun at 3000 RPM (2000 G) for one half hour. Following the centrifugation, the supernatant acid was discarded and distilled water was added to each bottle. The quartz sediment was resuspended and the bottles recentrifuged at 3000 RPM for one half hour. The supernatant liquid was discarded and the quartz was resuspended in distilled water and recentrifuged.

This procedure was repeated 11 times.

At the end of the washing manipulations, the quartz was resuspended in enough water to form a slurry which could be transferred from the bottles. This slurry was a clean white, and had the appearance of milk.

Isotonicity was secured by the method described by Tait and Elvidge[4]. A quartz suspension was made up in the following manner:

Into a 100 cc. volumetric flask was introduced 1.6 g. of the quartz slurry. To this was added 6.0 g. of dextrose. As a modification of the method described by Tait and Elvidge, 0.2 cc. of Triton (Reg.) * was added in order to keep the quartz particles discreet. The mixture was diluted to the fiducial mark with distilled water and autoclaved in a suitable container.

*Triton is a trade-mark of Rohm and Haas Co., Philadelphia, for oxyethylated tertiary octyl phenol formaldehyde polymer and supplied as entsufon (WR-1339) by Winthrop Laboratories, New York. Aas and Gardner[11] have found that surface active agents can control platelet agglutination. Since this particular agent has been proved safe for intravenous use at concentrations of 0.2%, it was felt that it might be of some use in these experiments in keeping the quartz particles discreet.

3. Platelet Counting

The method used for platelet counting was essentially that described by Feissly and Ludin[13]. The blood was drawn up to the 0.1 mark on a blood diluting pipette and then diluted 1:10 with a 1% ammonium oxalate solution. The pipette was then placed in a Burton electric pipette shaker, where it remained for about 10 minutes.

The hemacytometer used was an American Optical type 1475 especially designed for use in phase microscopy. The platelets were counted with phase microscopy.

4. Anticoagulant - The anticoagulant used was Na_2EDTA because it has been shown that platelets are discreet and do not have a tendency to clump in the presence of this anticoagulant.[14,15]

RESULTS

Platelet Survival

By a technique similar to that of Morgan, Keating and Reisner[16], platelet lifespan studies were undertaken as preliminary experiments to evaluate the feasibility of using the rabbit as an experimental model. A typical survival curve is given in figure 1. The percent radioactivity corresponds to measurements made on the washed platelets isolated from 6 cc. of rabbit artery blood. Significant activity was recovered for about 3 days. This is in agreement with the results obtained by Morgan Keating and Reissner.

Sequestration Followed with Platelet Counts

The drop in platelet count following transfusion of 25 cc. of quartz suspension is illustrated for a typical experiment in figure 2.

Loci of Sequestration Evaluated with Cr^{51}

A tabulation of the relative activities of the various organs is given in table 1. The values have been corrected for background and differences in weight. Values found in the presence of quartz are compared with those values for which no quartz was administered. The two runs marked PPP correspond to blank runs in which the $Na_2Cr^{51}O_4$ was added to

platelet poor plasma which was prepared by centrifuging the rabbit blood at 4° C. for 30 minutes at 3000 RPM (1700 G) and was essentially free of platelets. Two of these runs were made, only one of which included a quartz transfusion. The data in table 1 are graphically represented in figure 3.

One run was made in which the fall in platelet count was determined along with simultaneous measurements of blood radioactivity following a transfusion of quartz suspension. The results are given in figure 4. This same rabbit was sacrificed 24 hours after receiving the quartz transfusion. The organ activity is given in the last column of table 1.

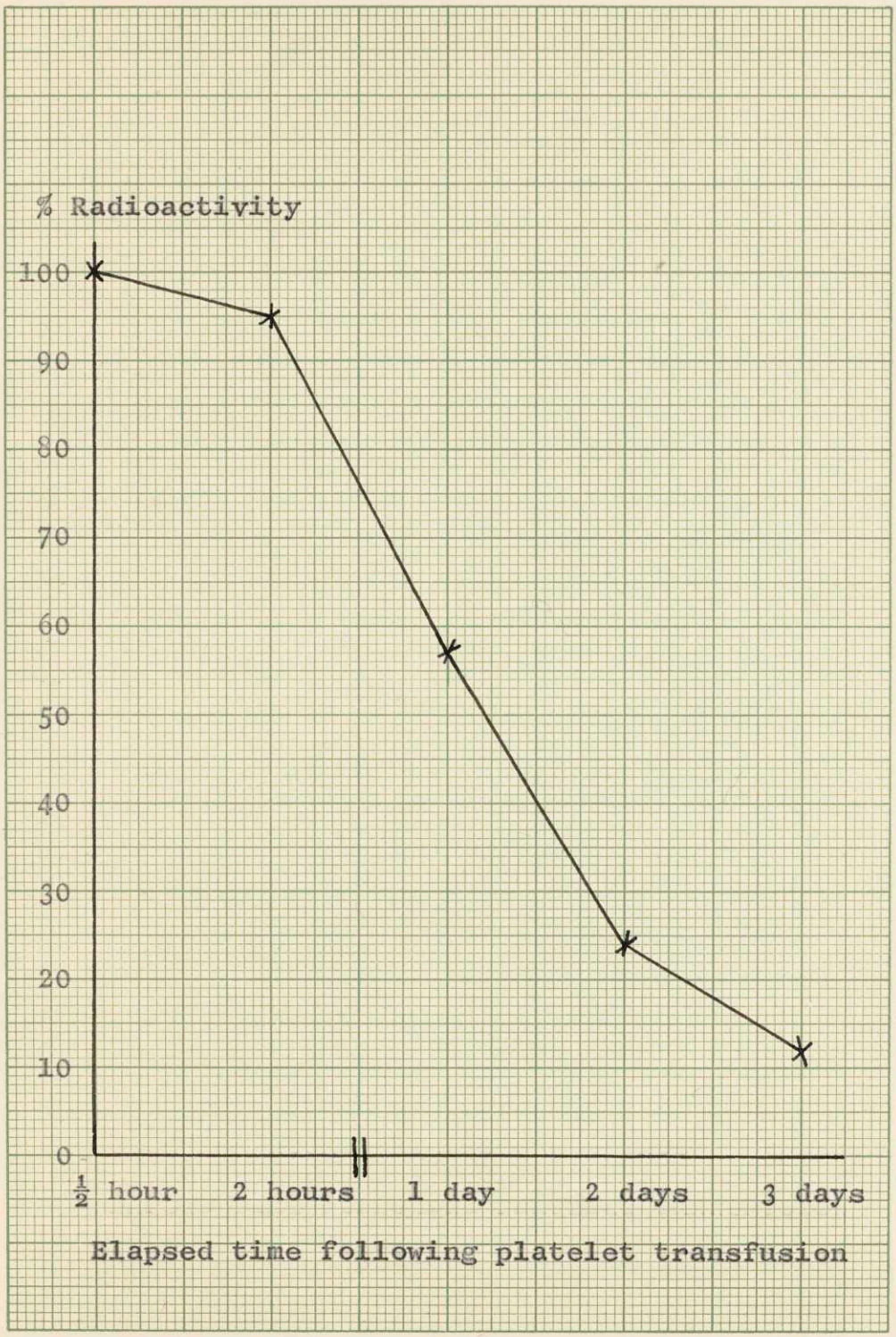
Table 1

The first number is the relative activity for the unwashed organ.
The second number is the relative activity for the washed organ.
The third number represents the % activity lost by washing.

Organ	with quartz	with quartz	with quartz	with quartz	with-out quartz	with-out quartz	with-out quartz	PPP with quartz	PPP with-out quartz	with quartz after 24 hours
BLOOD	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AORTA	1.50	1.62	1.79	1.44	0.73	0.88	0.65	0.30	0.55	0.92
	1.38	1.55	1.56	1.31	0.62	0.71	0.43	0.19	0.23	0.90
	8.0%	4.3%	12.8%	9.0%	15.0%	19.3%	33.8%	36.7%	58.2%	2.2%
LIVER	0.29	0.49	0.37	0.56	0.38	0.51	0.52	0.46	0.40	0.78
	0.27	0.43	0.33	0.48	0.35	0.44	0.38	0.40	0.31	0.61
	6.9%	12.2%	10.8%	14.3%	7.9%	13.7%	26.9%	13.0%	22.5%	21.8%
SPLEEN	2.32	2.03	2.27	2.66	2.40	2.55	2.72	2.20	2.10	1.17
	2.23	1.99	2.19	2.59	2.32	2.40	2.62	2.06	1.88	1.10
	3.9%	2.0%	3.5%	2.6%	3.3%	5.9%	3.7%	6.4%	10.5%	6.0%
KIDNEY	1.98	2.09	1.96	1.81	1.30	1.72	1.59	1.33	1.21	1.21
	1.75	1.94	1.84	1.69	1.24	1.57	1.46	1.02	0.94	1.13
	11.6%	7.2%	6.1%	6.6%	4.6%	8.7%	8.2%	23.3%	22.3%	6.6%
LUNG	0.65	0.61	0.88	0.74	0.73	0.69	0.59	0.86	0.93	0.96
	0.55	0.51	0.59	0.63	0.69	0.58	0.33	0.77	0.44	0.94
	15.4%	16.4%	33.0%	14.8%	5.5%	15.9%	44.0%	10.5%	52.7%	2.1%
URINE	1.06	1.20	1.48	1.33	0.86	1.41	1.27	2.83	3.48	0.54

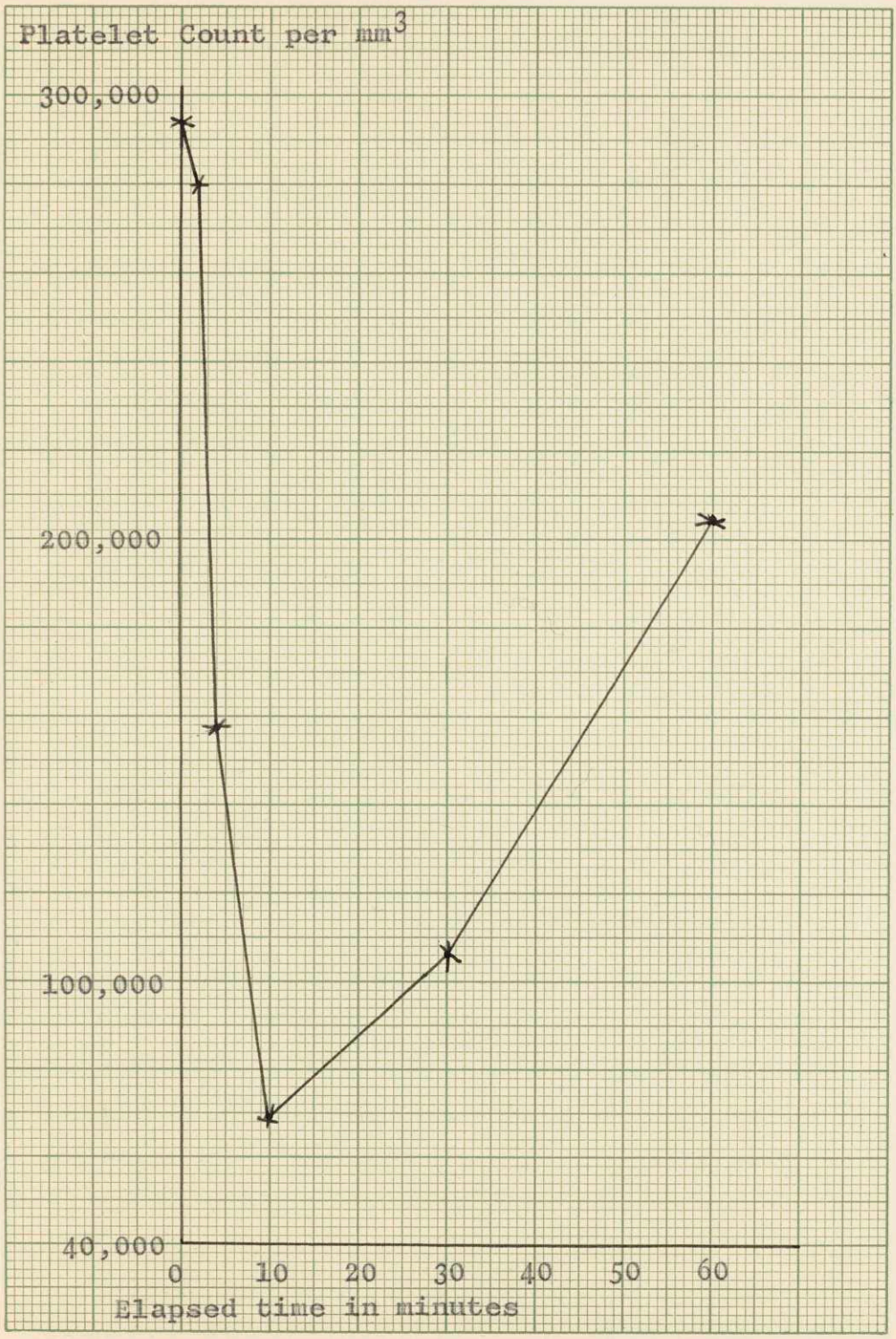
Activity values relative to blood for sequestration experiments. Each vertical column represents one rabbit.

Figure 1



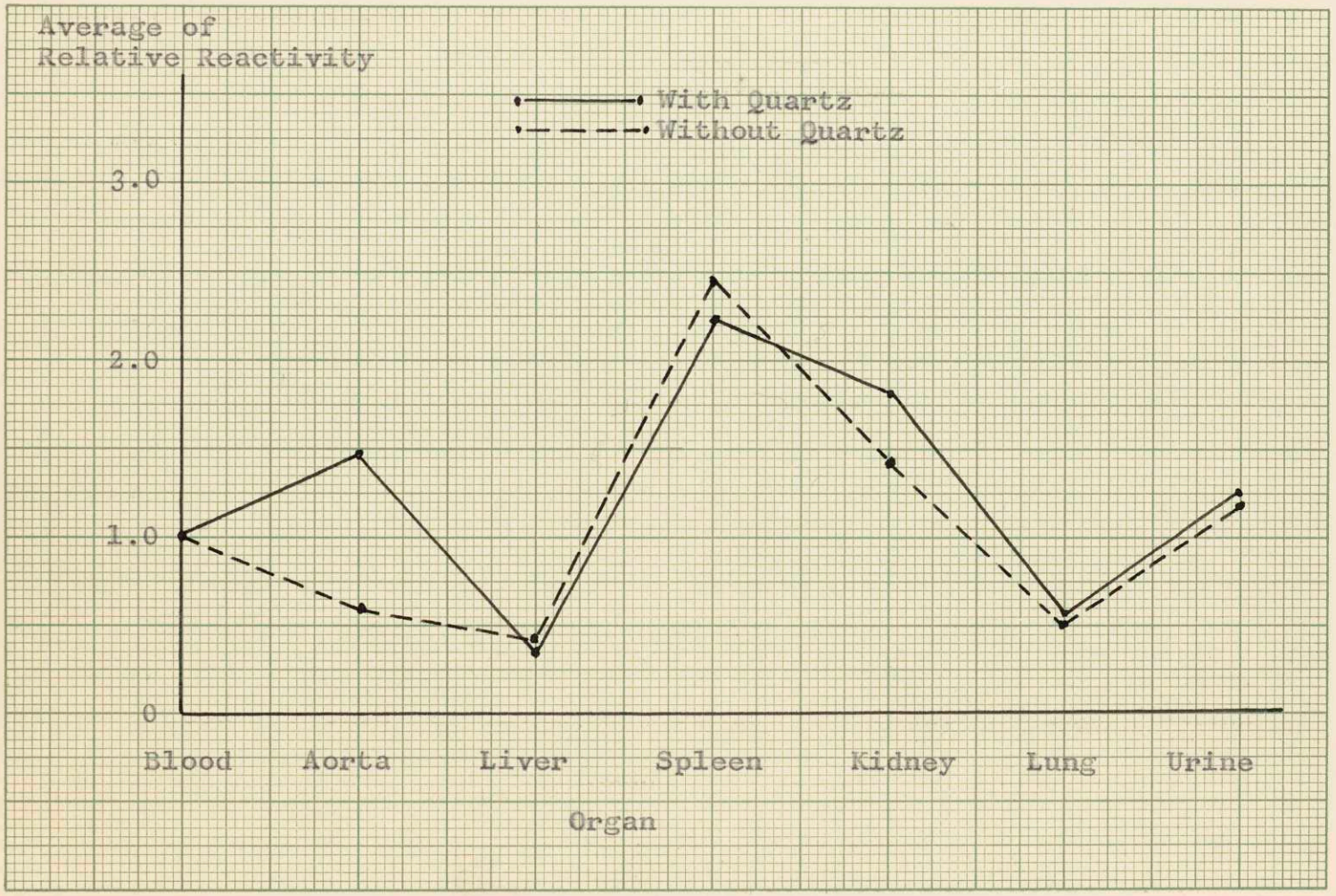
Graph of % radioactivity of washed platelets as a function of time. All values were corrected to day 0.

Figure 2



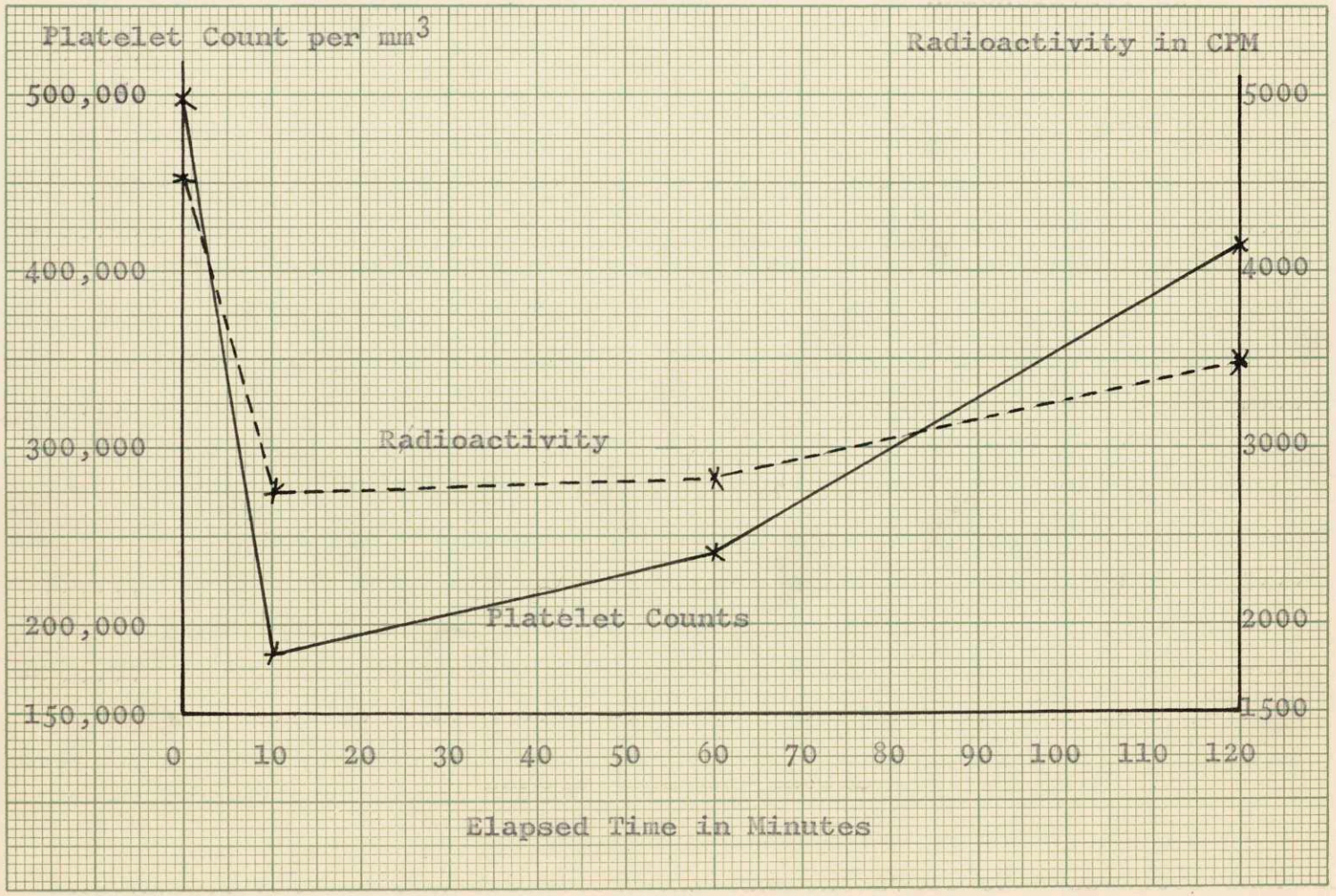
Typical drop in platelet count in a rabbit following a transfusion of 25 cc. of quartz suspension.

Figure 3



Average Values for Radioactivity Relative to Blood for Washed Organs in the presence and intthe Absence of Quartz as Represented in Table 1.

Figure 4



Simultaneous plot of platelet count and radioactivity of 2 cc blood samples in a rabbit with circulating radioactive platelets as a function of time following a transfusion of 25 cc. of quartz suspension.

DISCUSSION

It is apparant from the results of these experiments that platelets labelled with radioactive chromate tend to be deposited on the aorta in the presence of quartz particles. Since the aorta samples were stripped of the adventitia, it seems apparant that these platelets were deposited on the intima. Furthermore, the platelets appear to be fairly well bound, since there was only a small amount of elution in the single washing. There was also some retention of radioactivity over a 24 hour period as indicated by the results shown in the last column of table 1.

It is not without possibility that the observed results are due to exchange of radioactivity between the platelet and the wall of the aorta; that is, the chromium bound to the platelet could have become bound to the aorta wall. However, the possibility that some moiety of the blood other than platelets is being labelled by the chromate is ruled out by the results of the platelet poor plasma experiments. In these cases, there was very little activity in the organs and also a high percent of elution from washing. Also, there

was little difference between the platelet poor plasma experiment in the presence of quartz and the experiment performed in the absence of quartz.

The spleen activity is consistently high, even in the runs employing platelet poor plasma. Since this organ is involved in removing dead platelets, it would tend to have a high activity following a radioactive platelet transfusion; there does not, however, seem to be any satisfactory explanation at present for the high values obtained where only radioactive platelet poor plasma was administered.

Understandably, the kidney has a slightly elevated activity since it is involved in excreting the unbound radioactivity.

Many investigators [17,18,19] have observed a thrombocytopenia during lipaemia which is similar to the thrombocytopenia associated with quartz. Although quartz particles are certainly not fat globules, it may be that a deposition on the intima of the aorta occurs in the presence of these fat globules similar to the deposition observed in these quartz experiments. If this were so, it might have implications in the genesis of atherosclerosis.

It has been suggested by Rokitansky[20] as early as 1842 that atheroma may be due to mural thrombosis. Duguid[21], in 1954, reiterated this hypothesis. At the International Symposium on the Blood Platelets held in Detroit in March 1960, Mustard[22] reported that he had found that platelets are more adhesive in lipaemia.

Conclusions associating the results of the experiments reported here with atherosclerosis are only conjecture. Experiments will have to be performed using an intravenous fat preparation such as lipomul. Perhaps autoradiography or fluorescent antibody techniques can be employed to elucidate better the character of the deposition of platelets on the wall of the aorta.

SUMMARY

Studies with autogenous transfusions of rabbit platelets labelled with $Na_2Cr^{51}O_4$ have shown that these platelets are deposited on the wall of the aorta in the presence of quartz particles of the order of one micron. This phenomenon is coincident with a transient thrombocytopenia; a drop in the radioactivity of the peripheral blood followed by a slight increase correlates with a drop in platelet count in the first 10 minutes after the quartz administration, followed by a gradual return to the normal value. The radioactivity measured on the aorta resists elution by a single washing with isotonic saline. The deposition is not observed in the presence of quartz particles with platelet poor plasma tagged with $Na_2Cr^{51}O_4$.

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