# TRANSFER OF ADENINE NUCLEOTIDES BETWEEN THE RELEASABLE AND NONRELEASABLE COMPARTMENTS OF RABBIT BLOOD PLATELETS

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## ABSTRACT

The metabolic pool of adenine nucleotides in platelets can be labeled by incubating platelets for 1 h in vitro with [$^{14}$C]adenosine or [$^{32}$P]orthophosphate. When these platelets are treated with thrombin, the adenine nucleotides released are not labeled. Because of this, Holmsen's suggestion of a metabolically inert pool of granule nucleotides has been generally accepted. We have found that upon incubation of labeled rabbit platelets for longer times (up to 6 h) in vitro, or upon reinjection and reharvesting at times up to 66 h, the releasable pool of adenine nucleotides becomes labeled. Because the rates of $^{32}$P and $^{14}$C incorporation into this releasable pool are similar, it seems likely that these labels enter the granules as ATP. Equilibrium between the metabolic and granule pools is complete by 18 h.

When rabbit platelets are labeled in vivo by intravenous injection of [$^{32}$P]orthophosphate, peak labeling occurs between 60 and 70 h; this corresponds to their maturation time. The platelets probably incorporate $^{32}$P during their production in the megakaryocytes. The specific radioactivity of the adenine nucleotides in the releasable (granule) pool of these platelets is the same as the specific radioactivity in the nonreleasable (metabolic) pool. Since inorganic phosphate in platelets (and undoubtedly in the megakaryocytes) exchanges with inorganic phosphate in plasma, and since the radioactivity of the latter decreases rapidly, the adenine nucleotides in the two pools must exchange to maintain the same specific radioactivity.

Transfer of adenine nucleotides into storage granules may represent a general phenomenon because it has been observed in the chromaffin cells of the adrenal medulla also.

We have previously observed that, if rabbit platelets are labeled in vivo by injection of [$^{32}$P]orthophosphate ($^{32}$P$_i$), platelet ATP and ADP contain $^{32}$P, and considerable amounts of the labeled adenine nucleotides are released from the platelets upon treatment with thrombin (13, 20, 25). Since thrombin causes the selective extrusion (release) of the contents of the storage granules of platelets (11), the amine storage granules presumably contain some of the labeled nucleotides. This is in contrast to the results obtained with platelets labeled in vitro. A number of investigators (8–10, 12) have found that platelets incubated in vitro with $^{32}$P$_i$, [$^{14}$C]adenosine, or [$^{14}$C]adenine form

labeled adenine nucleotides which are not released upon addition of collagen or thrombin. Holmsen et al. (10) showed that the labeled adenine nucleotides are in the cytoplasm, the mitochondria, and the membranes, and concluded that only these metabolic pool nucleotides become labeled during a 3-h incubation in vitro. They proposed that the releasable adenine nucleotide pool is metabolically inert. More recently, DaPrada and Pletscher (5) and Kotelba-Witkowska et al. (15) have obtained evidence that there may be some transfer or exchange of adenine nucleotides from the cytoplasm into the amine storage granules.

We have now examined in more detail whether adenine nucleotides or components thereof are exchanged between the granule (releasable) and extragranule (nonreleasable) compartments of rabbit platelets by use of $^{32}P_i$ or [$^{14}C$]adenosine as in vitro labels and $^{32}P_i$ as an in vivo label for the adenine nucleotides.

## MATERIALS AND METHODS

### Nonradioactive Compounds

The thrombin used was crude bovine thrombin (Parke, Davis & Co., Detroit, Mich.). p-Tosyl-L-arginine methyl ester (HCl) (TAMe) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Prostaglandin $E_1$ (PGE$_1$) was a gift from the Upjohn Company, Kalamazoo, Mich. Pyruvate kinase and phosphoenolpyruvate were obtained from Boehringer Mannheim GmbH, New York. Luciferin-luciferase was purchased from E.I. Dupont de Nemours & Co., Wilmington, Del.

### Radioactive Compounds

Carrier-free $^{32}P_i$ in 0.02 N HCl was obtained from New England Nuclear-Canada, Ltd, Montreal, P.Q. [8-$^{14}C$]adenosine ([$^{14}C$]adenosine), sp act ~50 mCi/ mmol, and Na$_2$$^{51}$CrO$_4$ in sterile isotonic saline, sp act ~160 μCi/μg Cr, were obtained from Amersham/Searle Corporation, Arlington Heights, Ill.

### Platelet Suspensions

Suspensions of washed platelets from rabbits were prepared in Tyrode's solution containing albumin (0.35%), after the method described by Ardlie et al. (1).

### Labeling of Platelets with Radioactive Compounds

#### IN VITRO

[$^{14}C$]ADENOSINE: Platelet adenine nucleotides were labeled by incubating the platelets suspended in Tyrode's-albumin solution with [$^{14}C$]adenosine (10 μCi/ 10$^{10}$ platelets) for 60 min at 37°C. After the incubation

period the platelets were resuspended in fresh Tyrode's-albumin solution unless stated otherwise.

$^{32}P_i$: Platelets were labeled with [$^{32}P$] orthophosphate (0.5 mCi/10$^{10}$ platelets) in Tyrode's-albumin solution from which unlabeled phosphate had been omitted. N-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES buffer) (5 mM) was included in the suspending medium to keep the pH at 7.35 during the incubation with $^{32}P_i$ for 60 min at 37°C. After the incubation period the platelets were washed once in modified Tyrode's-albumin solution containing no calcium and resuspended in Tyrode's-albumin solution. (Platelet count: 1.0–1.5 × 10$^6$ μl).

DOUBLE LABELING: When platelets were labeled with both $^{51}Cr$ and [$^{14}C$]adenosine, Na$_2$$^{51}$CrO$_4$ (100 μCi/10$^{10}$ platelets) was added to the first washing fluid. Platelets were incubated for 30 min at room temperature, resuspended in Tyrode's-albumin solution, incubated with [$^{14}C$]adenosine, and resuspended as described above.

#### IN VIVO

Platelets were labeled in vivo by intravenous injection into rabbits of 2 mCi [$^{32}P$]orthophosphate/kg, 1 mCi at 56 h and 1 mCi at 60 h before the collection of blood. Platelet suspensions were prepared as described. The final platelet count was adjusted to 1.0–1.5 × 10$^6$/μl.

### Platelet Release Reaction

1 ml of platelet suspension (1.0 × 10$^6$ platelets/μl) was warmed at 37°C for 5 min and then incubated with the control solution (0.1 ml Tyrode's) or with the thrombin solution (0.1 ml) for 3 min at 37°C in a shaking device. The suspension was transferred rapidly to an Eppendorf centrifuge tube and centrifuged for 1 min at 12,000 g in an Eppendorf Microcentrifuge 3200 (Brinkmann Instruments, Rexdale, Ont.). The supernate was stored in an ice bath and analyzed for adenine nucleotides, lactate dehydrogenase activity, and radioactivity.

### Repeated Thrombin Treatment

Platelets were treated three times with thrombin (0.05 U/ml) and recovered as single platelets after each treatment, as described elsewhere (20).

### Assays of Radioactivity

The amount of radioactivity in platelet suspensions (for total radioactivity) or in the supernate after centrifugation was determined as described previously (13) if a single label had been used. In experiments in which platelets were labeled with $^{51}Cr$ and $^{14}C$, 0.1 ml of the platelet suspension or the supernatant fluid was dissolved in 0.5 ml NCS solubilizer (Amersham/Searle Corp.) before 10 ml of the toluene-based scintillation fluid were added (5 g 2,5-diphenyloxazole and 0.3 g 2,2-1-phenylenebis-[5-phenyloxazole] in 1 liter of toluene). $^{51}Cr$ and $^{14}C$ were measured simultaneously (21) in a Philips liquid scintillation counter. The settings of the

⁵¹Cr channel and the ¹⁴C channel were such that 2.5% of the ⁵¹Cr was counted in the ¹⁴C channel and 10% of the ¹⁴C in the ⁵¹Cr channel. Counts were corrected for the spillover.

## Adenosine Triphosphate Assay

For determination of the platelet ATP content, 10 $\mu$l of 20% Triton X-100 in ethanol was added to 1.0 ml platelet suspension (1.0 × 10⁶ platelets/$\mu$l). Platelet ATP and released ATP were assayed by the luciferin-luciferase assay (13, 26).

¹⁴C-labeled adenine nucleotides were separated by paper chromatography and counted by the methods described by Packham et al. (17). ³²P-labeled adenine nucleotides were separated from each other and from inorganic ³²P by low voltage paper electrophoresis for 16 h at 4°C with 0.12 M citrate buffer, pH 4.78.

## Lactate Dehydrogenase

Lactate dehydrogenase (LDH) was assayed by the method of Bergmeyer et al. (4).

## Platelet Survival Studies

Platelets were labeled in vitro with ⁵¹Cr, or [³²P] orthophosphate as described above. Between 6 × 10⁹ and 10¹⁰ platelets in 2-3 ml Tyrode's-albumin solution were injected into each rabbit. The number of platelets injected corresponded to approximately 10-15% of the total number of platelets in the recipient rabbit. 1, 18, 42, 66, and 90 h after the injection of the labeled platelets, 6 ml of blood were withdrawn from an ear vein into 1 ml of the acid-citrate-dextrose solution of Aster and Jandl (2). Platelets were prepared from each sample, washed in calcium-free Tyrode's-albumin solution, and resuspended in 1 ml of calcium-free Tyrode's-albumin solu-

tion. Platelets were counted microscopically, and the amount of radioactivity in 0.1 ml of the platelet suspension was measured. The specific radioactivity of the platelets (cpm/10⁸ platelets) was calculated. In experiments in which platelets were labeled with ³²P$_i$, aliquots were taken for electrophoretic separation of the ³²P-labeled adenine nucleotides. The results were expressed as counts per minute in ATP and P$_i$ per 10⁸ platelets. The biological half-lives of platelet radioactive labels were computed as described previously (19).

## RESULTS

### Release of [³²P]ATP from Washed Rabbit Platelets

IN VIVO LABELING: ³²P$_i$ injected intravenously (1 mCi/kg) disappeared rapidly from the plasma. In an experiment in which the ³²P$_i$ was given as a single injection, 90% of it disappeared from the plasma between 4 min and 1 h after the injection. In experiments in which the rabbits were given two injections of ³²P$_i$ 4 h apart, the amount of ³²P in the platelets reached a maximum about 60-70 h after the first injection (Fig. 1). Rabbit platelets harvested 56 h after the second intravenous injection of ³²P$_i$ contained [³²P]ATP, [³²P]ADP, and [³²P]AMP (Table I). After stimulation with thrombin, these platelets released ³²P-labeled nucleotides into the suspending fluid. The specific radioactivity of the released ATP was not significantly different from that of the platelets before thrombin treatment (Table II).
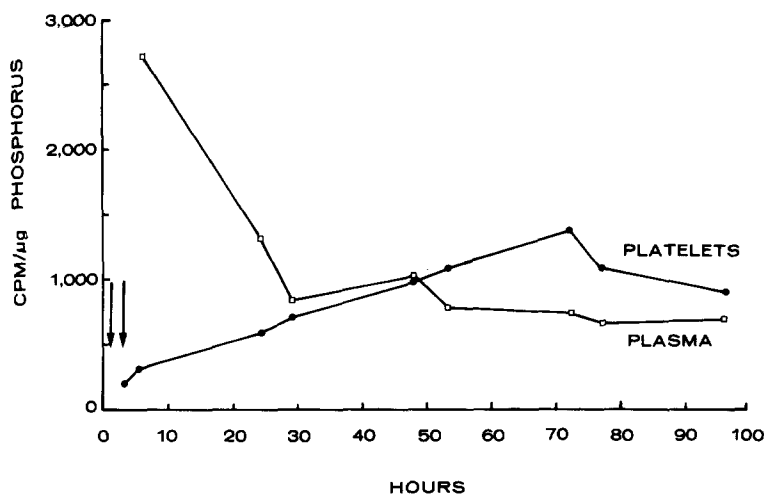


FIGURE 1 Specific radioactivity of ³²P in platelets after intravenous injections of ³²P$_i$ at 0 and 4 h (●——●). Arrows at left indicate the times of injection. The specific radioactivity of the ³²P in the platelet-poor plasma is also shown (□——□).

TABLE I

Distribution of $^{32}P$ between Adenine Nucleotides in Perchloric Acid Extracts of Platelets Labeled In Vivo or In Vitro with $^{32}P_i$

| | Total $^{32}P$ in perchloric acid extract | | | | |
| | ATP | ADP | AMP | $P_i$ | Not identified |
|---|---|---|---|---|---|
| | % | % | % | % | % |
| In vivo* | 44.4 ± 3.6 | 8.1 ± 0.3 | 3.8 ± 1.3 | 14.8 ± 1.6 | 28.9 |
| In vitro‡ | 45.3 ± 4.3 | 9.3 ± 1.2 | 0.6 ± 0.3 | 38.5 ± 2.2 | 6.4 |

* Means and SEM of three experiments.
‡ Means and SEM of five experiments.

TABLE II

Specific Radioactivity of [$^{32}P$]ATP Released by Thrombin (0.5 U/ml) from Washed Rabbit Platelets Labeled In Vivo with $^{32}P_i$

| | [$^{32}P$]ATP | ATP | Specific activity |
|---|---|---|---|
| | cpm/$10^8$ platelets | nmol/$10^8$ platelets | cpm/nmol |
| In platelets before thrombin treatment | 896 ± 62 | 4.8 ± 0.6 | 194 ± 10 |
| In supernate after thrombin treatment | 468 ± 29 | 2.6 ± 0.2 | 184 ± 11 |
| Significance of difference between means | | | $p < 0.3$ |

Means and SEM of nine experiments. Paired $t$ test analysis. The suspensions contained between 30,000 and 35,000 cpm/ml suspension. The platelet count was $10^6/\mu l$.

IN VITRO LABELING: The distribution of $^{32}P$ between ATP and ADP after in vitro incubation of platelets with $^{32}P_i$ was very similar to that after in vivo labeling with $^{32}P_i$ (Table I). However, a much smaller percentage of the total $^{32}P$ was found in the AMP of platelets labeled in vitro (Table I).

In contrast to in vivo labeled platelets, washed rabbit platelets labeled with $^{32}P_i$ in vitro showed very little release of $^{32}P$-labeled ATP upon treatment with thrombin (0.5 U/ml) (Table III). Repeated treatment of these platelets with thrombin led to a large increase in the specific radioactivity of the [$^{32}P$]ATP remaining within the platelets (Fig. 2). This is in contrast to the much smaller increase in specific radioactivity in [$^{32}P$]ATP found with the in vivo labeled platelets upon thrombin treatment (Fig. 2).

One explanation for the difference between the in vitro and in vivo results with the $^{32}P$-labeled platelets could be that exchange of label between the cytoplasm and the granules does occur but is so slow that it is not readily apparent in short-term in

vitro studies. This possibility was examined in the following experiments.

Transfer of Adenine Nucleotide Components from the Nonreleasable Compartment into the Releasable Compartment

TRANSFER OF PHOSPHATE: To determine whether transfer or exchange of phosphate between the cytoplasmic and the granule adenine nucleotides of platelets occurs, platelets were incubated with $^{32}P_i$ in vitro, washed, and resuspended in fresh medium. They were then kept at 37°C and the percentage of labeled ATP that could be released upon stimulation with thrombin (0.5 U/ml) was determined at various times (Fig. 3). This figure shows that about 9% of the labeled ATP in the washed rabbit platelets was releasable 6 h after the addition of the $^{32}P_i$ to the platelet suspension. The presence of labeled adenine nucleotides in the supernate of the thrombin-treated

TABLE III

Specific Radioactivity of [$^{32}$P]ATP Released by Thrombin (0.5 U/ml) from Washed Rabbit Platelets Labeled
In Vitro with $^{32}P_i$*

| | [$^{32}$P]ATP | ATP | Specific activity |
|---|---|---|---|
| | cpm/$10^8$ platelets | nmol/$10^8$ platelets | cpm/nmol |
| In platelets before thrombin treatment | 574,000 | 4.2 | 137,000 |
| In supernate after thrombin treatment | 12,800 | 2.3 | 5,600 |

* Platelet suspension contained 7.50 × $10^6$ cpm/ml. The platelet count was $10^6/\mu$l.
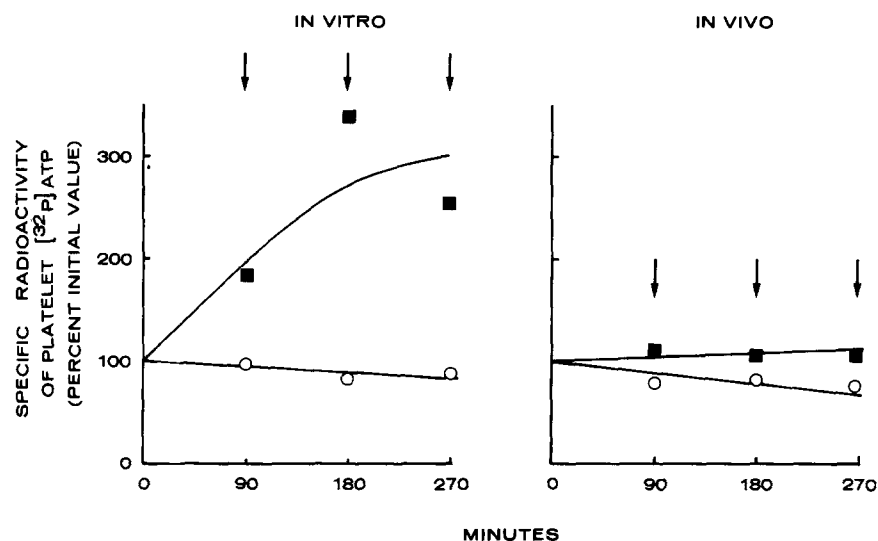


FIGURE 2    Specific radioactivity of [$^{32}$P]ATP remaining in platelets after three treatments with thrombin (0.05 U/ml) (■——■). On the left, platelets labeled in vitro; on the right, platelets labeled in vivo. Also shown are control values (O——O) for platelets treated three times with Tyrode's solution in the in vitro and in vivo experiments. Thrombin or Tyrode's solution was added at the times indicated by the arrows. Specific radioactivities are expressed as a percentage of the specific radioactivity of the original platelet ATP (counts per minute per nanomoles ATP). Platelet radioactivity as $^{32}$P at the beginning of the experiment was 34,732 cpm/ml of suspension when platelets had been labeled in vivo, and 1,030,000 cpm/ml of suspension when platelets had been labeled in vitro ($10^6$ platelets/$\mu$l).

platelet suspension could not be accounted for by platelet lysis. Less than 1% of the lactate dehydrogenase activity (LDH) of the total platelet suspension appeared in the supernate at the same time as the adenine nucleotides.

To provide a longer time for equilibration of cytoplasmic and granule phosphate, an experiment was done in which rabbit platelets labeled with $^{32}P_i$ in vitro were infused into rabbits and harvested 18 h later. A suspension of washed platelets was prepared in the usual manner. When these platelets were treated with thrombin (0.05 U/ml) they released 46% of their labeled ATP in conjunction

with 46% of their total ATP. Thus, the specific radioactivity of the released ATP (8,040 cpm/ nmol) was similar to that of the unstimulated platelets (8,120 cpm/nmol) (means of three observations). More than 90% of the radioactivity of the released adenine nucleotides was present as [$^{32}$P]ATP.

Since it was not possible to determine whether the $^{32}$P was entering the releasable pool as phosphate or as adenine nucleotides, we examined whether the adenosine moiety would cross the amine storage organelle membrane as well.
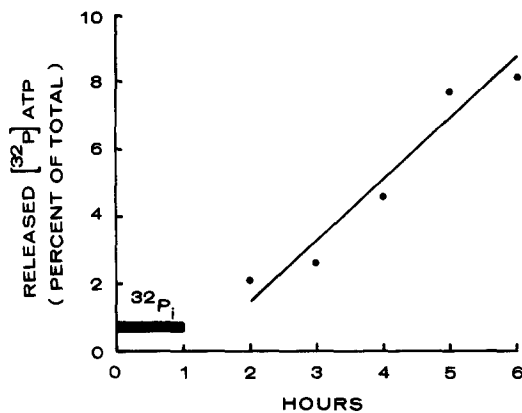
TRANSFER OF ADENOSINE:   When washed rab-

FIGURE 3 Percentage of thrombin-releasable $^{32}$P in the form of [$^{32}$P]ATP after storage of in vitro labeled platelets in Tyrode's-albumin solution. Platelets were exposed to $^{32}$P$_i$ for 1 h (black bar) and then resuspended in fresh medium containing unlabeled phosphate. Amount of releasable [$^{32}$P]ATP was determined at 2, 3, 4, 5, and 6 h by addition of 0.5 U/ml of thrombin to aliquots of the platelet suspension. The platelet suspension contained 7.50 × 10$^8$ cpm/ml. The platelet count was 10$^6$/µl.

bit platelets were incubated with 4 µM [$^{14}$C]adenosine, it disappeared rapidly from the suspending medium. 1 h after the addition of the label to the platelet suspension, nearly 40% of the $^{14}$C was recovered as [$^{14}$C]ATP inside the platelets (Fig. 4 a). When an aliquot of this platelet suspension was treated with thrombin (0.5 U/ml) 1 h after the addition of [$^{14}$C]adenosine to the platelet suspension, very little radioactivity was released as [$^{14}$C]ATP (Fig. 4 b). Aliquots were treated with thrombin in this way at various times up to 5 h. At 5 h, 4–5% of the radioactivity originally added was released by thrombin as [$^{14}$C]ATP. This constitutes about 9% of the labeled [$^{14}$C]ATP in the platelets before thrombin treatment. If the platelets labeled in vitro with [$^{14}$C]adenosine were injected into rabbits and harvested 18 h later, 52% ± 2% (mean and SEM of six experiments) of the platelet [$^{14}$C]ATP was released upon treatment with thrombin (0.5 U/ml).

The ratio of [$^{14}$C]ATP to [$^{14}$C]ADP in the released material was 6.4 ± 0.7, whereas the [$^{14}$C]ATP to [$^{14}$C]ADP ratio in the platelet suspen-
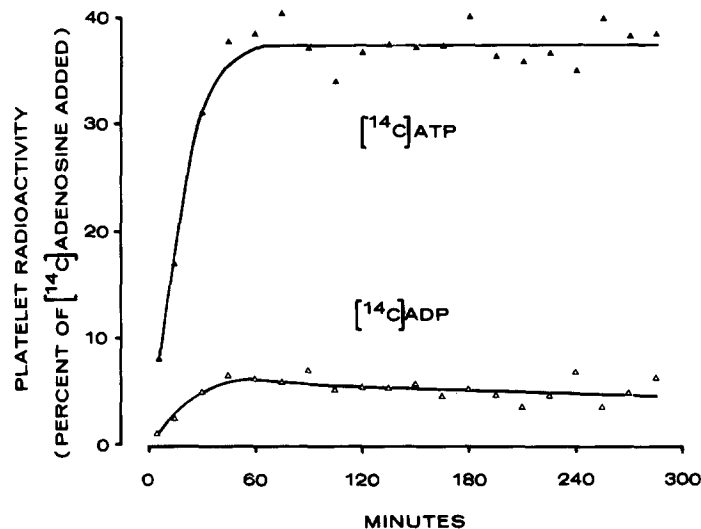


FIGURE 4 Percentages of $^{14}$C as [$^{14}$C]ATP and [$^{14}$C]ADP within platelets at various times after addition of [$^{14}$C]adenosine. At the times indicated, aliquots of the platelet suspension were removed and extracted with perchloric acid. The radioactive ATP and ADP in the perchloric acid extracts were separated by paper chromatography and counted. Results were expressed as percentages of the counts per minute added to the platelet suspension. (Appropriate corrections were made for small amounts of [$^{14}$C]ATP and [$^{14}$C]ADP in the supernate of the platelet suspension.) The suspension contained 184,000 cpm/ml. The platelet count was 10$^6$/µl.

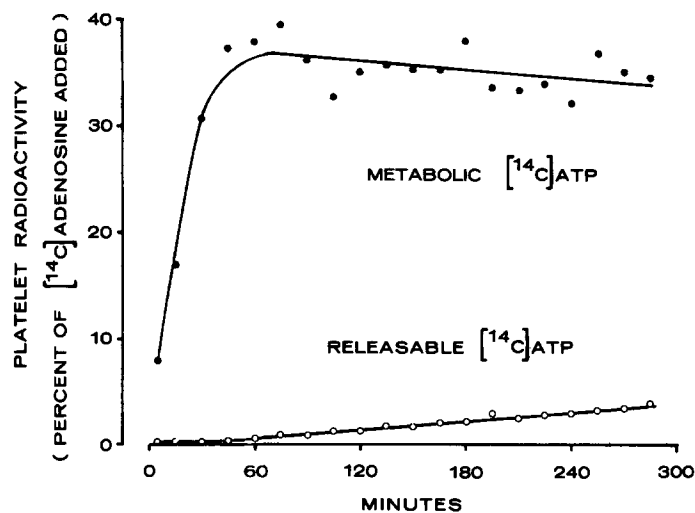FIGURE 4 a Percentages of radioactivity as [$^{14}$C]ATP (▲——▲) and [$^{14}$C]ADP (△——△) in the platelets.

FIGURE 4 *b* Each point represents the result of the treatment of an aliquot of the platelet suspension with 0.5 U/ml of thrombin. The metabolic [$^{14}$C]ATP (●——●) was calculated as the difference between total platelet [$^{14}$C]ATP (before induction of the release reaction) and the [$^{14}$C]ATP released (O——O) into the supernatant fluid after the addition of thrombin.

8.9 ± 0.8. This difference was significant ($P <$ 0.025; means and SEM of seven experiments).

In separate experiments it was found that a similar percentage of radioactive ATP was released by thrombin if platelets were isolated 18, 42, or 66 h after infusion of the labeled platelets into rabbits (Table IV).

To ensure that the appearance of labeled ATP in the supernatant fluid upon treatment with thrombin was not the result of platelet lysis rather than a specific release reaction, platelets were labeled in some experiments with both [$^{14}$C]adenosine and $^{51}$Cr before their injection into rabbits. $^{51}$Cr was used as a platelet label so that it would be possible to detect platelet lysis (22) if it occurred when the reharvested platelets were treated with thrombin. Table IV shows that 18 h after the original labeling, about 50% of the labeled ATP was released upon thrombin treatment. However, very little $^{51}$Cr appeared in the supernate at the same time. Similar results were obtained if the platelets were reharvested 42 h after the infusion of the labeled platelets. However, the proportion of $^{51}$Cr lost from the platelets upon exposure to thrombin 42 h after the labeling was somewhat greater than that after 18 h.

### Retention of [$^{32}$P]ATP and $^{51}$Cr in Platelets In Vivo

The following experiments were done to ascertain whether there is exchange between platelet phosphate and plasma phosphate, with corresponding changes in the specific radioactivity of ATP during the circulation of platelets in vivo. When the platelets were labeled in vitro with $^{32}$P or $^{51}$Cr and injected into rabbits, the total platelet $^{32}$P decreased more rapidly than the $^{51}$Cr associated with the platelets (Fig. 5). However, the platelet $^{32}$P$_i$ disappeared more rapidly than the platelet [$^{32}$P]ATP (Fig. 5). The rate of disappearance of total platelet $^{32}$P in all forms was intermediate between these two rates.

### DISCUSSION

Releasable, i.e. granule-bound, adenine nucleotides do not become labeled upon in vitro incubation of human platelets with $^{32}$P$_i$, [$^{14}$C]adenosine, or [$^{14}$C]adenine for a short period of time, although the nonreleasable metabolic adenine nucleotides become radioactive under the same conditions (10). This has led to the conclusion that the granule pool does not readily incorporate adenine nucleotides from the metabolic pool. The adenine nucleotides in the granules have therefore been considered as a metabolically inert pool which can be released upon exposure of platelets to thrombin, collagen, or other release-inducing agents (10). In rabbit platelets, however, DaPrada and Pletscher (5) were able to demonstrate a small but significant amount of newly formed [$^{14}$C]ATP in isolated amine storage granules upon exposure of intact platelets to [$^{14}$C]adenosine for 2 h. The authors

TABLE IV

*Thrombin-Induced Release of [¹⁴C]ATP and Loss of ⁵¹Cr from Platelets Labeled In Vitro and Injected into Rabbits*

| Experiment | Time of reharvesting of platelets after their injection | Release or loss (percent of total) | |
| --- | --- | --- | --- |
| | | [¹⁴C] ATP | ⁵¹Cr |
| | *h* | | |
| 1 | 18 | 51 | — |
| | 42 | 61 | — |
| | 66 | 57 | — |
| 2 | 18 | 50 | 6 |
| | 42 | 60 | 13 |

Experiment 1: platelets were isolated and incubated with [¹⁴C]adenosine. The platelets were resuspended in a fresh medium and injected into three rabbits. At 18, 42, and 66 h, one of the rabbits was exsanguinated and the platelets were isolated, washed, and resuspended at a platelet count of $10^8/\mu$l. Samples of the platelet suspension were treated with thrombin (0.5 U/ml) for 3 min and then centrifuged. The supernates were analyzed for released [¹⁴C]ATP after chromatographic separation. Platelet suspensions after reharvesting: 18 h, 43,060 cpm/ml; 42 h, 8,360 cpm/ml; 66 h, 6,260 cpm/ml.

Experiment 2: platelets were isolated and incubated with [¹⁴C]adenosine and Na₂⁵¹CrO₄. The platelets were resuspended in fresh media and injected into rabbits. Three experiments were done in which the platelets were harvested at 18 h and two in which the platelets were harvested at 42 h. At these times, suspensions of washed platelets were prepared and samples were treated with thrombin (0.5 U/ml) for 3 min and then centrifuged. The supernates were analyzed for ⁵¹Cr and for released [¹⁴C]ATP after chromatographic separation. (Mean values are shown.) Platelet radioactivity as ¹⁴C at 18 h was between 25,310 and 90,700 cpm/ml of suspension; at 42 h it was between 10,600 and 15,470 cpm/ml suspension. Platelet radioactivity as ⁵¹Cr at 18 hr was between 13,050 and 56,560 cpm/ml of suspension; at 42 h it was between 8,080 ·and 19,250 cpm/ml of suspension.

concluded that some of the triphosphonucleotides formed in the metabolic pool could be transferred into the storage organelles.

The results of the present experiments indicate that prolonged incubation in vitro of rabbit platelets that have been labeled in vitro with either $^{32}P_i$ or [¹⁴C]adenosine results in the gradual appearance of labeled ATP that can be released from the platelets when they are exposed to thrombin. Similar observations were made when platelets were labeled with these substances in vitro, rein-

fused into rabbits and reharvested after a number of hours. There are two possible sources of this labeled nucleotide: the cytoplasm and the amine storage organelles. Released material could not have come from the cytoplasm because the percentages of ⁵¹Cr or of lactate dehydrogenase activity which were lost from the cytoplasm were much less than the percentage of labeled ATP lost. In addition, shortly after labeling in vitro, when almost all of the label is in the metabolically active pool (10), addition of thrombin did not cause any release of labeled ATP. Thus, the storage granules must be the source of the radioactive ATP released from the platelets after prolonged incubation in vitro, or from platelets that had been reinjected and reharvested after 18–66 h.

Since both labels used in our study ($^{32}P$ and ¹⁴C) appeared in the releasable pool at a similar rate it seems likely that labeled ATP formed in the metabolic pool of the platelets was transferred as an intact molecule into the granule pool. It seems likely that ATP or its breakdown products can also be transferred from the granules to the cytoplasm since equilibration of the cytoplasmic and granule pool ATP appeared to be complete by 18 h; platelets harvested 18, 42, or 66 hours after the infusion into rabbits released practically the same percentage of radioactive ATP at all these times, upon addition of thrombin.

Further studies were done to investigate the transfer of labeled ATP or its products from the granules to the cytoplasm. After the injection of $^{32}P_i$ into rabbits, labeled inorganic phosphate is not incorporated rapidly into platelet adenine nucleotides because the labeling of the platelets is low when the plasma level of $^{32}P$ is highest. Peak labeling of the platelets does not occur until 2–3 days after the injection of $^{32}P_i$ and appears to coincide with the known maturation time for rabbit platelets as derived from studies in which [³⁵S]sulfate (7) or [⁷⁵Se]selenomethionine was used (6). Thus, it appears reasonable to conclude that the majority of the $^{32}P$ labeling of the platelets in the in vivo experiments occurred at some stage during the maturation of platelets in the megakaryocytes. Presumably, at some point during this process, both the cytoplasmic and granule nucleotides incorporate labeled phosphate by de novo synthesis. It seems likely that during the maturation of the platelets the specific radioactivity of the ATP in the cytoplasmic pool and the granule pool is the same. We found that the specific radioactivity of the ATP in
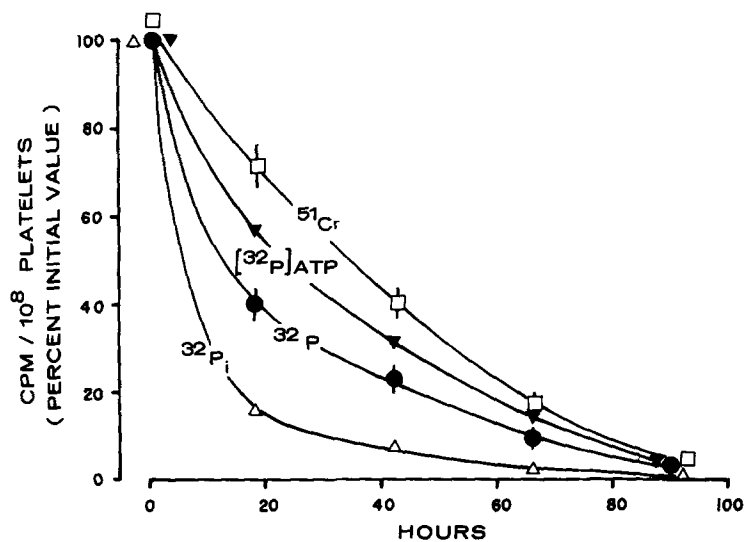
FIGURE 5 Platelet radioactivity after in vitro labeling with $^{51}$Cr (□——□) or $^{32}$P$_i$. Total platelet radioactivity as $^{32}$P (●——●), platelet radioactivity as inorganic phosphate $^{32}$P$_i$ (△——△), and platelet radioactivity as [$^{32}$P]ATP (▼——▼) (means ± SEM of eight experiments). Labeled platelets were injected into rabbits, and blood samples were taken at the times indicated. Platelets were isolated, and labeled compounds were separated and counted. Specific radioactivities (cpm/10$^8$ platelets) are plotted as a percentage of the initial specific radioactivity of the corresponding $^{32}$P-labeled material. The half-life for the disappearance of platelet-bound $^{51}$Cr from the circulation was 24.9 ± 2.0 h.

both pools was also the same 60 h after injection of $^{32}$P$_i$. If the ATP in the granule pool could not be transferred to the cytoplasm, one would expect that the specific radioactivity of the ATP in the two pools would differ at 60 h because the specific radioactivity of the plasma phosphate falls and there is an exchange of phosphate between platelets and plasma. (This is demonstrated by the fact that when $^{32}$P-labeled platelets were reinjected into rabbits, the specific radioactivity of platelet-bound $^{32}$P decreased more rapidly than that of platelet-bound $^{51}$Cr, and by Holmsen's [8] demonstration that rabbit platelets in plasma take up $^{32}$P-labeled inorganic phosphate.) However, 60 h after intravenous injection of $^{32}$P (at a time when the specific radioactivity of the plasma phosphorus was less than that of the platelet phosphorus), the ATP in the metabolic pool and the ATP in the releasable pool had the same specific radioactivity.[1] Therefore it seems likely that the ATP (or its metabolites) in the granules can be transferred to

The cytoplasm. Thus, ATP appears to exchange in both directions across the granule membrane.

From the results of the present experiments the absolute amount of ATP transported per unit time from the metabolic pool into the releasable pool can be estimated. From Table II it can be calculated that the metabolic pool contains 2.2 nmol of ATP per 10$^8$ platelets; from Fig. 3 and Fig. 4 it is apparent that about 2% of the labeled adenine nucleotides in the metabolic pool are transferred into the granules per hour. Thus, the rate of transfer of ATP can be calculated to be approximately 44 pmol/10$^8$ platelets/h. This may be compared to the maximum rate of serotonin transfer from the cytoplasm into the granules of 17.2 nmol/10$^8$ platelets/h that was observed in experiments reported previously (18). Since serotonin is transferred into the granules at a rate that is at least 300 times greater than the rate of transfer of ATP, it seems unlikely that transfer of serotonin into the granules is linked to ATP transport.

Cells other than platelets also possess granules which contain adenine nucleotides (16). Stevens et al. (23) demonstrated that radioactive adenosine is taken up by the isolated perfused cat adrenal gland, and that radioactive ATP accumulates in

[1] The small increase in the specific radioactivity after repeated thrombin-induced release reactions may be due to some preferential loss of an older (less labeled) platelet population. During repeated thrombin-induced aggregation, about 30% of the platelets are lost compared to 10% loss in the control suspension (20).

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the adrenal chromaffin granules and can be re-
leased upon stimulation with carbachol. This may
indicate that labeled nucleotides formed in the
metabolic pool of these cells can be transferred
into the chromaffin granules. Nucleotide transfer
from the metabolic pool into the storage granules
of cells may thus be a phenomenon which is not
limited to platelets. There is other evidence for
translocation of adenine nucleotides across mem-
branes. Among the best known is the transport of
adenine nucleotides across the mitochondrial
membrane via a specialized carrier system (14).
Adenine nucleotides can also diffuse across artifi-
cial bimolecular lipid membranes, a process that is
stimulated by divalent metal cations (24). Further-
more, isolated chromaffin granules have recently
been shown to take up small amounts of radioac-
tive ATP (3).

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