# TRANSPORT AND STORAGE OF SEROTONIN
# BY THROMBIN-TREATED PLATELETS

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ABSTRACT

Repeated thrombin treatment of washed platelets prepared from rabbits can
decrease the serotonin content of the platelets by about 80%. When these platelets
are deaggregated they reaccumulate serotonin but their storage capacity for
serotonin is reduced by about 60%. If thrombin-pretreated platelets are allowed to
equilibrate with a high concentration of serotonin (123 $\mu$M), they release a smaller
percentage of their total serotonin upon further thrombin treatment, in comparison
with the percentage of serotonin released from control platelets equilibrated with
the same concentration of serotonin. Calculations indicate that in thrombin-treated
platelets reequilibrated with serotonin, two-thirds of the serotonin is in the granule
compartment and one-third is in the extragranular compartment, presumably the
cytoplasm. Analysis of the exchange of serotonin between the suspending fluid and
the platelets showed that thrombin treatment does not alter the transport rate of
serotonin across the platelet membrane, and does not cause increased diffusion of
serotonin from the platelets into the suspending fluid. The primary reason for the
reduced serotonin accumulation by the thrombin-treated platelets appears to be
loss of amine storage granules or of the storage capacity within the granules.

Rabbit platelets that have been degranulated and
aggregated by treatment with thrombin in vitro
can be recovered as single platelets (Reimers et al.,
1973). These thrombin-treated platelets aggregate
upon the addition of low concentrations of ADP,
and upon reinfusion into rabbits they survive for a
normal length of time. In these experiments the
platelets lost more than 60% of the serotonin and
adenine nucleotides from their amine storage gran-
ules, but it appeared that the thrombin treatment
of the platelets did not lead to irreversible damage
which caused changes in platelet survival or loss of
responsiveness to ADP. We have now examined
the transport and storage of serotonin by these
thrombin-degranulated platelets.

Several investigators have postulated that sero-
tonin interacts with a receptor on the platelet
membrane, and have provided evidence that it is
transported across the membrane by an active
process, and appears in the amine storage granules
(Hardisty and Stacey, 1955; Born and Bricknell,
1959; Born and Gillson, 1959; Hughes and Brodie,
1959; Tranzer et al., 1966; Pletscher, 1968;
Pletscher et al., 1971; Baumgartner and Born,
1969; Baumgartner, 1969; Okuda and Nemerson,
1971; Sneddon, 1973). Serotonin also diffuses
from the platelets into the suspending medium
(Hughes and Brodie, 1959; Okuda and Nemerson,
1971). All of the platelet serotonin is freely ex-
changeable with serotonin in the medium (Born

and Gillson, 1959; Massini, 1970; Holmsen et al., 1973).

The experiments reported in this paper were designed to examine whether the thrombin treatment of platelets interfered with the transport of serotonin across the platelet plasma membrane into the cytoplasm, the diffusion from the cytoplasm into the suspending fluid, or the uptake from the cytoplasm into the amine storage granules.

## MATERIALS AND METHODS

### Nonradioactive Compounds

Thrombin was crude bovine thrombin (Parke, Davis & Company, Detroit, Mich.). p-Tosyl-L-arginine methyl ester (HCl) (TAMe) was obtained from ICN Pharmaceuticals Inc., Life Sciences Group (Cleveland, Ohio). Prostaglandin $E_1$ (PGE$_1$) was generously supplied by the Upjohn Co. (Kalamazoo, Mich.). ADP and 5-hydroxytryptamine creatinine sulfate complex (5HT; serotonin) were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Trypsin-activated pig plasmin was obtained from Novo-Fabrik, Copenhagen, and was dissolved in 0.9% NaCl (0.5 g/100 ml). Apyrase was prepared according to Molnar and Lorand (1961). Minimum essential medium (Eagle's medium; catalog no. F-11) was purchased from Grand Island Biological Co., Grand Island, N. Y. Reserpine (Serpasil) was obtained from Ciba Co. Ltd. (Dorval, Quebec); 1 ml aqueous solution contained 2.5 mg reserpine. Imipramine (Tofranil) was obtained as powder from Geigy Canada Ltd. and dissolved in 0.85% saline.

### Radioactive Compounds

[G-$^3$H]5-Hydroxytryptamine creatinine sulfate ([$^3$H]serotonin; [$^3$H]5HT) in aqueous solution containing 2% ethanol (specific activity approximately 11 Ci/mmol) and [3'-$^{14}$C]5-hydroxytryptamine creatinine sulfate ([$^{14}$C]serotonin; [$^{14}$C]5HT) (specific activity approximately 50 mCi/mmol), were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). The radioactive purity of the [$^{14}$C]5HT was determined by the paper chromatographic method of Pletscher et al. (1968) and was found to be between 82 and 91% at the time of its use. [carboxy-$^{14}$C]5-Hydroxy-3-indoleacetic acid ([$^{14}$C]5-hydroxyindoleacetic acid; [$^{14}$C]5HIAA) was purchased from New England Nuclear (Boston, Mass.). The specific activity of the [$^{14}$C]5HIAA was 10 mCi/mmol.

### Platelet Suspensions

Suspensions of washed platelets in Tyrode solution containing albumin (0.35%) and apyrase were prepared from rabbit blood as described by Ardlie et al. (1971). In experiments in which the pH was expected to fall, Eagle's medium was used instead of Tyrode solution. Suspensions of thrombin-treated platelets were prepared as described previously (Reimers et al., 1973): platelets in 20 ml of Tyrode-albumin solution (1.5 × 10$^9$ platelets/ml) were aggregated by the addition of thrombin (0.05 U/ml). After large aggregates had formed, PGE$_1$ (1 μM) and TAMe (1 mM) were added. Within 30 min at 37°C the platelets deaggregated. Platelets were washed once in calcium-free Tyrode-albumin and then resuspended in Tyrode solution containing apyrase and albumin. The procedure was repeated twice.

### Platelet Release Reaction

This reaction was studied using platelets labeled with [$^{14}$C]5HT as described previously (Packham et al., 1967). 1 ml of platelet suspension (1.0 × 10$^9$ platelets/ml) was warmed at 37°C for 5 min and then incubated with the control solution (Tyrode) or with the thrombin solution (0.1 ml) for 3 min at 37°C in a shaking device. The suspension was transferred rapidly into an Eppendorf centrifugation tube and centrifuged for 1 min at 15,000 g in an Eppendorf microcentrifuge 3200. The supernate was stored in an ice bath. Samples were taken for radioactivity determination.

### Studies of [$^{14}$C]Serotonin Accumulation

[$^{14}$C]5HT (0.8 μCi/μmol) in 0.2 ml Tyrode solution was added to 10-ml platelet suspension (1.0 × 10$^6$ platelets/μl) to give a final concentration of 123 μM. The radioactivity in an aliquot of the total platelet suspension and in an aliquot of the 15,000 g supernate (Eppendorf centrifuge 3200; 45 s) was determined at various times. The difference between the two radioactivity measurements was taken as a measure of [$^{14}$C]serotonin accumulation within the platelets. In studies with inhibitors of serotonin accumulation, the inhibitor (or Tyrode solution in the control) was added 5 min before the addition of [$^{14}$C]5HT to the platelet suspension. Experiments were performed at 37°C.

The accumulation of [$^{14}$C]5HIAA (final concentration 0.5 and 5.0 μM) was studied in the same way.

### Studies of [$^{14}$C]Serotonin Reaccumulation after the Release Reaction

Thrombin (0.05 U/ml) was added to 20 ml of a suspension of washed platelets (1.5 × 10$^9$ platelets/ml) that had been labeled with [$^{14}$C]5HT in the first washing fluid. After large aggregates had formed, aliquots were taken and the released radioactivity was determined in the supernatant fluid. Disappearance of the radioactivity from the surrounding medium upon addition of Tyrode solution (control) or PGE$_1$ (1 μM) was measured. All experiments were performed at 37°C.

If aggregation and release of $^{14}$C were induced by addition of a high thrombin concentration (0.5 U/ml), fibrin formed and bound the platelets together. To

prevent fibrin from binding the platelets together, plasmin (0.5 mg/ml) was added with $PGE_1$.

## Assays of Radioactivity

The amount of radioactivity in the platelet suspensions (for total radioactivity) or in the supernatant solutions was determined as described previously (Jenkins et al., 1971). In experiments in which a double-labeling technique was used the specific activities of the [$^3$H]5HT (8.8 mCi/mmol) and [$^{14}$C]5HT (176 μCi/mmol) were adjusted so that in each sample in which the radioactivity was measured the number of cpm in the $^3$H channel was approximately 5–10 times greater than that in the $^{14}$C channel. Sufficient counts were accumulated to obtain a radioactivity determination, in each sample, with an error of less than ±2%.

## Platelet Serotonin and Serotonin Metabolites

Platelet serotonin was measured fluorometrically using the method described by Weissbach et al. (1958). In experiments in which serotonin accumulation or the platelet storage capacity for serotonin was measured, platelets were incubated with serotonin for different lengths of time and the serotonin concentration was determined in the total platelet suspensions as well as in the supernatant fluid. Platelet serotonin was calculated from the difference between these values. Although the fluorometric method used measures all 5-hydroxyindoles, the platelet serotonin content may be estimated with reasonable accuracy from the difference between total 5-hydroxyindoles and supernatant 5-hydroxyindoles since it could be shown in experiments with [$^{14}$C]5HT that only small amounts of serotonin metabolites (5HIAA; 5-hydroxytryptophol [5HT'ol]) were within the platelets under all experimental conditions (less than 10% of the total platelet radioactivity). Most of the serotonin metabolites were found in the supernatant fluid. Serotonin in the washed platelet pellet was not determined directly because, according to Born and Gillson (1959), there is a partial loss of serotonin from human platelets into the surrounding medium during each washing step, and it was found that this occurred also with rabbit platelets when they had been incubated with serotonin at a high concentration.

Radioactive serotonin and its metabolites were separated by paper chromatography (Pletscher et al., 1968) and quantitated by liquid scintillation counting.

## Analysis of Serotonin Exchange Experiments

The exchange of serotonin between platelets and the suspending medium was carried out with two radioactive tracers, $^{14}$C and $^3$H. At the beginning of the experiments the [$^3$H]serotonin is within the platelet, and the [$^{14}$C]serotonin is in the suspending medium. The serotonin exchange data are obtained as counts of $^{14}$C and $^3$H

radioactivity in the surrounding medium. These data may be converted to the fraction of total $^{14}$C and $^3$H radioactivity in the platelet suspension. It is assumed that the platelet suspension contains three compartments: suspending medium (compartment 1); "cytoplasm" (compartment 2); and storage granules (compartment 3). Compartments 1 and 3 are connected through compartment 2. The fraction of the serotonin in any compartment that is transferred to another compartment in 1 min is the fractional turnover rate between these two compartments. The fractional turnover rate from compartment 1 to compartment 2 is $a_{21}$, etc. The fractional turnover rates are assumed to be equal for radioactive and nonradioactive serotonin. The fraction of tracer in compartment 1 (the surrounding medium) is $m_1$ and may be described by the following equations:

$$[^{14}C]m_1 = A + Be^{\lambda_1 t} + Ce^{\lambda_2 t} \qquad (1)$$

$$[^3H]m_1 = A + De^{\lambda_1 t} + Ee^{\lambda_2 t} \qquad (2)$$

where the preexponential constants $A$, $B$, $C$, $D$, and $E$ are functions of the initial tracer distribution and the fractional turnover rates, whereas the exponential constants, $\lambda_1$ and $\lambda_2$, are functions of the fractional turnover rates alone.

The initial distribution of $^{14}$C being known, all this tracer lying within the surrounding medium, it is possible to determine the fractional turnover rates from the $^{14}$C data set by fitting to Eq. 1. The platelets being at equilibrium with respect to serotonin, and the amount of serotonin within the surrounding medium being known, the total amount of serotonin in the two platelet compartments may be calculated as follows: let $S_1$ be the total amount of serotonin in compartment 1, $S_2$ the total amount of serotonin in compartment 2, and $S_3$ the total amount of serotonin in compartment 3, then (amount of serotonin in compartment 1) × (fraction of serotonin in compartment 1 transferred to compartment 2 every minute)

$$= \text{flux from 1 to 2} = a_{21}S_1$$

similarly:

$$\text{flux from 2 to 1} = a_{12}S_2.$$

At equilibrium:

$$a_{21}S_1 = a_{12}S_2$$

or:

$$S_2 = S_1\frac{a_{21}}{a_{12}}$$

similarly:

$$S_3 = S_2\frac{a_{32}}{a_{23}}.$$

Although the $^3$H data set cannot be used to calculate the fractional turnover rates, its initial distribution within the platelet being unknown, this data set is valuable in that the equation describing it, Eq. 2, has constants in common with Eq. 1 ($A$, $\lambda_1$, and $\lambda_2$).

The simultaneous fitting of both data sets to the Eqs. 1

and 2 thus provides a more precise estimate of these constants. This fitting employs a Bayesian multivariate technique of analysis. This method takes into account the correlation between the errors in both data sets. Details of this analytical technique will be published elsewhere (Allen, Reimers, Feuerstein, and Mustard, manuscript submitted for publication).

The analysis of the serotonin exchange experiments is based on the assumption that radioactive serotonin is not converted to other compounds to an appreciable extent during the experiment. However, it has been shown by Pletscher (1968) that part of the endogenous serotonin is metabolized in the presence of platelets agglutinated by thrombin (0.8% of the released serotonin was recovered as 5HT'ol after 30 min of incubation and 12.1% after 2 h of incubation; 5HT'ol was identified as the major metabolite in his studies). We confirmed Pletscher's finding that the formation of metabolic breakdown products is enhanced by treating platelets with thrombin. However, we determined that the breakdown products of serotonin would introduce errors only in the presence of much lower concentrations of serotonin than the 123 $\mu$M concentration used in the exchange experiments. For example: upon addition of a low concentration of [$^{14}$C]5HT (2 $\mu$M), 3.5% of the added serotonin was metabolized within 1 h to the major breakdown products 5HIAA and 5HT'ol in the control platelets, whereas 34.8% was metabolized in the presence of thrombin-treated platelets. However, upon the addition of the much higher concentration of serotonin used in the exchange experiments (123 $\mu$M), there was no statistically significant breakdown of serotonin into its metabolites (expressed as a percentage of the added serotonin) during the time-course of the experiments. The highest calculated (but not significant) breakdown of 123 $\mu$M serotonin in the presence of thrombin-treated platelets was 1.3% per hour. It was calculated that a breakdown at this rate had only a small effect (less than 15%) on the values calculated for the serotonin transport rates compared to those calculated by assuming no breakdown of serotonin.

In addition, the analysis of the serotonin exchange experiments assumes that the exchange of radioactivity represents exchange of serotonin and not of radioactive breakdown products. However, the [$^{14}$C]5HT used had some radiochemical impurities as shown by paper chromatography. The largest impurity was due to 5HIAA (up to 6% of the total radioactivity). An experiment was done, therefore, in which the uptake of [$^{14}$C]5HIAA by platelets was examined. It was found that, independent of the [$^{14}$C]5HIAA concentration used (0.5 or 5.0 $\mu$M), about 10% of the radioactivity became associated with both the control platelets and the thrombin-treated platelets within 2 min. There was no further accumulation of radioactivity within the platelets during the next hour. Since the 5HIAA impurity was less than 6% in the serotonin used, nonspecific uptake of radioactivity could not exceed 0.6% of the added radioactivity. Furthermore,

the exchange process was only analyzed between 2 and 50 min after addition of [$^{14}$C]5HT to the suspending medium.

It was also calculated that an error of $\pm 5\%$ in the determination of the purity of the [$^{14}$C]5HT did not appreciably alter the values calculated for the serotonin exchange rates, provided the breakdown products do not participate in the exchange process (see above).

## Electron Microscopy

Platelets for electron microscopy were fixed in 2.5% glutaraldehyde at 4°C for 30 min, washed in Millonig's buffer, and postfixed in 1% osmium tetroxide (4°C, 20 min). The fixed platelets were dehydrated in increasing concentrations of ethanol, and embedded in Spurr's resin. Sections were cut with an LKB microtome, stained with uranyl acetate and lead citrate, and examined on a Philips 300 electron microscope.

## RESULTS

### Reaccumulation of Serotonin after a Single Thrombin-Induced Release Reaction

Washed rabbit platelets labeled with [$^{14}$C]5HT (1.2 $\mu$M) aggregate upon addition of thrombin (0.05 U/ml) and release between 20 and 40% of their radioactivity. In the absence of apyrase in the suspending medium these platelets remain aggregated for at least 15 min and the amount of the radioactivity in the surrounding medium remains almost unaltered over this period (Fig. 1). However, upon the addition of PGE$_1$ (1 $\mu$M), platelets deaggregate rapidly and the majority of the radioactivity is taken up from the surrounding medium within approximately 30–60 min (Fig. 1). PGE$_1$ (1 $\mu$M) by itself does not influence serotonin uptake into rabbit platelets under the conditions of these experiments.

The effect of the concentration of serotonin used to label platelets on their ability to accumulate serotonin after the release reaction was investigated. A suspension of washed rabbit platelets (10$^9$/ml) was incubated with a high serotonin concentration (123 $\mu$M) for 60 min. (This time was chosen because no further net uptake of serotonin occurred after 20 min.) During this incubation the serotonin content of these platelets rose from approximately 75 nmol/10$^9$ platelets to 123 nmol/10$^9$ platelets. Upon addition of thrombin (0.05 U/ml), these platelets released approximately 25% of their [$^{14}$C]5HT. This is similar to the percentage [$^{14}$C]5HT released by thrombin treatment of platelets incubated with a low concentration of serotonin (1.2 $\mu$M). (These platelets contained 80 nmol

of serotonin/$10^9$ platelets before thrombin treatment.) The percentage of serotonin reaccumulated by the platelets during the first 30 min after deaggregation was greater if the platelets had been incubated with a low serotonin concentration before the release reaction (Fig. 2). The rate of serotonin reaccumulation was calculated from the specific activity of the serotonin present in the platelets before the release reaction and from the

disappearance rate of $^{14}C$ from the surrounding medium. The rate of serotonin reaccumulation was 334 pmol/min/$10^9$ platelets for the suspension of platelets labeled in the presence of 123 $\mu$M [$^{14}C$]5HT and 584 pmol/min/$10^9$ platelets for the platelets labeled in the presence of 1.2 $\mu$M [$^{14}C$]5HT.

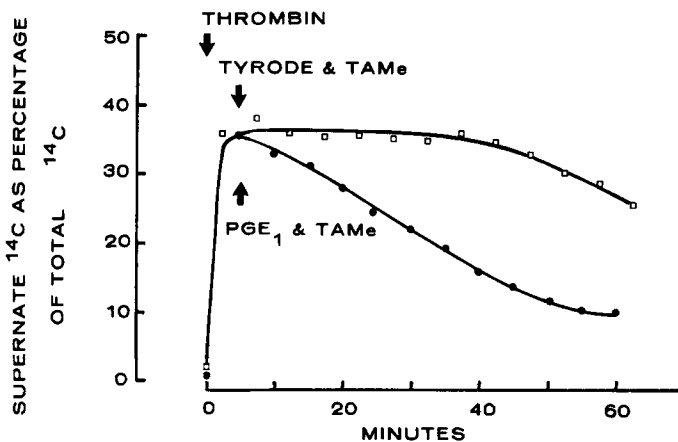The effect of the concentration of thrombin on the ability of platelets to reaccumulate serotonin



FIGURE 1   Comparison of reuptake of [$^{14}C$]5HT by thrombin-treated platelets. Platelet aggregation and release was induced with thrombin (0.05 U/ml). 5 min after the addition of thrombin, either PGE$_1$ (1 $\mu$M) and TAMe (1 mM) or Tyrode solution (control) and TAMe (1 mM) were added to the platelet suspension. The amounts of [$^{14}C$]5HT in the supernates of aliquots of the suspension in which deaggregation had been induced by PGE$_1$ (●——●) and in the control suspension (□——□) were determined.
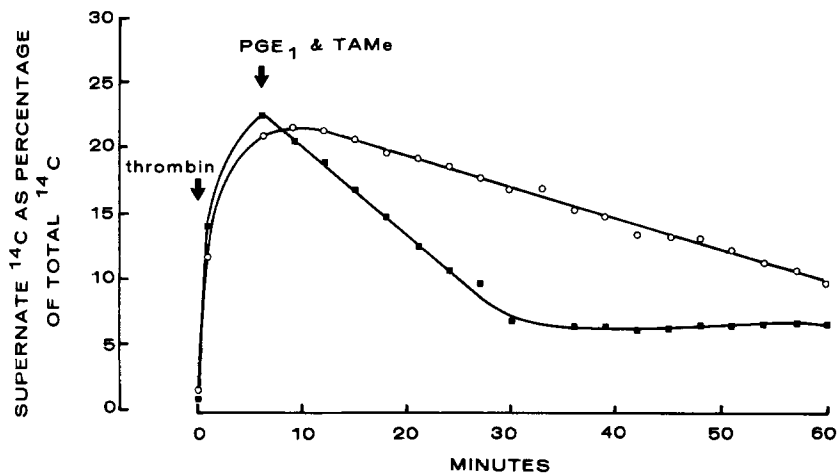


FIGURE 2   Reuptake of [$^{14}C$]5HT after thrombin-induced release of [$^{14}C$]serotonin from rabbit platelets prelabeled with different amounts of serotonin. Platelets were labeled in the presence of either 1.2 $\mu$M [$^{14}C$]5HT (■——■) or 123 $\mu$M [$^{14}C$]5HT (O——O). Platelet aggregation and release were induced in both suspensions with thrombin (0.05 U/ml). After 5 min, PGE$_1$ (1 $\mu$M) and TAMe (1 mM) were added to the suspension to cause platelet deaggregation. The percentages of radioactivity in the supernates from aliquots of the two suspensions were determined.

after undergoing release was also studied. Fig. 3 shows one of three similar experiments in which platelets that had been incubated in the presence of a high concentration of [$^{14}$C]5HT (123 $\mu$M) were stimulated with two different concentrations of thrombin (0.5 and 0.05 U/ml). The percentage of [$^{14}$C]5HT reincorporated per minute into the platelets after deaggregation with PGE$_1$ was identical under these conditions.

## Reaccumulation of Serotonin after Repeated Thrombin-Induced Release Reaction

When platelets were treated with thrombin (0.05 U/ml) three times, their serotonin content fell to about 20% of the initial value (Table I). Upon incubation with serotonin (123 $\mu$M), these platelets were still able to accumulate serotonin against a concentration gradient of approximately 50:1. This was calculated on the assumption that 10$^9$ platelets occupy a volume of approximately 10 $\mu$l and the observation that thrombin-treated platelets contain 56 nmol of serotonin/10$^9$ platelets after they have been incubated with 123 $\mu$M serotonin. The total amount of serotonin that could be accumulated by these thrombin-treated platelets was approximately 40% of the amount that could be accumulated by the untreated control platelets

(Table I). When thrombin-treated platelets were incubated with lower concentrations of serotonin (0.4 $\mu$M), they were able to accumulate serotonin against a concentration gradient of greater than 3,000:1. The total amount of [$^{14}$C]serotonin accumulated in 30 min by thrombin-treated platelets and untreated control platelets was less in the presence of either imipramine (20 $\mu$M) or reserpine (2 $\mu$M) (Fig. 4). Reserpine decreased the apparent initial rate of serotonin uptake by the untreated control platelets but not by thrombin-treated platelets: initial rates of serotonin uptake were similar with both types of platelets. The apparent initial rates of serotonin uptake in the presence of imipramine were similar with thrombin-treated platelets and untreated control platelets (Fig. 4).

## Storage Capacity and Subcellular Compartmentation of Serotonin after the Thrombin-Induced Release Reaction

There are three possible reasons why thrombin-treated platelets do not store as much serotonin as the unstimulated control platelets: (a) thrombin damages the uptake mechanism for serotonin (influx), (b) there is an increased rate of loss (efflux) of serotonin because of increased mem-
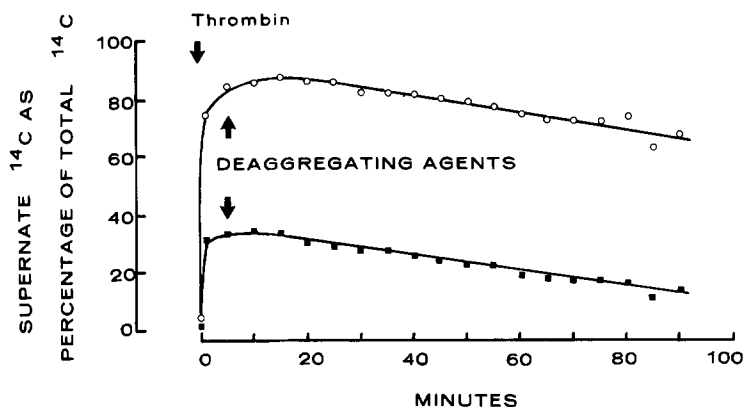


FIGURE 3 Reuptake of [$^{14}$C]5HT by rabbit platelets after induction of the release reaction with two thrombin concentrations. Platelet aggregation and release were induced with either 0.05 U/ml thrombin (■———■) or 0.5 U/ml thrombin (O———O). 5 min after the addition of thrombin, PGE$_1$ (1 $\mu$M), TAMe (1 mM), and plasmin (0.5 mg/ml) were added (at the points indicated by the arrows) to facilitate deaggregation. The percentage of [$^{14}$C]5HT in the supernates of aliquots of the suspensions was measured. Linear regression was employed to determine the fractional rate of reuptake of serotonin from the suspending medium into the platelets. The regression was significant in all three experiments done ($P$ < 0.025, $F$-test after analysis of variance). When the data obtained with the two different thrombin concentrations were compared, it was found that the mean square for departures from parallelism was less in all cases than the estimate of variance of the data. Therefore the fractional rate of reuptake of $^{14}$C was the same, independent of the thrombin concentration used.

| | Control platelets* | Thrombin-treated platelets | Significance of difference between means |
|---|---|---|---|
| | nmol/10⁹ platelets | | |
| Platelet serotonin content before incubation with exogenous serotonin | 74 ± 16 (3) | 15 ± 2 (4) | P <0.02 |
| Platelet serotonin content upon incubation with exogenous serotonin (123 μM) | 132 ± 7 (4) | 56 ± 9 (4) | P <0.001 |

Means and standard error of means. The number of experiments is indicated in parentheses.
* The serotonin content of platelets before any treatment was 74 ± 4 nmol/10⁹ platelets (mean ± SE of 10 experiments).

brane permeability of the platelet plasma membrane, or (c) there is a decreased storage capacity within the platelets after the thrombin-induced release reaction.

These three possibilities were examined in experiments in which the influx of serotonin into the platelets and the efflux of serotonin from the platelets were determined simultaneously. Platelets were equilibrated with [³H]5HT at a concentration of 123 μM in the suspending fluid for 60 min and then resuspended in fresh Tyrode solution containing albumin and apyrase. The suspension was divided into two parts. One part was treated three times with thrombin (0.05 U/ml), and the other part (control) was treated with Tyrode solution instead of thrombin. After the third deaggregation, thrombin-treated platelets and control platelets were again equilibrated with a high concentration of [³H]5HT (123 μM) for 30 min at 37°C. Platelets were recovered by centrifugation and resuspended in Eagle's medium containing albumin (0.35%) and apyrase so that [³H]5HT was essentially only inside the platelets. [¹⁴C]5HT at the same final concentration (123 μM) was added to the suspending medium. Therefore no further net loss or net uptake could occur. However, exchange of serotonin between the surrounding medium and the platelets continued under these conditions (Fig. 5). [¹⁴C]5HT was taken up by the platelets and [³H]5HT accumulated in the suspending medium. This exchange did not occur at 4°C (Fig. 5). All experiments were stopped 50 min after addition of the [¹⁴C]5HT because in the suspension of thrombin-treated platelets the pH

fell below 7.2 (thrombin-treated platelets produce about twice as much lactate per minute under these conditions as unstimulated control platelets [Reimers et al., 1973]).

The data were analyzed using the hypothesis that at equilibrium the amount of serotonin transported per minute from the surrounding medium into the cytoplasm equals the amount of serotonin transported from the cytoplasm into the surrounding medium. Similarly, the amount of serotonin transported per minute across the amine storage organelle membrane is the same in both directions. The results of our analysis of these experiments using [³H]5HT and [¹⁴C]5HT are shown in Tables II and III. The amount of serotonin transported per minute from the surrounding medium into the cytoplasm did not differ significantly in thrombin-treated platelets and in unstimulated control platelets. In contrast, the amount of serotonin transported per minute from the cytoplasm into the amine storage granules in thrombin-treated platelets was approximately 40% of that in the unstimulated control platelets. In addition, it can be seen in Table II that the percentage of the amine storage granule serotonin that was transferred per minute into the cytoplasm was not significantly greater in the thrombin-treated platelets than in the unstimulated control platelets. The percentage of the cytoplasmic serotonin transferred per minute into the surrounding medium was not greater in the thrombin-treated platelets than in the control platelets. The absolute amounts of serotonin in the cytoplasm and in the amine storage granules were calculated from the amount of serotonin in the suspending medium and the fractional turnover rates of serotonin between these compartments for the equilibrium conditions (Table III). The amount of serotonin in the cytoplasm was similar in thrombin-treated platelets and unstimulated control platelets, whereas the serotonin bound in the amine storage granules was reduced by approximately 70-75% in the thrombin-treated platelets.

## Release of Serotonin from Platelets Treated Three Times with Thrombin and Incubated with Serotonin

The difference in the amounts of serotonin held in the storage granules of platelets treated three times with thrombin and control platelets should be reflected in the percentage of serotonin that can be released upon maximal stimulation with thrombin. Whereas after reloading with serotonin (123
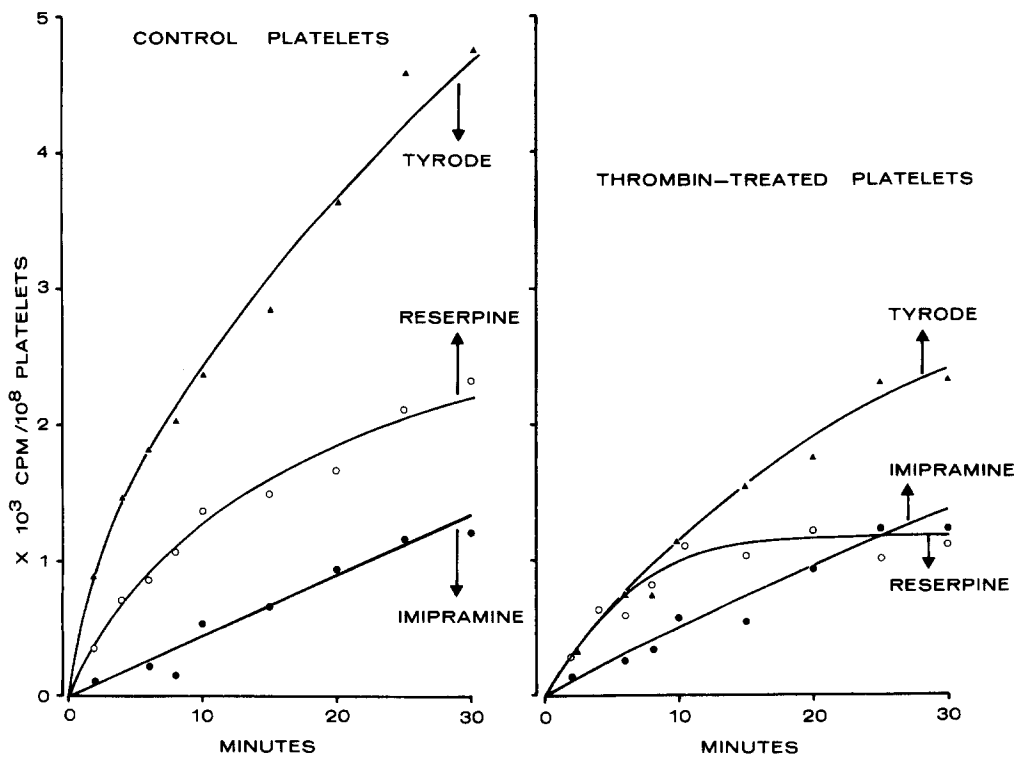
FIGURE 4   Comparison of [¹⁴C]serotonin accumulation by thrombin-treated platelets and untreated control platelets in the presence of reserpine (2 $\mu$M) or imipramine (20 $\mu$M). The [¹⁴C]serotonin concentration in the suspending medium was 123 $\mu$M. Less than 15% of the added radioactivity was accumulated by the platelets in all experiments; thus substrate depletion was negligible.
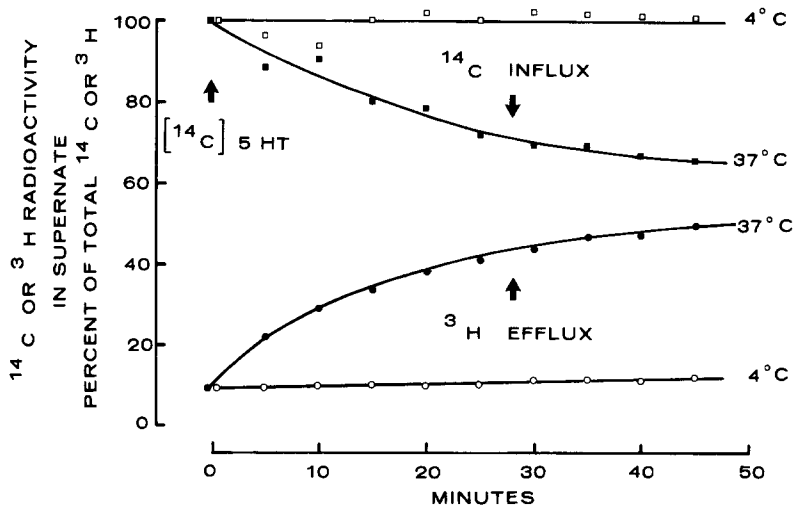


FIGURE 5   Exchange of platelet serotonin ([³H]5HT) with [¹⁴C]5HT in the suspending medium. Platelets had been treated three times with a low concentration of thrombin (0.05 U/ml), deaggregated, resuspended in fresh medium, and equilibrated with [³H]5HT. After equilibration, platelets were resuspended in a fresh medium containing [¹⁴C]5HT at the same concentration. The percentages of radioactivity (³H and ¹⁴C) in the supernates expressed as a percentage of the total ³H or ¹⁴C in the suspension were measured in this experiment at 5-min intervals in aliquots of the two suspensions kept at either 37° or 4°C.

TABLE II

Effect of Thrombin Treatment of Washed Rabbit Platelets on Serotonin Exchange between Different Platelet Compartments and Suspending Medium

| | Control platelets | Thrombin-treated platelets | Significance of difference between means* |
|---|---|---|---|
| Percent of serotonin in the suspending medium transferred into the cytoplasm per minute | 7.0 | 6.8 | $P > 0.05$ (Not significant) |
| Percent of serotonin in the cytoplasm transferred into the suspending medium per minute | 64.8 | 49.7 | $P > 0.05$ (Not significant) |
| Percent of serotonin in the cytoplasm transferred into the amine storage granules per minute | 28.0 | 7.6 | $P < 0.05$ (Significant) |
| Percent of serotonin in the amine storage granules transferred into the cytoplasm per minute | 2.0 | 2.6 | $P > 0.05$ (Not significant) |

Means of three experiments.

* In these studies, serotonin transport has been described by nonlinear equations containing several constants. These constants are highly correlated. Significance of the change in their values upon thrombin treatment was assessed through the following tests: (a) paired t-test, (b) t-test upon the estimates of the values of the constants for each experiment, the covariance matrix for the constants being determined by linearization in the region of the maximum likelihood estimate of the constants, (c) direct determination of 95% joint confidence contours for the estimated values in each experiment.

$\mu$M) the control platelets released 88% of their [$^{14}$C]5HT, thrombin-pretreated platelets released significantly less (68%) of their radioactivity upon incubation with a high thrombin concentration (0.5 U/ml) (significance of difference between mean percentage release for control [88 ± 3] and mean percentage release for thrombin-treated platelets [68 ± 2]: $P < 0.001$, mean of six experiments). On the assumption that the released radioactivity represents the serotonin in the amine storage granules, whereas the retained radioactivity represents primarily cytoplasmic serotonin (and possibly some of its metabolic breakdown products), one can calculate from the data in Table I that the cytoplasmic compartment contains 18 nmol of serotonin/$10^9$ platelets in the case of thrombin-treated platelets and 16 nmol of serotonin/$10^9$ platelets in unstimulated control platelets ($P < 0.7$).

## Morphology of Thrombin-Treated Platelets

Platelets that had been treated three times with a low concentration of thrombin (0.05 U/ml) and untreated control platelets were prepared for electron microscopy. Fig. 6 shows that the thrombin-treated platelets had lost most of their electron-dense (amine storage) organelles and many of their $\alpha$-granules, whereas their mitochondria appeared to be retained.

Thrombin-treated platelets and untreated control platelets were incubated for 45 min with a high concentration of serotonin (123 $\mu$M) and then fixed, stained, sectioned, and examined by electron microscopy. The numbers of electron-dense (amine storage) organelles in sections of platelets before and after exposure to serotonin were compared (Table IV). The control platelets (not treated with thrombin) had an increased number of visible amine storage organelles after they had been exposed to a high concentration of serotonin whereas thrombin-treated platelets showed little increase. It can be calculated from Table IV that the number of visible amine storage organelles in thrombin-treated platelets was reduced by 79% in this experiment.

## DISCUSSION

We have shown previously (Reimers et al., 1973) that thrombin-induced platelet aggregates can be deaggregated by the addition of PGE$_1$ and hirudin or TAMe, even if the platelets have released 30–40% of their amine storage granule contents. In the present experiments, it was observed that the released serotonin was almost completely taken up again by the platelets as they deaggregated. Even after three treatments with low concentrations of thrombin which resulted in loss of 70–80% of the

*Effect of Thrombin Treatment of Washed Rabbit Platelets on Serotonin Storage Capacity and on Serotonin Exchange Rate between Different Platelet Compartments and Suspending Medium*

| | Control platelets | Thrombin-treated platelets | Significance of difference between the means |
|---|---|---|---|
| | *nmol/10⁹ platelets* | | |
| Cytoplasmic compartment | 12 ± 3 | 16 ± 4 | $P < 0.2$ |
| Amine storage granule compartment | 146 ± 10 | 40 ± 6 | $P < 0.01$ |
| | *nmol/min/10⁹ platelets* | | |
| Serotonin exchange rate between cytoplasm and amine storage granules | 2.86 ± 0.52 | 1.06 ± 0.20 | $P < 0.05$ |
| Serotonin exchange rate between suspending medium and cytoplasm | 8.60 ± 3.23 | 8.39 ± 0.52 | $P < 0.95$ |

Means ± SE of means of three experiments; paired *t*-test.

endogenous serotonin, platelets were able to take up serotonin from the surrounding medium.

Degranulation of platelets by thrombin might be expected to decrease the ability of platelets to accumulate serotonin. This is based on the assumption that when platelets have been exposed to thrombin they may have a decreased granule capacity for storing serotonin, or a decreased ability to take up serotonin due to damage of the serotonin uptake mechanism. Alternatively, an increased diffusion of serotonin from the platelets into the surrounding medium could result in a decrease in platelet serotonin.

A fundamental consideration in the design of the experiments to investigate these possibilities is the relationship between serotonin uptake and exchange. When platelet serotonin is in equilibrium with the serotonin in the suspending medium, exchange of serotonin between the platelets and the surrounding medium continues; the rate of exchange is the same as the rate at which serotonin is taken up initially (Born and Gillson, 1959). In experiments in which we studied serotonin transport, the platelets were first incubated with [³H]5HT until no further net uptake occurred. Because these studies were done under equilibrium conditions, it was possible to estimate the trans-

port rates of serotonin across the platelet plasma membrane as well as to estimate the absolute amounts of serotonin in the cytoplasm and the amine storage organelles (Allen, Reimers, Feuerstein, and Mustard, manuscript submitted for publication).

Analysis of the serotonin exchange experiments with thrombin-treated platelets and untreated platelets showed that serotonin transport across the platelet membrane into the cytoplasm was not altered by thrombin treatment. Normal human platelets can take up serotonin against a concentration gradient of more than 1,000:1 (Born and Gillson, 1959). In our experiments, thrombin-stimulated rabbit platelets were also found to take up serotonin against a concentration gradient of more than 1,000:1 (we did not determine the upper limit of the gradient against which serotonin could accumulate). These calculations are based on the assumption that the total platelet serotonin is present in a single compartment, and do not indicate the actual serotonin gradient across the platelet plasma membrane against which serotonin is taken up from the suspending medium. In the experiments reported here, we were able to estimate the serotonin concentration in the intermediate (cytoplasmic) compartment. From these data it can be calculated that, under our experimental conditions, serotonin was transported across the platelet plasma membrane against a gradient of approximately 10:1. Furthermore, serotonin exchange by the thrombin-treated platelets was nearly completely inhibited if they were kept at 4°C. These observations are in keeping with the hypothesis that, at 37°C, serotonin uptake by these platelets is at least in part an active process.

The studies with inhibitors of serotonin accumulation in platelets provided additional evidence that the mechanism and rate of serotonin transport across the plasma membrane of platelets are not altered by thrombin treatment. Imipramine, a drug known to affect serotonin uptake at the plasma membrane (Da Prada and Pletscher, 1968), reduced the apparent initial rate of serotonin accumulation by thrombin-treated platelets and by untreated control platelets; in the presence of imipramine, the apparent initial rates of serotonin uptake were similar. Likewise, the apparent initial rates of serotonin accumulation by thrombin-treated platelets and untreated control platelets were similar in the presence of reserpine, a drug that specifically inhibits the incorporation of serotonin into platelet granules but does not inhibit
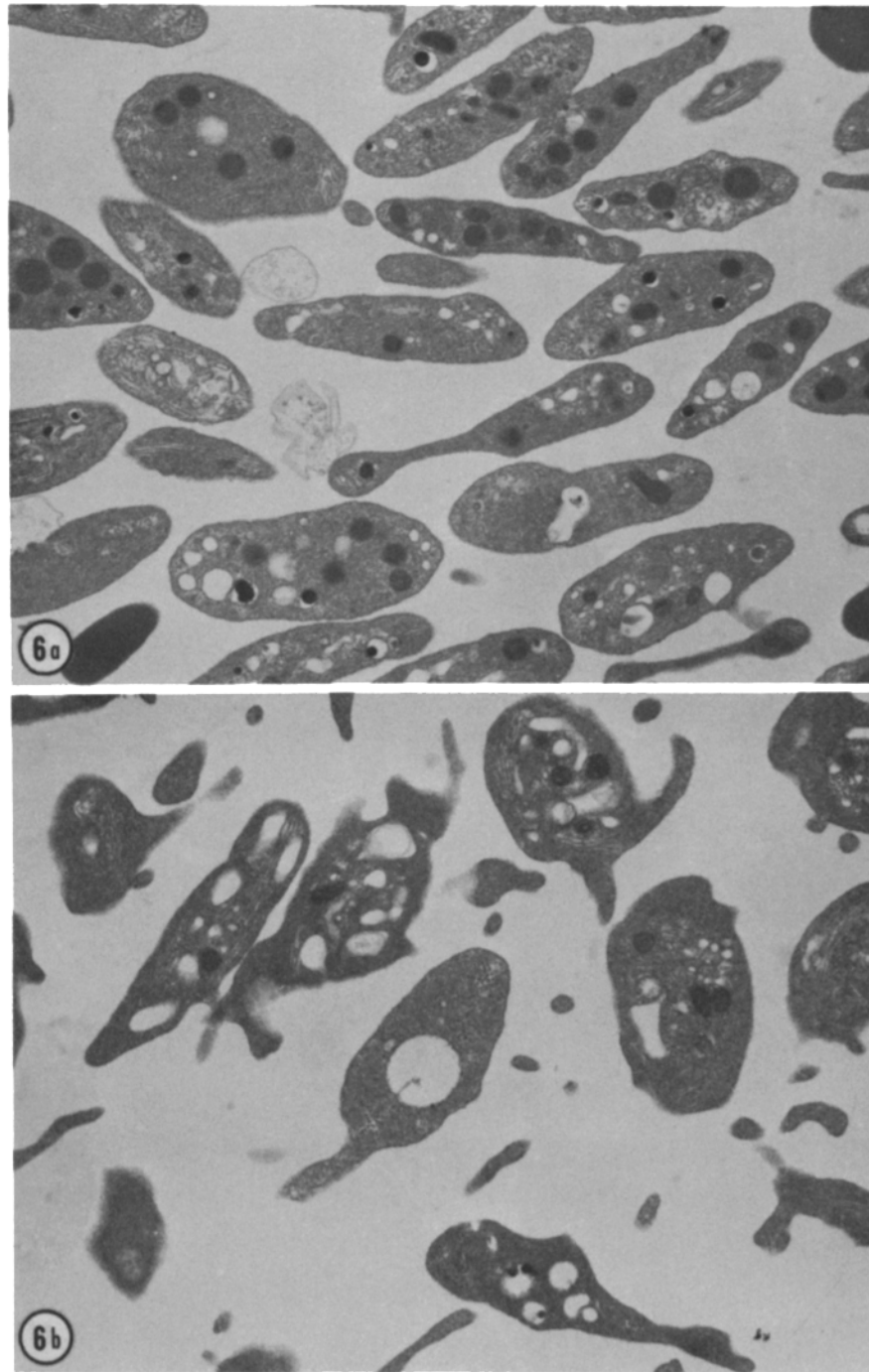
FIGURE 6 Electron micrographs of platelets after exposure to a high concentration (123 μM) of exogenous serotonin: (a) control platelets, (b) platelets that had been treated three times with 0.05 U/ml thrombin before incubation for 45 min in the serotonin-containing medium. (a) × 9,800; (b) × 11,100.

| Electron-dense organelles | Mean number of electron-dense organelles per 100 platelet sections* | |
| | Control platelets | Thrombin-treated platelets‡ |
| --- | --- | --- |
| Before incubation with exogenous serotonin | 101 | 28 |
| After incubation with exogenous serotonin (123 μM) | 159 | 33 |

serotonin transport across the platelet plasma membrane (Da Prada and Pletscher, 1968; Weiss et al., 1974).

Analysis of the data from these experiments showed that thrombin treatment of platelets did not significantly change the fraction of cytoplasmic serotonin which diffused into the suspending medium per unit time. Thus, it appears that thrombin treatment under the conditions of these experiments does not alter the membrane uptake mechanism, nor does it increase the diffusion of serotonin into the suspending medium.

The main reason for the diminished accumulation of serotonin in the thrombin-treated platelets is a decreased transfer rate of serotonin from the cytoplasm into the platelet amine storage organelles. The most likely explanation for this is that, after thrombin-induced degranulation, there are fewer granules or granule-binding sites available for serotonin. Two-thirds of the serotonin storage capacity of the thrombin-treated platelets appeared to be located in the granules because only two-thirds of the serotonin could be released upon the addition of a high concentration of thrombin. The results from the serotonin exchange experiments using thrombin-treated platelets also indicated that, under the present experimental conditions, about one-third of the serotonin is in the small compartment (cytoplasm) and two-thirds in the large compartment (amine storage granules). These results do not imply that there is this much serotonin present in the platelet cyto-

plasm under physiological conditions in vivo. In the experiments reported here, high serotonin concentrations were present in the suspending medium (123 μM). The high serotonin concentration was chosen to ensure that there be no appreciable net movement of serotonin across the platelet plasma membrane and to minimize the (relative) formation of serotonin breakdown products during the exchange experiments (see Materials and Methods). These conditions were essential for later analysis. There is no doubt that the concentration of serotonin in the platelet cytoplasm will be lower under physiological conditions because the concentration of serotonin in the blood plasma is extremely low and a different equilibrium situation between cytoplasmic serotonin and serotonin in the blood plasma will be established. In addition, it should be pointed out that the cytoplasmic serotonin pool probably does *not* reflect an alternative serotonin storage compartment since small amounts of serotonin are relatively rapidly metabolized by thrombin-treated ("degranulated") platelets (see Materials and Methods) or by unstimulated platelets in the presence of reserpine (Pletscher, 1968). However, the high concentrations of serotonin used in these experiments allowed us to estimate the maximum transport rates of serotonin between the different compartments which would otherwise be impossible with presently available techniques.

Analysis of the data from the serotonin exchange experiments indicated that the serotonin capacity of the larger platelet pool (granule pool) was reduced by approximately 73% upon thrombin treatment. Electron micrographs showed that the number of electron-dense granules (amine storage organelles) in sections of thrombin-treated platelets was reduced by approximately 79% when compared with the untreated control platelets.

It is possible that with thrombin-treated platelets some of the granule membranes are reutilized but that the binding affinity for serotonin may be diminished because of the lack of ATP with which serotonin is thought to form complexes (Berneis et al., 1971). This hypothesis of partial reutilization of amine storage granule membranes could explain the observations in the present study that some uptake of [$^{14}$C]5HT occurred after the platelets had been exposed to a high concentration of thrombin. This reuptake of [$^{14}$C]5HT took place after thrombin stimulation even if the platelets had been incubated with a high concentration of serotonin before stimulation. "Preloading" the plate-

lets with serotonin was intended to reduce reuptake into amine storage granules which had not discharged contents upon thrombin stimulation.

We have previously shown that platelets which have been repeatedly stimulated with thrombin survive in the circulation for the same length of time after reinfusion as platelets which have not been subjected to thrombin treatment. We have also obtained evidence (Reimers et al., 1974) that cytoplasmic ATP can be transferred into the amine storage granules and that exchange between cytoplasmic and granule ATP is complete within 24 h. Taken together, the results raise the possibility that amine storage granules or granule contents may be reconstituted in vivo after the release reaction.

The mechanism by which serotonin is handled by these thrombin-treated platelets appears to be similar to that described for platelets from some patients with albinism or storage pool disease (Hardisty and Mills, 1972; Weiss et al., 1974). It is possible that some conditions of acquired storage pool disease may represent the consequence of in vivo release reactions. There has been a report indicating that this does occur in man (Zahavi and Marder, 1974).

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