Subcellular Localization of $^{111}$In in Human and Rabbit Platelets


In order to examine the subcellular distribution of $^{111}$In in $^{111}$In-oxine-labeled human and rabbit platelets, we employed a hypothetical grain technique of EM autoradiograph analysis. The results indicated that in the rabbit $^{111}$In was concentrated within the platelet dense bodies, particularly when the platelets had been labeled in a plasma-free system. Under comparable conditions of labeling, human platelets appeared to accumulate almost all the radiolabel within the cytosol. Using inhibitors of 5-hydroxytryptamine (5-HT) uptake, i.e., cimomipramine, ouabain, sodium fluoride, p-chloromercuribenzoate, and reserpine, we were unable to demonstrate an active uptake process in either species. Both collagen and thrombin were able to cause dose-dependent release of radioactivity from the labeled rabbit platelets only. In the case of collagen, this mimicked endogenous 5-HT release and was inhibited by indomethacin. These results and their implications are discussed.

RECENTLY it was suggested by Thakur et al. that $^{111}$In, when complexed with 8-hydroxyquinoline (oxine), could replace $^{51}$Cr as a platelet label, which up to now has been recommended as the only satisfactory agent currently available for platelet studies in man by the International Panel on Diagnostic Applications of Radioisotopes in Haematology. The physical characteristics of $^{111}$In have the advantages over $^{51}$Cr of a shorter half-life and of gamma photons that are well suited to external imaging. Furthermore, the high efficiency of the $^{111}$In labeling procedure means that a much smaller volume of blood is required for labeling than in the case of $^{51}$Cr.

The lifespan of $^{111}$In-labeled platelets was found to be comparable with $^{51}$Cr-labeled platelets in animals and man, while the recovery of injected $^{111}$In-labeled platelets was found to be greater. Using the imaging properties of $^{111}$In, $^{111}$In-labeled platelets have been used to detect experimentally induced vascular lesions and experimental infective endocarditis in animals and to locate vascular thrombi in man. The use of $^{111}$In-oxine has been extended to labeling of other circulating blood cells, including granulocytes for kinetic studies, leukocytes for kinetic studies and abscess localization, and lymphocytes for lymph node scanning.

It was decided to investigate the usefulness of $^{111}$In-oxine-labeled rabbit platelets as a means of evaluating the effect of platelet-active drugs in an animal model. However, it was essential to establish the subcellular location of the nuclide in case retention within the platelet was affected by the platelet secretory process. The evidence so far available indicated that the $^{111}$In is bound to the cytoplasm, since ADP, thrombin, and collagen failed to elicit its release from the internal organelles of labeled human platelets, and trypsinization showed that the label was not surface bound.

A direct method of establishing the subcellular location of $^{111}$In was required that could provide quantification of the results. A possible solution was to utilize the soft Auger electrons, which are produced during the radioactive decay of $^{111}$In in conjunction with analytical electron microscopic (EM) autoradiography. This report describes the use of a hypothetical grain analysis that permitted the study of subcellular localization of radioactivity in intact human and rabbit platelets, since the method takes into account the range distribution of Auger electrons.

The hypothetical grain analysis predicted that in rabbit platelets the radiolabel would be largely secretable. The use of complimentary biochemical techniques to test this prediction allowed a rare opportunity to verify data and conclusions obtained from EM autoradiographs.

MATERIALS AND METHODS

Preparation of $^{111}$In-Oxine Complex for Labeling Rabbit Platelets

The complex was prepared by a modification of the method of Thakur et al. $^{111}$InCl$_3$ was obtained in solution in 0.04 M HCl from the Radiochemical Centre, Amersham, U.K. at a concentration of 10 mCi/ml (specific activity of $^{111}$InCl$_3$, 5 Ci/mg). This solution (100 µl) was added to 1 ml of water containing 50 µl of a solution of oxine in ethanol (1 mg/ml) and 200 µl of 0.3 M acetic acid buffer, pH 5. After mixing the solutions, 75% of the radioactivity was extracted as oxine into 2 ml of chloroform. The chloroform solution was evaporated to dryness under a stream of nitrogen; 100 µl of ethanol was then added and the evaporation repeated to remove the last traces of chloroform. Finally, the $^{111}$In-oxine was redissolved in 50 µl of ethanol.

Rabbit Platelet Isolation and Labeling in Plasma

New Zealand white rabbits were bled through a carotid cannula into 3.8% trisodium citrate (1 part citrate: 9 parts blood), and...
platelet isolation was carried out by the method of Butler et al. The labeling was performed using a final $^{111}$In-oxine concentration in the platelet suspension of 100 $\mu$Ci/ml. Two different platelet suspensions were used for labeling: concentrated (8 x $10^8$ ml) and dilute (0.3 x $10^9$/ml).

**Rabbit Platelet Isolation and Labeling in Calcium-Free Tyrode (CFT)**

Conditions for the maximal efficiency of platelet labeling with $^{111}$In-oxine were established using a range of concentrations from 0.22 to 4.42 $\mu$Ci/ml (containing 0.04-0.88 ng/ml) and the time course of uptake determined using a range of time points from 30 sec to 10 min.

Platelet isolation was as described by Baker and Pay. Washed platelets were routinely labeled using a 10 min 25°C incubation at a final $^{111}$In-oxine concentration of 0.44 $\mu$Ci/ml (containing 0.09 ng/ml). This method of platelet isolation and labeling is essentially the same as the method described above for plasma except that platelets are resuspended in CFT.

**Preparation of $^{111}$In-Labeled Human Platelets**

The method used was the optimal method described by Hawker et al., using the $^{111}$In-oxine solution supplied by the Radiochemical Centre, Amersham, U.K. for cell labeling.

**Inhibition of Uptake Experiments**

Quantities of 0—100 $\mu$M ouabain, sodium fluoride, clomipramine, and p-chloromercuribenzoate were added to the suspension of washed platelets 5 min before the addition of the $^{111}$In-oxine. In separate experiments, reserpine and 5-HT were added 30 min before the radioactivity and the platelets then incubated at 37°C.

**Release Experiments**

Bovine thrombin was purchased from Sigma and Horm collagen from Hormon-Chemie, München GMBH. All aggregations were performed in a Payton aggregometer at 37°C. All platelet harvesting after labeling and/or aggregation was carried out in an Eppendorf 5412 fixed speed centrifuge.

**Measurement of Endogenous 5-HT**

This was carried out using the fluorometric automated assay of Hardeman et al., using the $^{111}$In-oxine solution supplied by the Radiochemical Centre, Amersham, U.K. for cell labeling.

**Gamma Counting**

Half milliliter aliquots of all solutions and platelet pellets from 0.5 ml of suspension were counted using a Beckman Biogamma gamma counter.

**Preparation of Platelets for Electron Microscopy**

Since the half-life of $^{111}$In is 68 hr, the preparation schedule has to be carried out quickly. Approximately 2 ml of rabbit platelet suspension was added to 4 ml of 2.5% glutaraldehyde in 0.1 $M$ cacodylate buffer (pH 7.2) and left at room temperature for 5 min. The samples were then centrifuged at 300 g for 20 min. In the case of human platelets, the concentration of glutaraldehyde was 0.5%. Coulter counting of the suspension before centrifugation and the supernatant afterwards showed that 85% of platelets were harvested. The supernatant was discarded and the pellet resuspended in a minimum volume of bovine serum albumin and rapidly gelled with glutaraldehyde according to the method of Bullock and Christian, but instead of siliconized slides, coverslips in Coplin jars were used for further processing. Postfixation was in a mixture of 50/50 osmium tetroxide (1%) and glutaraldehyde (2.5%) in 0.1 $M$ cacodylate for 30 min. The specimens were then rinsed in 0.1 $M$ cacodylate for 2 min and dehydrated in a graded ethanol series starting at 70%—each change being of 15 min duration. Infiltration was with 50/50 propylene oxide/Epon-Araldite for 2 hr and then Epon-Araldite alone at room temperature for 4 hr. The resin was polymerised at 70°C for 16.5 hr.

**Preparation of Specimens for Electron Microscopic Autoradiography**

The methods used to prepare electron microscopic autoradiographs has been described previously. In the present study, however, exposure was for 2 wk and the specimens were developed with Kodak D19. When rabbit platelets washed in CFT (i.e., those essentially free of plasma proteins) were labeled with $^{111}$In-oxine at a concentration of 0.3 x $10^9$/ml and autoradiographs prepared with the same developer (D19), the density over almost all platelets was so great as to make interpretation and analysis impossible. The use of Microdot X at 10°C, which at 20°C is between 2 and 3 times less efficient than D19, gave autoradiographs of the type illustrated in Fig. 2, which were used for analysis. The final print magnification obtained was x25,000.

**Analysis of EM Autoradiographs**

The technique applied was a hypothetical grain analysis that uses a computer program to compare hypothetical grain distributions with the real grain distribution. In this case, uniformly distributed hypothetical sources of activity were established by applying to the autoradiographs an overlay screen with computer-predicted distances that generate the hypothetical silver grains (sites) emanating from these sources. The directions of these hypothetical decays are, of course, random. The computer-predicted distances are based on experimentally measured range-distribution values of the type first reported by Salpeter for a source of tritium. This technique allocates estimates of activity to organelles, taking “crossfire” of the decay particles into account. The activity estimates derive from systematic modification of the hypothetical source values by a minimising sub-routine until the hypothetical and real grain distributions fit optimally as assessed by the chi-square test.

The overlay screen used to plot hypothetical grains was produced for $^{35}$S, whose range distribution properties are almost identical to those of $^{111}$In. The number of micrographs analyzed for rabbit platelets varied from 33 to 137, depending on the labeling density in a given experiment. The numbers of hypothetical and real grains used were between 1022 and 1100, and 577 and 694, respectively. For human platelets the number of micrographs analyzed was 26 and the number of hypothetical and real grains were 2245 and 728, respectively.

**RESULTS**

**Grain Density of Autoradiographs**

When rabbit platelets concentrated in plasma (8 x $10^9$/ml) were incubated with $^{111}$In-oxine, radioactivity, as represented by silver grains, appeared in 35% of platelet profiles when selected randomly (Fig. 1). When the concentration of cells was lower (0.3 x $10^9$/ml), the radioactivity was associated with only 17% of platelets. However, the labeled platelets in
Fig. 2. EM autoradiograph of rabbit platelets incubated in calcium-free Tyrode solution (CFT) with $^{111}$In-oxine. Development was with the less efficient Microdol X at 1°C to reduce grain density to acceptable levels. Most platelets appear labeled and many silver grains are apparently associated with dense bodies. (x20,000)

these experiments were so infrequent that fields for photography had to be selected. Hence the figure of 17% is an overestimate and not strictly comparable with the other figure of 35%.

Human platelets labeled in a plasma-free acid citrated buffer (Hawker et al.20) were well labeled, but autoradiographs required no modification of development (Fig. 1). As already mentioned, the platelets labeled in C.F.T. produced autoradiographs with a high grain density, even after low efficiency development (Fig. 2).

Fig. 3. EM autoradiograph of human platelets incubated in a plasma-free buffer with $^{111}$In-oxine; the platelets are well labeled. An obvious feature is the smaller number of dense bodies that can be seen in rabbit platelets. Development with D19 at 20°C. (x20,000) ag, alpha-granules; db, dense bodies; m, mitochondria; mt, microtubules; scs, surface connected system (may include some dense tubular system).

Hypothetical Grain Analysis

The potential sources of radioactivity investigated in this analysis were cytoplasm, surface connected system (SCS), alpha granules, dense bodies, and mitochondria. The absence of certain platelet organelles from Tables 1-4 reflects allocation of zero activity to these sources in order to minimize the chi-square value between hypothetical and real grain distributions, and thus to establish the “best fit” between these distributions. “Grains per grid point” is a measure of specific radioactivity and indicates the relative abilities of subcellular compartments to concentrate radioactivity, whereas “relative activity” indicates the percentage of total label associated with a compartment.

Rabbit platelet plasma concentrate ($8 \times 10^8$/ml) incubated with $^{111}$In-oxine gave approximately 70% labeling of cytoplasm, with 17% of the label associated with the dense bodies (Table 1). In terms of relative specific activity, however, the concentration of label in dense bodies was 10 times greater than that in cyto-

Table 1. Hypothetical Grain Analysis of EM Autoradiographs of Rabbit Platelets ($8 \times 10^8$/ml) Incubated With $^{111}$In-Oxine in Plasma

<table>
<thead>
<tr>
<th>Source</th>
<th>Specific Radioactivity as Grains/Grid Point</th>
<th>Percentage of Total Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>0.54 ± 0.06</td>
<td>70.9 ± 8.0</td>
</tr>
<tr>
<td>Surface connected system</td>
<td>0.30 ± 0.31</td>
<td>6.7 ± 6.7</td>
</tr>
<tr>
<td>alpha-Granules</td>
<td>0.51 ± 0.41</td>
<td>5.4 ± 4.1</td>
</tr>
<tr>
<td>Dense bodies</td>
<td>5.70 ± 2.37</td>
<td>17.1 ± 4.9</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.000 ± 0.30</td>
<td>0.0 ± 0.5</td>
</tr>
</tbody>
</table>

Chi-square = 1.6; Df = 3; p > 0.50.
plasma. Reducing the platelet concentration to 0.3 x 10^9/ml gave similar results (Table 2), with 82% of the label in the cytoplasm but a relatively lower concentration of the label in the dense bodies.

When rabbit platelets were washed in CFT prior to labeling with \(^{111}\)In-oxine, the largest portion of radioactivity (circa, 45%) was associated with dense bodies, while the cytoplasm appeared to be unlabeled to any significant extent (Table 3). A further 30% of radioactivity was located in the SCS, although this was still 5 times less concentrated than that in the dense bodies. This may represent a strongly bound plasma residue of label that remained after the washing procedure.

Washed human platelets appear to retain by far the majority of label (ca, 79%) within the cytosol and a nonsignificant 2% within the dense bodies (Table 4). In fact, when the 12.7% of activity in the matrix is excluded, the proportion attributable to the cytosol is over 90%.

**Labeling Parameters for Rabbit Platelets**

The curves in Fig. 4 show that rabbit platelets can become saturated with \(^{111}\)In-oxine at concentrations in excess of 0.6 ng/ml (3 μCi/ml). In order to achieve high labeling efficiencies, it was necessary to use concentrations of less than 0.2 ng/ml (1 μCi/ml).

Platelet labeling was very rapid initially, being maximal after 5~10 min at 25°C (Fig. 5).

**Inhibition of Labeling in Rabbit Platelets**

Various attempts were made to inhibit \(^{111}\)In-oxine uptake by platelets using the following compounds, clomipramine, p-chloromercuribenzoate, ouabain, and sodium fluoride each in concentrations from 0 to 100 μM. The results showed that none of these had
any effect, and high labeling efficiencies were still achieved even using very high concentrations of these inhibitors.

In another experiment rabbit platelets were preloaded with 5-hydroxytryptamine (5-HT) prior to \(^{111}\)In labeling. The conditions used were 0.5 \(\mu M\) 5-HT for 30 min at 37\(^\circ\)C. In this case control platelets (also preincubated but without 5-HT) accumulated 90.7\% of the radiolabel (\(n = 2\)), whereas the 5-HT preloaded platelets accumulated 91.4\% (\(n = 2\)) showing that 5-HT labeling had no effect on the efficiency of \(^{111}\)In uptake into platelets. Analogous experiments were performed using reserpine. Again various concentrations from 1 to 14 \(\mu M\) had no effect on platelet \(^{111}\)In-oxine uptake.

When 6\% bovine serum albumin (BSA) was included in the labeling medium (CFT), the labeling efficiency was reduced from 81.4\% (control) to 12.3\% (\(n = 2\)). EDTA (2 \(mM\)) reduced the labeling efficiency in rabbit platelets by 90\% (IC\(_{50}\) = 0.3 \(mM\)).

**Release Characteristics of \(^{111}\)In in Rabbit Platelets**

When rabbit platelets were labeled in CFT and treated with thrombin, using a 5 min incubation at 37\(^\circ\)C, a small dose-dependent release of radioactivity without any dose-dependent release of lactate dehydrogenase (LDH) activity occurred (Table 5). However, when the CFT-labeled platelets were chilled in ice, to aid resuspension, harvested by centrifugation at 15,000 \(g\) for 30 sec, and then resuspended in CFT containing 6\% BSA prior to the addition of thrombin, an apparently much larger dose-dependent release of radioactivity was seen (Table 6). Whereas the radioactivity was released in both cases (i.e., both CFT and CFT + 6\% BSA containing media), only the media containing the BSA was able to retain the \(^{111}\)In-oxine outside the cell by binding of the label and therefore preventing reuptake. Again no dose-dependent LDH release occurred. These results show that thrombin can cause rabbit platelets to secrete \(^{111}\)In label without lysis of the plasma membrane. We also showed that rabbit platelets suspended in CFT containing 6\% BSA, when allowed to stand at room temperature, slowly lose radioactivity into the milieu at a rate of approximately 3\% per hour. This effect was not observed with human platelets.

In further experiments platelets labeled in CFT and

<table>
<thead>
<tr>
<th>Thrombin Dose (U/ml)</th>
<th>Percent Total Added Radioactivity</th>
<th>Percent LDH Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant (CFT)</td>
<td>Platelet Pellet</td>
</tr>
<tr>
<td>5.0</td>
<td>28.0</td>
<td>72.0</td>
</tr>
<tr>
<td>2.5</td>
<td>24.1</td>
<td>75.9</td>
</tr>
<tr>
<td>1.0</td>
<td>21.2</td>
<td>78.8</td>
</tr>
<tr>
<td>0.5</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>0</td>
<td>12.8</td>
<td>87.2</td>
</tr>
</tbody>
</table>

Results are expressed as mean of duplicates.

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**Table 5. The Release of \(^{111}\)In and Lactate Dehydrogenase (LDH) From Labeled Platelets by Thrombin**
Results are expressed as mean of duplicates.

Table 6. Effect of the Presence of 6% Bovine Serum Albumin in CFT on Thrombin-Induced $^{111}$In and LDH Release

<table>
<thead>
<tr>
<th>Thrombin Dose (U/ml)</th>
<th>First Supernatant</th>
<th>Second Supernatant</th>
<th>Platelet Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Added Radioactivity</td>
<td>Percent Total Added Radioactivity</td>
<td>Percent Added Radioactivity</td>
</tr>
<tr>
<td>5</td>
<td>14.3</td>
<td>52.8</td>
<td>32.8</td>
</tr>
<tr>
<td>2.5</td>
<td>16.6</td>
<td>43.5</td>
<td>30.0</td>
</tr>
<tr>
<td>1.0</td>
<td>18.8</td>
<td>41.0</td>
<td>30.6</td>
</tr>
<tr>
<td>0.5</td>
<td>19.8</td>
<td>35.9</td>
<td>48.3</td>
</tr>
<tr>
<td>0</td>
<td>10.3</td>
<td>7.0</td>
<td>82.7</td>
</tr>
</tbody>
</table>

Table 7. Effect of Indomethacin on Collagen-Induced 5-HT and $^{111}$In Release Into the Media From Platelets

<table>
<thead>
<tr>
<th>Collagen Concentration (µg/ml)</th>
<th>Control Percent Total Radioactivity</th>
<th>Indomethacin Treated Percent Total Radioactivity</th>
<th>5 µg/ml</th>
<th>10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5-HT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>111In</td>
<td>5-HT</td>
<td>111In</td>
</tr>
<tr>
<td>0</td>
<td>2.1</td>
<td>0.5</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>1.25</td>
<td>6.6</td>
<td>7.3</td>
<td>4.3</td>
<td>3.6</td>
</tr>
<tr>
<td>2.5</td>
<td>60.9</td>
<td>59.8</td>
<td>33.6</td>
<td>37.2</td>
</tr>
<tr>
<td>5.0</td>
<td>66.4</td>
<td>61.4</td>
<td>34.5</td>
<td>36.7</td>
</tr>
</tbody>
</table>

Results are expressed as mean of duplicate.

ND, not determined.

subsequently resuspended in CFT containing 6% BSA were incubated in a Payton aggregometer with various concentrations of collagen. After the completion of aggregation, the samples were decanted and centrifuged for 2 min at 15,000 g and the supernatants analyzed for both radioactivity and secreted endogenous 5-HT. Controls were carried out using platelets in CFT with no collagen present. Table 7 shows that collagen induced a dose-dependent release of both the $^{111}$In and 5-HT in rabbit platelets. Also shown are the results of experiments carried out in the presence of indomethacin (added 2 min prior to the collagen), which inhibited both the secretion of 5-HT and radioactivity.

In another experiment using collagen aggregation, the time course for $^{111}$In and endogenous 5-HT secretion was examined. Samples of labeled platelets resuspended in CFT containing 6% BSA were placed in an aggregometer. An intermediate dose of collagen (1.5 µg/ml) that gave a slow aggregation was added. This dose of collagen gave a submaximal release of 5-HT (25%). At various points during the aggregation, aliquots were removed and the supernatants prepared and analyzed as described above. The results, which are summarized in Fig. 6, show that $^{111}$In and 5-HT secretion follow a similar time course.

The ability of released radioactivity to relabel control platelets was also investigated. Labeled platelets in CFT were stimulated to release radioactivity with 5 µ/ml thrombin. After centrifugation, the supernatant or releasate was heated at 56°C for 45 min in order to destroy the thrombin activity. This releasate was then used to resuspend unlabeled control platelets that were incubated for 5 min at 25°C. As a control, CFT containing $^{111}$In-oxine was also heated and used to resuspend unlabeled control platelets prior to incubation for 5 min at 25°C.

These platelets were harvested, and it was found that 65.5% of the radioactivity in the releasate was present in the platelet pellet. In the controls this value was 77.5%. This demonstrates that the $^{111}$In released by thrombin from labeled rabbit platelets was capable of reabsorption.

In addition to the experiments described above, the rabbit platelets isolated in the normal way using CFT were labeled using the commercially available aqueous preparation of the complex. The labeling characteristics and subsequently the release of the label by thrombin were found to be the same as for those platelets labeled by the ethanolic $^{111}$In-oxine solution. Furthermore, Hawker (personal communication) has shown that using either the ethanolic or aqueous solution makes no difference to the uptake and release of $^{111}$In-oxine by human platelets.

Uptake and Release of $^{111}$In-Oxine by Human Platelets

As with the rabbit platelets, cloimipramine, reserpine, ouabain, sodium fluoride and para-chloromercuribenzoate were unable to inhibit the accumulation of $^{111}$In-oxine in human platelets. Only those compounds that bound the $^{111}$In extracellularly, i.e., BSA or EDTA, prevented the uptake. Lowering the temperature also reduced the rate of uptake. Neither collagen nor thrombin (5 µg/ml or 5 U/ml, respectively) were
able to release more than 4% of the label accumulated by the platelets.

**DISCUSSION**

The reason for investigating \(^{111}\)In-oxine platelet labeling was to evaluate the technique for studies of platelet survival and distribution in animal models. Goodwin et al.\(^9\) have demonstrated that autologous human platelets labeled with \(^{111}\)In in plasma when subsequently reinfused into patients show an average recovery of more than 50% as compared with only 5% when platelets were labeled in saline. However, more recently, Hawker et al.\(^2\) using a plasma-free labeling technique that minimizes the period for which the human platelets were deprived of plasma, reported a mean recovery of 69% in healthy subjects. Consequently, platelets labeled in both plasma and plasma-free media were investigated in the present study. The frequent observation that platelets are labeled more efficiently by \(^{111}\)In-oxine in protein-free media is reflected in the grain densities achieved in our EM autoradiographs. This was particularly striking in platelets labeled in CFT, where the grain density was so overwhelming as to necessitate reduction of development efficiency in order to allocate grains to structures for analysis.

A notable feature of platelets labeled in PRP was the very small number in which silver grains were seen. Those that were labeled, moreover, appeared to possess several grains per platelets. The explanation for this is unclear and may be related to platelet age.

When compared with the total activity lost following treatment of rabbit platelets with thrombin (Table 5), there would seem to be a short-fall in the combined \(\alpha\)-granule and dense body percentage revealed by autoradiography (Table 3). Previous observations on fixation of platelets\(^9\) have shown that aldehydes cause loss of 5-HT in the region of 10% from platelets previously labeled with \(^3\)H-5-HT creatinine sulphate. The present results show that \(^{111}\)In may be released from platelets in a manner similar to 5-HT secretion, and hence, we would suggest that the aldehydic fixatives could cause some specific release of radiolabel during EM processing resulting in an underestimate of dense body related radioactivity.

An equally plausible explanation is that at least some of the 31% radioactivity ascribed to the surface connected system may be available to the supernatant during thrombin-induced aggregation.

That intracellular \(^{111}\)In is primarily bound to cytoplasm has been reported for human platelets by Joist et al.\(^8\) and Hawker et al.\(^1\) and for human neutrophils by Thakur et al.\(^1\) The three conditions of labeling used in this study revealed a variable tendency of rabbit platelet dense bodies to accumulate radioactivity to concentrations considerably above those present in the cytoplasm. The ratio of dense body to cytoplasmic concentration of radiolabel was greatest in the case
of the washed platelets, the percentage of total radioactivity in cytoplasm was much greater when platelets were labeled in plasma. However, the observation that the overall labeling density in washed platelets was greater than in the plasma experiments means that the washed platelets had a far higher absolute specific radioactivity in the cytoplasm. This would appear to be an essential requirement prior to further uptake by the dense bodies within the cytoplasm.

Since rabbit platelets are able to absorb over 70% of added radioactivity at concentrations below 0.2 ng/ml (1 μCi/ml), it would appear that 111In, once inside the platelet, is no longer free to diffuse across the plasma membrane. Since the efficiency of labeling decreases with increasing exogenous 111In-oxine concentration, however, the platelets appear to have a limited capacity to absorb 111In-oxine. The rapid rate of uptake of radioactivity is presumably a consequence of the high lipophilicity of the 111In-oxine complex and the temperature dependence is consistent with this (see also Joist et al.28). In human platelets, it was suggested that temperature dependence is evidence of an active uptake mechanism. Nevertheless, in the present study we have examined several inhibitors of 5-HT uptake and metabolic function and found that none had any influence on the efficiency of labeling in either species. We therefore concluded that 111In labeling of washed platelets is an entirely passive process.

It is known, however, that dense body content of rabbit platelets is greater than that of other species, including human.29 Therefore, in a species that contains a higher dense body to cytoplasm ratio, it is not unreasonable to expect a concentration of 111In by dense bodies to be more conspicuous.

Reserpine has been shown to inhibit platelet 5-HT accumulation30,31 by inhibiting active transport at the "granule" membrane site. This drug, however, had no effect on 111In uptake into dense bodies, presumably because such accumulation also is not an active process. Furthermore, preloading of platelets with 5-HT had no effect on labeling efficiency, it would thus appear unlikely that the 111In-oxine competes for the amine storage sites.

The only agents studied that inhibited uptake of label were BSA and EDTA, which act by binding the 111In therefore rendering it unavailable for platelet labeling. Similar observations were made by Joist et al.28 using BSA and human serum. This binding to serum proteins (e.g., albumin, transferrin) is probably the major cause of low efficiency of labeling when incubations are carried out in plasma.

The small thrombin dose-dependent release of radioactivity from platelets suspended in CFT indicated that 111In could be released from rabbit platelets that had been labeled with high efficiency. This apparent release was greater in the presence of BSA with no apparent loss of the specific cytoplasmic marker lactate dehydrogenase. Since it is known that BSA binds 111In, we conclude that its presence prevents reuptake of the label. Some of the radioactivity released into CFT was capable of labeling further washed platelets and may possibly be available in the form of the oxine complex.

Collagen also causes 111In release from rabbit platelets, which parallels in kinetics and magnitude the release of endogenous 5-HT (Fig. 6). In addition, since indomethacin inhibits the release of 111In as well as 5-HT, this is further evidence that 111In is associated with the dense bodies and will undergo secretion.

It is noteworthy that the order of thrombin-induced radioactivity loss from washed rabbit platelets (> 50%) is in accordance with that predicted by the autoradiographic analysis. Similarly, for human platelets, less than 10% of intracellular activity was shown to be available for agonist-induced secretion by autoradiography, a result confirmed by challenge with collagen and thrombin.

In conclusion, we have shown autoradiographically and biochemically that washed rabbit platelets accumulate 111In significantly in dense bodies and to a lesser extent in α-granules by an uptake process that appears to be entirely passive, although the radiolabel can be released by active secretion. However, using the same technique, human platelets have been shown to accumulate in the cytosol most of the radiolabel, which is demonstrably resistant to active secretion. This result confirms the current view20 that human platelets labeled with 111In-oxine are suitable for survival studies but that in the rabbit at least, an alternative radiolabel must be used. Nevertheless, the rabbit model may offer an advantage in investigations (in vitro and in vivo) of the platelet secretion reaction.

First, this advantage is technical, permitting gamma counting instead of the more involved scintillation techniques required by 5-HT labeled with beta-emitting isotopes. Second, 111In has theoretical benefits since the presence of suitable protein(s) outside the platelet prevents its reuptake, while the use of 5-HT in secretion studies permits no such control.

ACKNOWLEDGMENT

We would like to express our thanks to I. H. M. Williamson and Dr. G. R. Bullock of these laboratories for assistance with preparation of the autoradiographs; to Dr. N. M. Blackett of the Institute of Cancer Research, Sutton, Surrey, for assistance with the hypothetical grain analysis; and to M. J. Kestett of the MRC Cyclotron Unit, Hammersmith Hospital, London, for preparing the 111In-oxine.
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Subcellular localization of 111In in human and rabbit platelets

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