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### Research Article

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Acute hemorrhage 24 hr before administration of  $^{75}\text{SeM}$  increased the incorporation of  $^{75}\text{SeM}$  into platelets. Transfusion-induced thrombocytosis reduced the incorporation of  $^{75}\text{SeM}$  to approximately 30% of that observed in control animals. Suppression of bone marrow function with nitrogen mustard resulted in decreased numbers of platelets in the circulation, and a decrease in incorporation of  $^{75}\text{SeM}$ . Delayed appearance of  $^{75}\text{SeM}$  was observed in circulating platelets during recovery from marrow suppression. Injection of 75-225 ml of plasma from thrombocytopenic donors into normal rabbits increased incorporation of  $^{75}\text{SeM}$  into platelets while normal plasma did not have this effect.

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# Measurement of Thrombopoiesis in Rabbits Using <sup>75</sup>Selenomethionine

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**ABSTRACT** Incorporation of <sup>75</sup>selenomethionine (<sup>75</sup>SeM) has been used to study platelet production in rabbits. Radioactivity of platelets was low after the intravenous administration of <sup>75</sup>SeM and rose to a maximum approximately 3 days after administration. Platelet radioactivity was independent of concurrent plasma levels. The life span of rabbit platelets, as estimated with this technique, was 4-5 days. In vivo reutilization of <sup>75</sup>SeM previously incorporated into plasma proteins was not detected. In vitro incorporation of <sup>75</sup>SeM by platelets in platelet-rich plasma was not demonstrated.

Acute hemorrhage 24 hr before administration of <sup>75</sup>SeM increased the incorporation of <sup>75</sup>SeM into platelets. Transfusion-induced thrombocytosis reduced the incorporation of <sup>75</sup>SeM to approximately 30% of that observed in control animals. Suppression of bone marrow function with nitrogen mustard resulted in decreased numbers of platelets in the circulation, and a decrease in incorporation of <sup>75</sup>SeM. Delayed appearance of <sup>75</sup>SeM was observed in circulating platelets during recovery from marrow suppression. Injection of 75-225 ml of plasma from thrombocytopenic donors into normal rabbits increased incorporation of <sup>75</sup>SeM into platelets while normal plasma did not have this effect.

The rate of appearance of <sup>75</sup>SeM in circulating platelets appears to provide a sensitive and specific method for the study of production of platelets by megakaryocytes. The data suggest more rapid entry of platelets into the circulation, and a sustained increase in incorporation of <sup>75</sup>SeM into platelet protein after stimulation of platelet production. The results are consistent with the concept of a humoral agent (thrombopoietin) that acts on megakaryocytes to regulate platelet production.

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## INTRODUCTION

Regulation of red cell production is mediated by a humoral factor, erythropoietin. The suggestion has been made that platelet production is controlled by a similar mechanism, but the existence of "thrombopoietin" has not been demonstrated convincingly. Thrombocytopenia in some members of two families was attributed to deficiency of a plasma component with thrombopoietic stimulating activity (2, 3); whether this factor is concerned with regulation of platelet production is unknown.

Experimental evidence favoring a humoral control mechanism has been reviewed recently (4, 5). Thrombocytopenia produced by exchange transfusions or platelet antiserum is followed by transient thrombocytosis (6-9), and thrombocytosis secondary to platelet hypertransfusion is followed by transient thrombocytopenia (10-12). These phenomena are compatible with a feedback mechanism. Injection of plasma from thrombocytopenic animals has not regularly caused measurable increases in the platelet counts of normal recipients (13, 14). However, platelet counts do not necessarily reflect alterations in rate of production since the concentration of circulating cells in the blood is the resultant of the rate of production and of the rate of destruction. Furthermore, there is considerable fluctuation of platelet counts of rodents (10, 15, 16), the animals used in most experimental studies; Odell, McDonald, and Howsden (17) have emphasized the possible misinterpretation of apparent variations in platelet counts.

Attempts to study platelet production more directly by use of isotopic labels have led to discordant results (11, 18-20). In preliminary experiments by Odell, Jackson, and Reiter (11), and Harker (20) employing Na<sup>25</sup>SO<sub>4</sub>, induced thrombocytopenia and thrombocytosis altered levels of the isotope in circulating platelets, suggesting a feedback control mechanism. Ebbe, Stohlman, Donovan, and Howard (19), studied the rate of fall of platelet radioactivity after labeling with diisopropylfluorophosphate-<sup>3</sup>H, and were not able to demonstrate altera-

tion of platelet production after platelet hypertransfusion in rats.

<sup>75</sup>Selenomethionine (<sup>75</sup>SeM) follows the metabolic pathway of methionine and is incorporated into proteins (21-23); it provides the technical advantage of producing gamma emissions. The isotope previously has been used to study the production of red blood cells (24, 25). Intravenously administered <sup>75</sup>SeM appears in circulating platelets presumably after its initial incorporation into megakaryocytes (26).

In the studies to be described, <sup>75</sup>SeM was employed to estimate platelet production. The results demonstrate that alterations in the rate of thrombopoiesis, as measured by changes in the rate of appearance and levels of <sup>75</sup>SeM in circulating platelets, can be detected after hemorrhage, hypertransfusion of platelets, or suppression of bone marrow function. Infusion of plasma from thrombocytopenic donors into normal recipients increased the rate of appearance and levels of <sup>75</sup>SeM in circulating platelets; this suggested that a humoral substance, thrombopoietin, is involved in the control of platelet production.

## METHODS

New Zealand white rabbits from a single local supplier were used. Their average weight was  $1.93 \pm 0.34$  kg; the mean hematocrit value was  $38 \pm 5\%$ , and mean platelet count was  $567,000/\text{mm}^3 \pm 140,000/\text{mm}^3$  (1 SD). Rabbits were received 1-3 days before use, caged individually, and received standard laboratory food and water ad lib. In vivo incorporation of <sup>75</sup>SeM<sup>1</sup> into platelets and plasma proteins was measured serially after the intravenous injection of  $15 \mu\text{C}$  of <sup>75</sup>SeM. Blood samples (4.5 ml) for preparation of platelets and plasma for measurement of radioactivity were obtained by venipuncture from the marginal ear vein, and collected in siliconized glassware<sup>2</sup> using buffered ethylenediaminetetraacetate (EDTA)<sup>3</sup> (one part EDTA:nine parts whole blood) as anticoagulant, except for studies of in vitro incorporation of <sup>75</sup>SeM by platelets, for which ACD<sup>4</sup> was used (one part ACD:nine parts whole blood). Blood samples for measurement of platelet count and hematocrit value were collected in capillary tubes<sup>5</sup> from the same venipuncture. Microhematocrit values were determined by the method of Strumia, Sample, and Hart (27), and platelets were counted by the method of Bull, Schneiderman, and Brecher (28), by use of a Coulter electronic counter.<sup>6</sup>

<sup>1</sup> <sup>75</sup>Selenomethionine, Sethotope, E. R. Squibb & Sons, New Brunswick, N. J. Specific activity 200-250 mc/mg.

<sup>2</sup> G. E. Silicone S.C.-87 Dri-Film, General Electric Company, Waterford, N. J.

<sup>3</sup> 0.054 M EDTA (2 g/100 ml in distilled H<sub>2</sub>O) buffered to pH 7.5 with Tham (Tris(hydroxymethyl)amino-methane). Fisher Scientific Co., Fair Lawn, N. J.

<sup>4</sup> ACD (acid-citrate dextrose) solution, USP, formula A, Fenwal Laboratories, Inc., Norton Grove, Ill.

<sup>5</sup> Drummond Capillary tubes (for platelet counts: treated with EDTA and heparin; for microhematocrit values: double oxalated). Drummond Scientific Co., Broomall, Pa.

<sup>6</sup> Coulter Electronic Counter, Model B, Coulter Electronics, Hialeah, Fla.

Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 g for 30 min at room temperature, and the volume and platelet count of the plasma were measured. PRP contained approximately  $1 \times 10^6$  platelets/mm<sup>3</sup>, less than 500 white blood cells (WBC)/mm<sup>3</sup>, and approximately 10,000 red blood cells (RBC)/mm<sup>3</sup>. Platelet-free plasma (PFP), containing less than 5000 platelets/mm<sup>3</sup>, was prepared by centrifugation of PRP for 5 min at 5500 g at 4°C, or for 40 min at 1100 g at room temperature.

To distinguish between incorporation of <sup>75</sup>SeM into plasma proteins and nonspecific binding, we added 0.05 ml 1 N NaHSO<sub>3</sub> to 0.5-ml portions of PFP to destroy selenosulfide bonds (29). These samples were incubated for 60 min at 37°C, along with paired 0.5-ml portions of PFP to which 1 N NaHSO<sub>3</sub> had not been added. Protein then was precipitated with an equal volume of 20% trichloroacetic acid (TCA), and the precipitate removed by centrifugation at 5500 g for 5 min. The supernatant was decanted and the precipitate washed twice with 2 ml of cold 10% TCA. The radioactivity of the supernatant, washings, and precipitate from each sample was determined.

*Preparation of platelets for counting of radioactivity by use of the Millipore filter method.* Platelets were sedimented by centrifugation of PRP, and resuspended in 5 ml of Tris(hydroxymethyl)amino-methane (THAM) buffer<sup>7</sup> using a Vortex mixer.<sup>8</sup> Washing platelets three to five times by differential centrifugation to remove plasma protein was time consuming, and resulted in variable loss of platelets which was difficult to quantify; a more efficient method was developed. The resuspended platelets were poured onto a 1.2 μ Millipore filter held in a Millipore prefilter and Swinnex holder.<sup>9</sup> Complete transfer of the platelets onto a Millipore filter was accomplished by rinsing the tube with 2 ml of buffer, and the platelets on the filter were immediately washed with 30 ml of THAM buffer. The entire filter was folded, enclosed in Parafilm,<sup>10</sup> and placed in a tube for measurement of radioactivity. Radioactivity of the washed platelets and of 1 ml of the PFP was determined by use of a gamma well scintillation counter.<sup>11</sup> Samples were counted for 30 min at an energy range from 0.350 to 0.450 Mev; background was 15 cpm.

Counts per minute (cpm) of <sup>75</sup>SeM retained on the filter were determined by isotope incorporated into platelets, the total number of platelets on the filter, and the radioactivity of the plasma (which varied from rabbit to rabbit and from day to day). Therefore, a correction for radioactive plasma protein trapped within the filter and retained with the platelets was necessary. Contamination of nonradioactive platelets by radioactive plasma protein was determined as follows: nonradioactive platelets were resuspended in radioactive PFP prepared from blood collected in EDTA from rabbits that had received <sup>75</sup>SeM at least 24 hr previously; these platelets were incubated for 15 min at 25°C and 45 min at 4°C. Radioactivity remaining on Millipore filters was measured when platelets from these in vitro incubation mixtures were placed on the filters and washed. Data included

<sup>7</sup> 72.8 g THAM, 18 g NaCl, 240 ml 0.2 N HCl, and 40 ml 4.8% EDTA in 4 liters of distilled H<sub>2</sub>O.

<sup>8</sup> Vortex-Genie, Scientific Industries, Inc., New York.

<sup>9</sup> Millipore filter #72AW P02500; Millipore prefilter #AP 2502200; and Swinnex-25 holder. Millipore Filter Corp., Bedford, Mass.

<sup>10</sup> Parafilm, American Can Company, Neenah, Wis.

<sup>11</sup> Picker-Nuclear Autowell II, Intertech, Inc., North Haven, Conn.

in this paper demonstrate that platelets did not significantly incorporate  $^{75}\text{SeM}$  in vitro, or reutilize  $^{75}\text{SeM}$  already in plasma protein in vivo. Plasma protein radioactivity in the incubation mixtures ranged from 430 cpm/ml to 20,000 cpm/ml and total platelets placed on the Millipore filter from  $8 \times 10^6$  to  $75 \times 10^6$ , values encountered under experimental conditions. More  $^{75}\text{SeM}$  was trapped within the filter at higher levels of plasma radioactivity, and the amount of  $^{75}\text{SeM}$ -labeled plasma protein trapped with the platelets was greater when more platelets were on the filter. The log of contaminant activity was directly proportional to the log of the total number of platelets in the incubation mixture and subsequently placed on the Millipore filter. The slopes of the isopleths formed when the plasma radioactivity was held constant and the number of platelets on the filter was varied, were calculated by linear regression analysis using a 7094 IBM Computer.<sup>13</sup> The mean slope ( $\Delta \log$  filter radioactivity/ $\Delta \log$  total platelets) was 0.486 with a SE of 0.055. The intercepts of the isopleths with the abscissa occurred at  $\log 0.0023 \times$  plasma radioactivity with a SE of 0.0003. Radioactive plasma protein trapped on the filter was equal to  $0.0023 \times$  total plasma radioactivity  $\times$  total platelets on the filter<sup>13,14</sup>. The contamination of the filter by radioactive plasma protein was calculated for each sample, and was approximately 4% of the total counts per minute on the filter. To allow for differences in circulating platelet mass, and to permit comparison between different experimental groups, we calculated incorporation of  $^{75}\text{SeM}$  as the per cent administered dose in circulating platelets as follows: (cpm/platelet (corrected)  $\times$  total circulating platelets<sup>13</sup>  $\times$  100)/(total cpm  $^{75}\text{SeM}$  injected).

Platelets for production of thrombocytosis in recipient animals were prepared in siliconized glassware. Blood was obtained by intracardiac puncture from normal, unanesthetized animals using acid-citrate dextrose as anticoagulant, as previously described. The platelets were sedimented from platelet-rich plasma by centrifugation at 1100 *g* for 20 min at room temperature, resuspended in 10–15 ml of the donors' fresh platelet-free plasma, and immediately injected into recipient rabbits.

Suppression of bone marrow function was accomplished by administration of nitrogen mustard,<sup>14</sup> using three dose schedules which will be described.

Platelet antiserum was prepared in guinea pigs by injecting an emulsion of washed rabbit platelets and complete Freund's adjuvant.<sup>15</sup> The guinea pigs received an initial injection in the footpads followed by three weekly subcutaneous injections. Serum was obtained 1 wk after the last injection, adsorbed with washed rabbit red blood cells, and frozen. Guinea pig antirabbit platelet serum, thus prepared, did not produce detectable anemia, hemolysis, or leukopenia when injected into rabbits. Rabbits were rendered thrombocytopenic (platelet count less than 35,000/mm<sup>3</sup>) by injection of 0.5–1.0 ml of the anti-rabbit platelet serum. Platelet-free plasma was prepared from blood collected in siliconized glassware

using acid-citrate dextrose as anticoagulant, and obtained by intracardiac puncture from thrombocytopenic, unanesthetized animals from 2 to 4 hr after administration of the platelet antiserum. Platelet-free plasma was prepared similarly from blood obtained from normal rabbits. Platelet-free plasma from thrombocytopenic or normal donor rabbits was pooled and stored at  $-20^\circ\text{C}$  for periods of up to 5 days. Pooled plasma, either from normal or thrombocytopenic donors, was administered via the marginal ear vein to normal rabbits; its effect on the incorporation into platelets of subsequently administered  $^{75}\text{SeM}$  was measured as an index of thrombopoietic activity of the infused plasma.

## RESULTS

*Disappearance of free  $^{75}\text{SeM}$  from the circulation and its incorporation into plasma protein, in vivo.* After injection of 15  $\mu\text{c}$   $^{75}\text{SeM}$  into each of three rabbits, free isotope rapidly disappeared from the circulation. The fraction of  $^{75}\text{SeM}$  remaining in the circulation decreased to less than 5% in 5 min, and was less than 0.3% after 24 hr. Essentially all circulating  $^{75}\text{SeM}$  had been incorporated into plasma protein by 6 hr, as demonstrated by its presence in TCA-precipitable material (Fig. 1), and was not circulating in a "free" form. Selenosulfide binding of  $^{75}\text{SeM}$ , as determined by incubation of cell-free plasma with  $\text{NaHSO}_3$  before precipitation with TCA, reached a peak of 14% approximately 1 hr after administration of the isotope, and then rapidly disappeared (Fig. 1). Selenosulfide binding was not measurable 1 day or 19 days after administration of isotope.

To determine whether  $^{75}\text{SeM}$  previously incorporated into plasma proteins was significantly reutilized by megakaryocytes or platelets, we collected blood from donor rabbits by intracardiac puncture 18 hr after intravenous injection of 15–30  $\mu\text{c}$   $^{75}\text{SeM}$ , at which time all isotope in the plasma had been incorporated into plasma protein. Labeled PFP (12–30 ml with 6550–6900 cpm/ml) was injected into three normal rabbits and 5 ml of PFP (16,000 cpm/ml) into two normal rabbits. The radioactive plasma protein circulated normally with a  $t_{1/2}$  of 6–8 days. Hematocrit values and platelet counts of the recipients remained within normal limits. Daily measurements during the 8 days after injection showed that radioactivity did not appear in the platelets, indicating that there was no detectable incorporation of  $^{75}\text{SeM}$  into platelets from isotope previously incorporated into plasma proteins.

*Incorporation of  $^{75}\text{SeM}$  into platelets, in vitro.* PRP was prepared from rabbit blood obtained by intracardiac puncture, by use of siliconized syringes wetted with heparin (10,000 U/ml). The blood additionally was anticoagulated with acid-citrate dextrose (one part ACD: nine parts whole blood).  $^{75}\text{SeM}$  was added to 1 ml volumes of PRP to give 5000 or 9000 cpm of  $^{75}\text{SeM}$ /ml of PRP. The levels of radioactivity equaled those achieved in circulating plasma protein under experimental con-

<sup>13</sup> A portion of the computations in this paper were carried out in the Computing Center of The Johns Hopkins Medical Institutions, which is supported by NIH Research Grant FR-0004 and by Educational Contributions from IBM.

<sup>14</sup> Whole blood platelet count/ml  $\times 0.06 \times$  body weight (kg).

<sup>15</sup> Mechlorethamine HCl for injection, USP, as Mustargen. Merck Sharp & Dohme, West Point, Pa.

<sup>16</sup> Freund's adjuvant, complete. Difco Laboratories, Detroit, Mich.

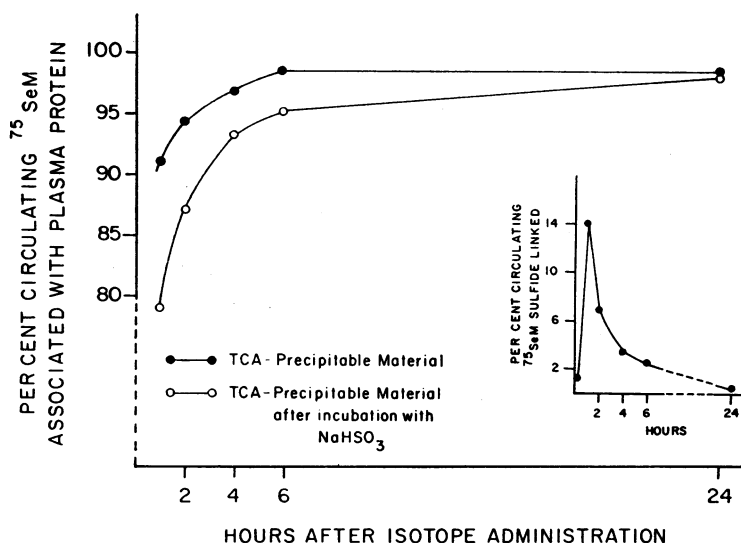


FIGURE 1 Rate of incorporation of  $^{75}\text{SeM}$  into plasma protein.  $15\ \mu\text{c}$  of  $^{75}\text{SeM}$  was injected into each of three rabbits. The rate of appearance of  $^{75}\text{SeM}$  associated with protein (TCA-precipitable material), and the amount bound to protein by selenosulfide binding (TCA-precipitable material after incubation with  $\text{NaHSO}_3$ ) are shown. The insert depicts the proportion of isotope that was sulfide linked. Results are the mean of these three animals.

ditions. The concentration of platelets ranged from  $827,000/\text{mm}^3$  to  $970,000/\text{mm}^3$ . The mixtures were incubated under aseptic conditions at  $37^\circ\text{C}$ . Samples were removed at 15 min, 2, 4, 6, and 24 hr, and incorporation of  $^{75}\text{SeM}$  into platelets determined.

Uncorrected  $\text{cpm}/10^8$  platelets never rose above 3 when the incubation mixture contained 9000  $\text{cpm}$  of  $^{75}\text{SeM}/\text{ml}$ . Uncorrected  $\text{cpm}/10^8$  platelets remained below 2 when there were 5000  $\text{cpm}/\text{ml}$  in the incubation mixture. Radioactivity associated with the washed platelets did not increase during the 24 hr period of observation in either series of experiments, and the total radioactivity remaining on the filter was approximately 0.4% of the total radioactivity in the incubation mixture. Furthermore, there was no uptake of  $^{75}\text{SeM}$  by platelets in vitro when the contamination of platelets by trapped plasma protein was calculated as described above.

*Incorporation of  $^{75}\text{SeM}$  into platelets in vivo.* The rate of appearance of  $^{75}\text{SeM}$  in the circulating platelets of 21 rabbits after the intravenous administration of  $^{75}\text{SeM}$  is shown in Fig. 2. Peak radioactivity/platelet occurred approximately 3 days after injection. The life span of rabbit platelets, calculated from the time of half-maximum radioactivity of circulating platelets was 4–5 days, in agreement with previous investigations (30, 31). Radioactivity associated with platelets began to decrease after 3 days, and by 8 days, radioactivity had fallen to the level observed 18 hr after administration of  $^{75}\text{SeM}$ . However, some "tailing" was

noted. Platelet counts (Fig. 3) of these rabbits subjected to serial withdrawal of blood (estimated total blood loss was 5 ml/sample) remained stable for 4 days but subsequently tended to rise, probably secondary to the effects of hemorrhage.

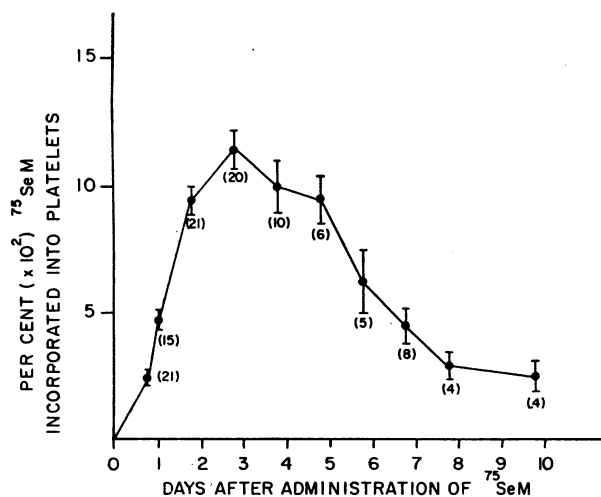


FIGURE 2 Per cent incorporation of  $^{75}\text{SeM}$  into circulating platelets in normal rabbits. Each rabbit received  $15\ \mu\text{c}$  of  $^{75}\text{SeM}$  intravenously. The mean  $\pm 1$  SE is shown, and the numbers in parentheses indicate the number of animals studied. Maximum  $^{75}\text{SeM}$  in circulating platelets occurred 3 days after administration of the isotope.

*Effects of normal plasma.* 41 rabbits served as controls. 20 rabbits received from 75 to 225 ml of platelet-free plasma from normal donor rabbits during the 32 hr period preceding administration of  $^{75}\text{SeM}$  to determine the effect of normal plasma on uptake of  $^{75}\text{SeM}$ . The remaining 21 rabbits received either no injection or an injection of 1 ml of normal saline daily, starting 7 days before administration of 15  $\mu\text{C}$  of  $^{75}\text{SeM}$ . The rate of incorporation of  $^{75}\text{SeM}$  into circulating platelets was not significantly different in rabbits that received normal plasma and in control animals that received either no infusions or only normal saline before administration of the isotope (Fig. 4). Maximum activity of  $^{75}\text{SeM}$  in circulating platelets occurred 3 days after administration of the isotope in both groups of animals. If thrombopoietin was present in the normal plasma, it was not detected by this technique.

*Effects of acute hemorrhage.* Six rabbits were bled 25 ml by intracardiac puncture. 24 hr later these and six control rabbits were given  $^{75}\text{SeM}$ , and blood samples were obtained during the next 5 days. Significantly more  $^{75}\text{SeM}$  was incorporated into the platelets of animals that had been bled than in the control group during the 42 hr period after administration of the isotope (Fig. 5). The difference remained significant even when corrected for the volume of blood removed, and when the assumption was made that the bled animals did not restore their blood volumes to normal. When the data are calculated as per cent change from control values, the increases at 18 and 42 hr were 115 and 36%, respectively. Furthermore, acceleration of incorporation of  $^{75}\text{SeM}$  was detected 5 days after administration of the isotope in the animals which had been bled. Removal of 25 ml of blood reduced the average hematocrit value from 39.3 to 27.5% but did not produce thrombocytopenia; it was followed by an increase in the incorporation of  $^{75}\text{SeM}$  into platelets,

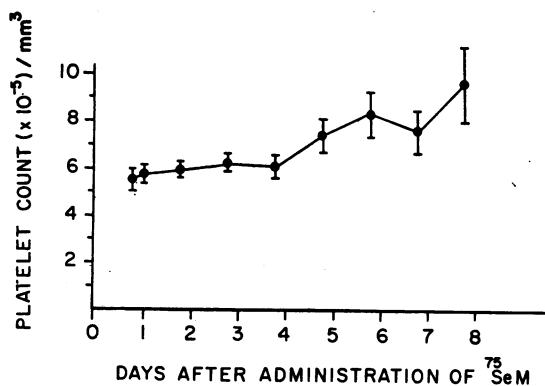


FIGURE 3 Platelet counts in rabbits during study of incorporation of  $^{75}\text{SeM}$ . Approximately 5 ml of blood was obtained at each venipuncture. Platelet counts remained stable for 4 days, and then tended to rise, probably secondary to blood loss. The mean  $\pm 1$  SE is shown.

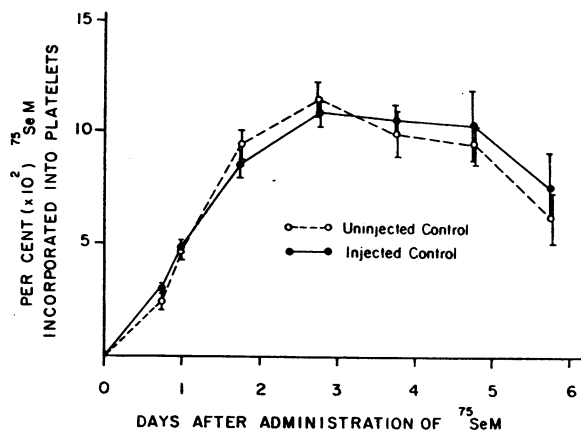


FIGURE 4 Effect of normal plasma on incorporation of  $^{75}\text{SeM}$  into platelets. Each of 41 rabbits received 15  $\mu\text{C}$  of  $^{75}\text{SeM}$  intravenously. 21 rabbits of this group (uninjected controls) received either no material or 1 ml of normal saline daily before administration of isotope. The remaining 20 rabbits (injected controls) received from 75 to 225 ml of platelet-free plasma from normal donors, in three equally divided doses, during the 32 hr period before isotope administration. The mean  $\pm 1$  SE is shown. There was no significant difference in per cent incorporation of  $^{75}\text{SeM}$  into platelets between the two groups.

even though the platelet counts remained stable until the 5th day. At that time, both the bled and control rabbits demonstrated a rise in platelet counts similar to that seen in other animals after repetitive blood sampling (Fig. 3). The results suggest that incorporation of isotope is

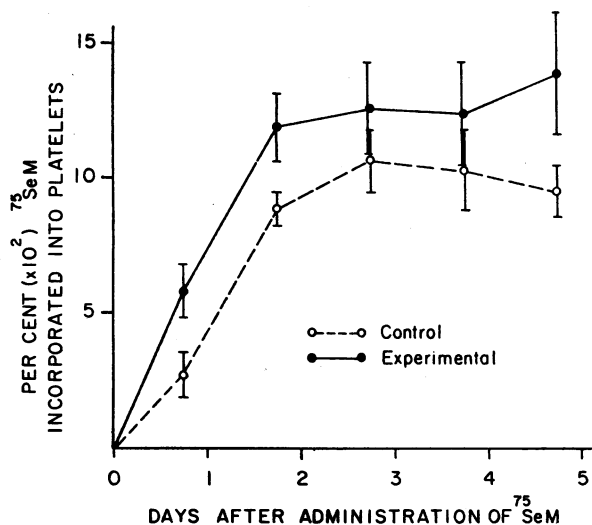


FIGURE 5 Effect of hemorrhage upon incorporation of  $^{75}\text{SeM}$  into platelets. Experimental animals were bled 25 ml, 1 day before administration of  $^{75}\text{SeM}$ . There were six animals in each group. The mean  $\pm 1$  SE is shown. The rabbits which had been bled incorporated more  $^{75}\text{SeM}$  into their platelets than did controls.

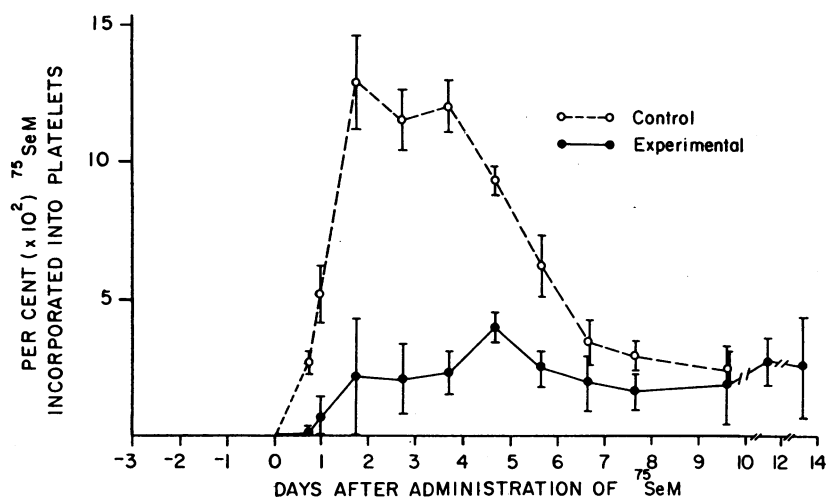


FIGURE 6 Effect of hypertransfusion of platelets upon incorporation of  $^{75}\text{SeM}$  into platelets. Two animals were hypertransfused with platelets, starting 3 days before administration of  $^{75}\text{SeM}$ . Four control animals received equal volumes of fresh, platelet-free plasma. Thrombocytosis was maintained by daily platelet transfusions for a total period of 6 days (Fig. 7). The mean  $\pm 1$  SE is shown. Hypertransfusion of platelets produced a marked decrease of incorporation of  $^{75}\text{SeM}$  into platelets.

a more sensitive indicator of alteration in platelet production than is the platelet count.

*Effects of hypertransfusion of platelets.* Thrombocytosis was produced and maintained in two rabbits by serial transfusion of fresh platelet concentrates. Initially,  $18.5 \times 10^{10}$  platelets were administered to each animal followed by  $8-10 \times 10^{10}$  platelets daily for an additional 3 days. After 3 days of sustained thrombocytosis (circulating platelet levels  $2-4 \times$  normal),  $^{75}\text{SeM}$  was administered, and  $7-10 \times 10^{10}$  platelets were administered daily for an additional 3 days. Four control animals received equal volumes of fresh platelet-free plasma.

The per cent of  $^{75}\text{SeM}$  incorporated into platelets was markedly decreased in rabbits with sustained thrombocytosis produced by hypertransfusion of platelets (Fig. 6). Relative thrombocytopenia occurred after the period of transfusion-induced thrombocytosis (Fig. 7), a phenomenon previously observed by others (10-12); this suggested suppression of platelet production. There appeared to be a rebound after the period of relative thrombocytopenia. The numbers of platelets sufficient to produce and sustain thrombocytosis during the first 3 days of the experiment did not maintain equally high platelet levels during the last 2 days of platelet transfusion (days 2 and 3 after administration of  $^{75}\text{SeM}$ ), further suggesting suppression of platelet production.

*Effects of depression of bone marrow function with nitrogen mustard.* Two rabbits received  $^{75}\text{SeM}$  1 day after a single dose of nitrogen mustard (2.6 mg/kg of body weight). Incorporation of  $^{75}\text{SeM}$  into platelets was decreased (Fig. 8). An initial brief peak of radioactivity

in platelets occurred 2 days after injection of the isotope, during the period of relative thrombocytosis (mean platelet count was  $800,000/\text{mm}^3$ ) which occurred 24-48 hr after the administration of nitrogen mustard to these animals.

Two rabbits received nitrogen mustard 44 hr before (2.6 mg/kg) and 6 hr after (1.3 mg/kg) the administration of  $^{75}\text{SeM}$ . Three other rabbits received four daily injections of nitrogen mustard (0.99 mg/kg),  $^{75}\text{SeM}$  on the 5th day, and an additional dose of nitrogen mustard (0.49 mg/kg) 6 hr after administration of the isotope. Significant levels of the isotope did not appear in the circulating platelets for 5-6 days (Fig. 8). In each experimental group, there was a delayed or secondary increase in level of  $^{75}\text{SeM}$  in platelets, 6-10 days after administration of the isotope, which correlated with rising platelet counts. Fig. 9 shows the rate of appearance of  $^{75}\text{SeM}$  in the circulating platelets of another rabbit that demonstrated a striking rebound in the platelet count during recovery from nitrogen mustard (2.6 mg/kg 44 hr before, and an additional 1.3 mg/kg 6 hr after the administration of  $^{75}\text{SeM}$ ). Significant levels of  $^{75}\text{SeM}$  were not observed in the circulating platelets until 5-7 days after administration of the isotope, at which time the levels approximated those normally seen at 2-3 days.

The increase in per cent of  $^{75}\text{SeM}$  associated with platelets, as the platelet count was rising, indicated entry into the circulation of a population of labeled platelets. Presumably  $^{75}\text{SeM}$ , which did not appear in circulating platelets at the usual time (i.e. 2-4 days), remained available in the marrow within megakaryocytes

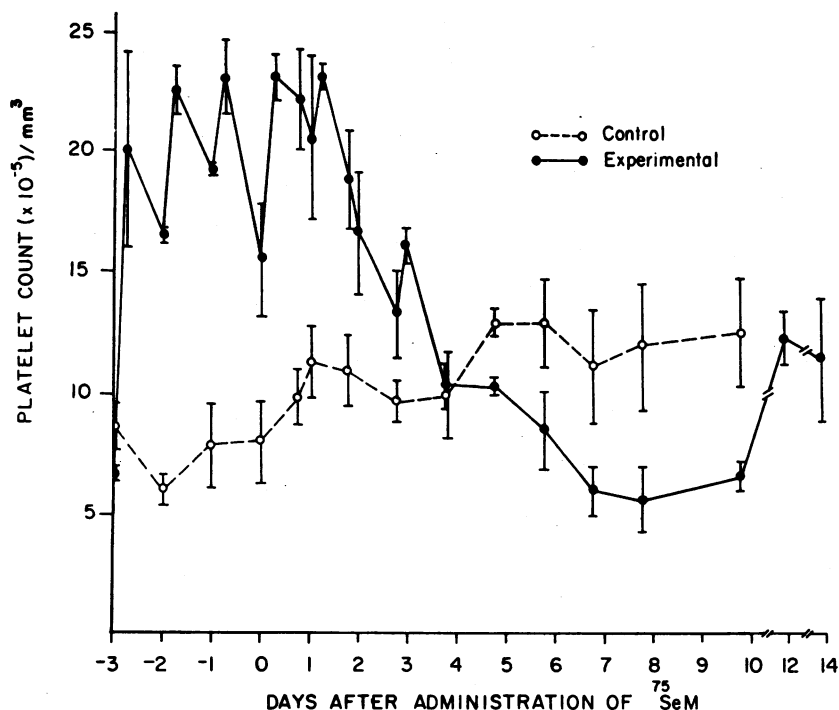


FIGURE 7 Effect of hypertransfusion of platelets upon peripheral platelet counts. The mean  $\pm 1$  SE is shown. Peripheral platelet levels were maintained from 2-4  $\times$  normal for a period of 6 days. Relative thrombocytopenia was observed after platelet transfusions were discontinued (days 7-10), at a time when control rabbits had elevated platelet counts secondary to repeated withdrawal of blood samples. A rebound above the original control level was seen after this period in the experimental animals.

or their precursors which were not productive; and subsequently appeared in the circulating platelets when marrow function was reestablished. Dosage schedules of nitrogen mustard were chosen to avoid the production of severe thrombocytopenia; the platelet counts of the rabbits usually were greater than 100,000/mm<sup>3</sup>. Therefore, it was possible to collect numbers of platelets adequate for measurement of radioactivity.

*Thrombopoietic activity in plasma from thrombocytopenic donors.* In each of three experiments six normal rabbits were given platelet-free plasma from rabbits rendered acutely thrombocytopenic by administration of anti-rabbit platelet serum. In each experiment six other rabbits were given platelet-free plasma from normal donor animals. Total volumes of 75, 150, or 225 ml of plasma were administered intravenously in three equally divided doses during the 32 hr period preceding administration of <sup>75</sup>SeM, which was given 6-8 hr after the last plasma infusion. In each experiment there was a significant increase in the uptake of <sup>75</sup>SeM into the platelets of animals that had received plasma from thrombocytopenic donors. There was no measurable dose response relationship in the range tested; the data were

pooled and the results are shown in Fig. 10. When these data are calculated as per cent change from control values, the increases at 18 and 24 hr were 39 and 32%, respectively; *P* was less than 0.005 during the 42 hr period after administration of <sup>75</sup>SeM.

Maximum radioactivity in circulating platelets occurred in both groups 3 days after administration of the isotope. The results suggest that the platelet-free plasma from thrombocytopenic animals contained a substance which increased the uptake of <sup>75</sup>SeM into the platelets of normal recipients. The platelet counts of recipients given plasma from thrombocytopenic donors did not increase or differ from those of rabbits given normal plasma.

## DISCUSSION

<sup>75</sup>Selenomethionine was removed rapidly from the circulation after intravenous administration. The phenomenon of selenosulfide bridging was short lived and the <sup>75</sup>SeM in the circulation 6 hr after injection was incorporated into plasma proteins (Fig. 1). These results are in agreement with previous observations of the rate of disappearance of amino acids from the circulation after



their intravenous administration (32), and the rapid incorporation of  $^{35}\text{S}$ -labeled (33, 34) and  $^{75}\text{Se}$ -labeled (25, 35) methionine into protein.

Platelet radioactivity remained at low levels immediately after the intravenous administration of  $^{75}\text{SeM}$  and maximum activity appeared in circulating platelets approximately 3 days later. Thus, maximum radioactivity in circulating platelets occurred at least 48 hr after the maximum levels of  $^{75}\text{SeM}$  in plasma proteins. Platelet radioactivity was rising at a time when the radioactivity of plasma proteins was rapidly decreasing. There was no detectable labeling of platelets already circulating at the time of isotope administration. The delay in peak radioactivity of  $^{75}\text{SeM}$  in circulating platelets was similar to the delayed appearance of sulfate- $^{35}\text{S}$  and formate- $^{14}\text{C}$  in platelets in rats (36, 37). The pattern of disappearance of circulating radioactive platelets (Fig. 2) resembled that obtained with  $\text{Na}_2^{35}\text{SO}_4$  (37), and formate- $^{14}\text{C}$  (36). All demonstrate "tailing"; this suggested either labeling of early megakaryocytic precursors or reutilization of the isotope. The life span of rabbit platelets, as measured with  $^{75}\text{SeM}$ , is in agreement with previous estimates (30, 31). The first portion of the uptake curve appears suitable for estimating the rate of production of

platelets at a time when limited delayed reutilization does not interfere with the interpretation. Appearance of labeled platelets in the circulation was almost linear during the 18–42 hr period after administration of  $^{75}\text{SeM}$ . Repeated withdrawal of samples of blood was accompanied by a rise in peripheral platelet counts (presumably secondary to blood loss [38]), and increased variation of  $^{75}\text{SeM}$  activity in circulating platelets during the latter portion of the uptake curves.

Hansson and Jacobsson (39) reported that intravenously injected  $^{75}\text{SeM}$  is available in the bone marrow for incorporation by megakaryocytes into cytoplasmic protein. In the present studies the increase in platelet radioactivity was independent of the concurrent plasma levels and indicated entry into the circulation of platelets that were labeled in another compartment. These observations are consistent with incorporation of  $^{75}\text{SeM}$  into the cytoplasm of megakaryocytes, subsequent division of the cytoplasm into platelets, and their release into the circulation; they suggest that  $^{75}\text{SeM}$  labels a cohort of platelets. Awwad, Adelstein, Potchen, and Dealy (40) have described the appearance of  $^{75}\text{SeM}$  in the red blood cells of rats given infusions of plasma proteins labeled with  $^{75}\text{SeM}$ ; however, their studies uti-

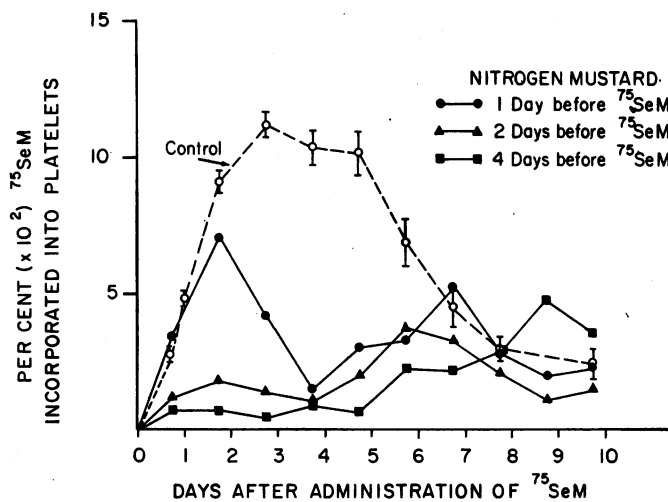


FIGURE 8 Effect of nitrogen mustard upon incorporation of  $^{75}\text{SeM}$  into platelets. Two rabbits received a single dose of nitrogen mustard one day before  $^{75}\text{SeM}$  (●). Initial dose of nitrogen mustard was given 2 days before  $^{75}\text{SeM}$  to two animals (▲), and 4 days before  $^{75}\text{SeM}$  to three animals (■) (see text). The mean is shown. The experimental groups significantly differed from normal animals until day 7. Appearance of  $^{75}\text{SeM}$  in platelets of normal animals is shown by open circles and broken line. In each group administration of nitrogen mustard in a dose sufficient to cause thrombocytopenia resulted in a decrease of incorporation of  $^{75}\text{SeM}$  into platelets.  $^{75}\text{SeM}$  subsequently reappeared in circulating platelets when marrow function was reestablished. A short period of thrombocytosis which occurred 24–48 hr after administration of nitrogen mustard in one group (●), was reflected by a brief peak of appearance of  $^{75}\text{SeM}$  in circulating platelets.

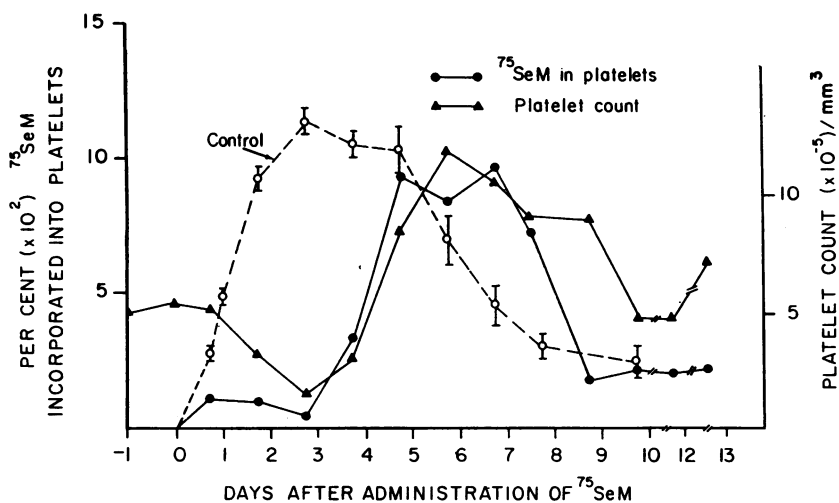


FIGURE 9 Effect of nitrogen mustard upon incorporation of  $^{75}\text{SeM}$  into platelets and peripheral platelet counts. One animal that had received nitrogen mustard 2 days before  $^{75}\text{SeM}$  demonstrated an unusual rebound phenomenon, with a period of marked thrombocytosis after thrombocytopenia. Delayed appearance of  $^{75}\text{SeM}$ , after the dose of nitrogen mustard, was seen during this recovery period. Appearance of  $^{75}\text{SeM}$  in platelets of normal animals is shown by open circles and broken line.

lized proteins which might have contained selenosulfide-bound  $^{75}\text{SeM}$ . In the present studies infusions of radioactive plasma, in which all of the isotope had been incorporated into proteins, did not result in detectable re-incorporation of  $^{75}\text{SeM}$  into platelets.

Incorporation of  $^{75}\text{SeM}$  into platelets did not occur when the isotope was incubated with platelet-rich plasma, *in vitro*. Chambers, Salzman, Neri, and Sivertsen (41) similarly were unable to demonstrate incorporation of radioactive amino acids into platelets suspended in platelet-rich plasma. *In vitro* incorporation of some labeled amino acids into washed platelets has been demonstrated (42, 43). The differences in experimental design may account for the apparent discrepancy, or perhaps a quantitative difference exists between transport mechanisms for different amino acids. This is suggested by the observation that the concentration of leucine in platelets (the amino acid used by Warshaw, Laster, and Shulman [43]) is five times greater than that of methionine (44).

Anemia and the rate of erythropoiesis have not been shown to have a direct effect on thrombopoiesis (5, 12, 45, 46). However, thrombocytosis after acute hemorrhage is well recognized (38, 47), suggesting an increase in the rate of platelet production after acute blood loss. In the present studies acute hemorrhage, which did not produce measurable thrombocytopenia or thrombocytosis, increased incorporation of  $^{75}\text{SeM}$  into platelets. Increase in the fraction of  $^{75}\text{SeM}$  that appeared in circulating platelets, without concomitant increase in plate-

let count, is consonant with an increase in turnover rate. These results are compatible with an increase in the rate of production of platelets or platelet protein after acute hemorrhage, and suggest that the rate of incorporation of  $^{75}\text{SeM}$  may be a more sensitive indicator of altered thrombopoiesis than are changes in the platelet count.

Conversely, transfusion-induced thrombocytosis, which was sustained for 6 days, produced a marked reduction in incorporation of  $^{75}\text{SeM}$  into platelets. Furthermore, after the period of thrombocytosis, the animals displayed a period of relative thrombocytopenia similar to that reported by Cronkite et al. (10), Odell et al. (11), and de Gabriele and Penington (12). These observations are compatible with a feedback mechanism by which circulating platelets affect the rate of thrombopoiesis.

Suppression of bone marrow function with nitrogen mustard resulted in decreased numbers of platelets in the circulation, and a decrease in incorporation of  $^{75}\text{SeM}$ . A delayed appearance of  $^{75}\text{SeM}$  was observed in circulating platelets during recovery from suppression of thrombopoiesis. There was excellent correlation between the platelet count and appearance of  $^{75}\text{SeM}$  in circulating platelets during this period. The late appearance of  $^{75}\text{SeM}$  in circulating platelets, when bone marrow function was reestablished, indicates that the isotope had remained available, presumably because it had been incorporated into megakaryocytes or their precursors which transiently were not producing platelets. Increased levels of  $^{75}\text{SeM}$  in circulating platelets, at the time that the plate-

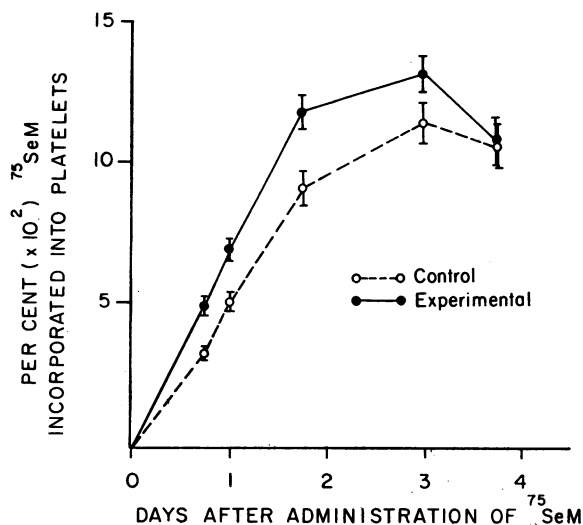


FIGURE 10 Effect of plasma from thrombocytopenic donors upon incorporation of  $^{75}\text{SeM}$  into platelets. 75–225 ml of platelet-free plasma from either normal or thrombocytopenic donors was injected into normal recipients in three equally divided doses during the 32 hr period preceding the administration of  $^{75}\text{SeM}$ . There were 18 animals in each group. The mean  $\pm 1$  SE is shown. There was a significant increase in incorporation of isotope into platelets of animals that received plasma from thrombocytopenic donors ( $P$  less than 0.005 at 18, 24, and 42 hr).

let count was rising, indicates entry into the circulation of a heavily labeled population of new platelets, reflecting return of megakaryocyte function and platelet production.

Some investigators (4, 13, 48) have reported that plasma from thrombocytopenic animals causes a temporary rise in circulating platelet levels in normal recipients. In the current studies the injection of plasma from thrombocytopenic donors into normal animals increased the fraction of  $^{75}\text{SeM}$  which appeared in the circulating platelets, without a concomitant rise in platelet count, again suggesting that the isotopic method may be a more sensitive measure of altered platelet production. The observed changes reflect the response to a relatively acute stimulus. A dose response relationship could not be detected in the range of volumes of plasma administered. The temporary decrease in platelet counts due to the dilutional effect of infusions of large volumes of plasma was observed in both experimental and control rabbits, and was not responsible for stimulation of the experimental groups, since a greater mean decrease in platelet counts was noted in control animals which did not show increased incorporation of  $^{75}\text{SeM}$ . All infused plasma was rendered platelet free to prevent potential inactivation of "thrombopoietin" by platelets (48). The results suggest that the plasma from thrombocytopenic donors contained a substance which in-

creased the uptake of  $^{75}\text{SeM}$  into the platelets of normal recipients, and are consistent with the concept of a humoral agent (thrombopoietin) that acts on megakaryocytes to regulate platelet production.

Changes in the rate of appearance or levels of  $^{75}\text{SeM}$  in circulating platelets were demonstrable in each experimental model designed to alter the rate of platelet production. The stability of peripheral platelet counts during the period of increased incorporation of  $^{75}\text{SeM}$  into platelets after hemorrhage or administration of plasma from thrombocytopenic donors indicates that alteration in levels of  $^{75}\text{SeM}$  in platelets does not reflect nonspecific redistribution of platelets existing at the time of stimulation. The data do not permit us to distinguish between release of increased numbers of platelets or release of young, large platelets which presumably contain more protein per platelet (49, 50). In none of the experiments did the levels of  $^{75}\text{SeM}$  incorporated into plasma proteins differ significantly; therefore, the changes observed in circulating platelets do not reflect a generalized effect on protein production. Thrombopoietic activity was not demonstrable by this method in normal rabbit plasma in the dose range tested. Other investigators similarly have been unable to demonstrate thrombopoietic activity in the blood of normal animals when the recipient and donor animals were of the same species (13, 51, 52).

It has been suggested that stimulation of thrombopoiesis produces increased entry of precursor cells into the megakaryocyte pool (5, 8, 31), an increase in the rate of maturation of megakaryocytes (5, 8, 31), and an increase in megakaryocyte number and size (5, 9, 20). In the current experimental models designed to stimulate platelet production (hemorrhage or transfusion of plasma from thrombocytopenic donors), maximum changes in the rate and levels of isotope incorporation were manifested during the 3 day period after administration of  $^{75}\text{SeM}$  or approximately 2–4 days after the initiation of the experimental stimulation. This interval of time is consistent with previous estimations of the transit time of megakaryocytes in rabbits (53) and rats (54). It is compatible with increasing entry of megakaryocyte precursors from an unidentified pool of stem cells into the compartment of recognizable megakaryocytes, their subsequent maturation, and release of platelets.  $^{75}\text{SeM}$  probably is incorporated into the cytoplasm of precursor cells and megakaryoblasts, as well as later stages. If  $^{75}\text{SeM}$  were incorporated only into the most mature megakaryocytes, one would predict very rapid release of labeled platelets into the circulation and a shift of the peak of appearance of labeled platelets in the circulation to the left (i.e. to 1 or 2 days after administration of  $^{75}\text{SeM}$ ). This was not observed and suggests that if labeling and early release of platelets from ma-

ture megakaryocytes did occur, it was not the only physiologic alteration. Our results are consistent with more rapid entry of platelets into the circulation and a sustained increase in incorporation of  $^{75}\text{SeM}$  into platelet protein.

$^{75}\text{SeM}$  appears to label a cohort of platelets, and is technically easy to measure. Our method of washing platelets, using Millipore filtration and relatively large volumes of fluid is efficient; it allows the handling of large numbers of samples rapidly and in a standardized manner. The technique provides a method which is feasible for the study of platelet production and of factors which affect thrombopoiesis. The rate of incorporation of  $^{75}\text{SeM}$  into platelets appears to reflect alterations in platelet production. It is more sensitive and probably more specific than are changes in circulating platelet levels, and should prove useful in studies of megakaryocytopoiesis and platelet production.

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