

# In Vivo Radiolabeling of Platelet Proteins: A New Method With Identification of Platelet Factor XIII

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**A method for radiolabeling platelets in vivo was developed in which  $^3\text{H}$ -arginine was injected into the bone marrow of normal dogs. On the third day after injection, a maximum of 6%–7% of the radioactivity had been incorporated into the total platelet mass. This method of isotope administration resulted in a 50–60-fold increase in maximum uptake of radiolabel by platelets, as compared to values obtained by others using intravenous injections of various radioactive compounds. Tritium-labeled platelets were harvested from the animals and then were washed to remove unbound  $^3\text{H}$ -arginine. On polyacrylamide gel electrophoresis 7 labeled protein bands, with molecular weights ranging from 29,000 to 132,000, were obtained from the platelet-soluble fraction. One  $^3\text{H}$ -containing protein with a molecular weight of 81,000 was identified immunologically and enzymatically as platelet factor XIII.**

**P**LATELETS have been labeled in vivo with a number of radioactive compounds.  $^{14}\text{C}$ -serotonin and diisopropylfluorophosphate ( $^3\text{H}$ -DFP and DF $^{32}\text{P}$ ), when injected intravenously, bind directly to circulating platelets.<sup>1–5</sup>  $^{14}\text{C}$ -serotonin is actively transported and concentrated within platelets, while DFP labels circulating platelets by attaching irreversibly to the active sites of serine proteases within the platelet.<sup>1–4</sup> Following intravenous injection,  $^{35}\text{S}$ -sodium sulphate,  $^{35}\text{S}$ -methionine, and  $^{75}\text{Se}$ -methionine are partially incorporated into megakaryocyte cytoplasm, which in turn is released into the circulation as newly formed platelets containing the radioactive label.<sup>6–8</sup> In studies with humans and other animal species, maximum incorporation of  $^{75}\text{Se}$ -methionine into the total platelet mass was 0.12% of the total radioactivity injected, which was achieved 3–7 days after intravenous injection, depending on the species studied.<sup>8–13</sup> Recently, Nachman et al. labeled platelet proteins in vitro by incubating megakaryocyte cultures with  $^3\text{H}$ -leucine and identified a  $^3\text{H}$ -labeled protein with a molecular weight of 42,000 as platelet actin.<sup>14</sup>

In this report a new method for labeling platelet proteins in vivo is described. By injecting  $^3\text{H}$ -arginine directly into the bone marrow of dogs, we have observed a 50–60-fold increase in uptake of label by the platelets. We obtained labeled platelet proteins that were identified by their migration in 7% SDS-polyacrylamide gels, and one of these was further identified enzymatically and immunologically as platelet factor XIII.

## MATERIALS AND METHODS

### Administration of $^3\text{H}$ -Arginine

Healthy female mongrel dogs, weighing 12.2–18.2 kg, were used in this study. All animals had normal erythrocyte, leukocyte, and platelet counts prior to administration of  $^3\text{H}$ -arginine. The dogs were lightly anesthetized with a 4.0% solution of thiamylal sodium (Surital, Parke-Davis and Company) according to the manufacturer's recommended dosage. The area around the hip was shaved and then cleansed with ethanol and iodine solutions. L-[2,3- $^3\text{H}$ ] arginine (specific activity 22.14 Ci/mole, from New England Nuclear) was

diluted in sterile saline. A 16-gauge, 1 $\frac{1}{16}$ -in biopsy needle (Becton-Dickinson), which was prerinced with heparin, was used to inject the  $^3\text{H}$ -arginine solution directly into the bone marrow of the iliac crest. Two different doses were tested, 100  $\mu\text{Ci}$  (6  $\mu\text{Ci}/\text{kg}$ ) and 2.0 mCi (115  $\mu\text{Ci}/\text{kg}$ ).

### Determination of $^3\text{H}$ -Labeling of Platelets

Blood samples were collected daily into 3.8% sodium citrate and were monitored for tritium activity and platelet number. Whole blood samples were centrifuged at 200 g for 10 min to obtain platelet-rich plasma (PRP), and the PRP was centrifuged at 2000 g for 20 min to obtain platelet-poor plasma (PPP) and a platelet pellet. Platelet counts on whole blood, PRP, and PPP were performed under phase-contrast microscopy.<sup>15</sup> The platelet pellet was carefully resuspended in 0.05 M Tris–0.1 M NaCl–0.001 M EDTA–Trasytol, 100 KIU/ml, pH 7.4, and then centrifuged at 100 g for 10 min to remove contaminating erythrocytes and leukocytes. The platelets were sedimented by centrifugation at 2000 g for 20 min. The platelet pellet was then alternately washed with the Tris–NaCl–EDTA–Trasytol buffer and centrifuged until the tritium activity in the supernatant approached background levels. With this washing procedure, contaminating labeled plasma proteins were removed from the platelets. Samples of PRP and PPP and the platelet pellet were counted for tritium activity; 50- $\mu\text{l}$  aliquots of PRP or PPP or the pellet from 0.7 ml PRP were mixed with 0.5–1.0 ml Protosol (New England Nuclear) to solubilize the cells and proteins. Then, 10 ml of toluene-based scintillation fluid were added to each vial, and the tritium activity was counted in a liquid scintillation spectrometer (Beckman LS-233).

### Analysis of Labeled Proteins in the Soluble Platelet Fraction

When the peak of tritium activity in the circulating platelets was obtained, larger volumes of blood were collected into 3.8% sodium citrate. Labeled platelets were separated from blood and washed in the same manner as described above for the test samples. Platelet number and tritium activity were determined for the final platelet

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suspension, which was then homogenized in a Sorvall Omni-Mixer and centrifuged at 18,000 g for 1 hr. The supernatant containing the soluble platelet proteins was divided into two aliquots. From one aliquot proteins were precipitated by adding saturated ammonium sulfate (ultrapure from Schwarz Mann) to a final concentration of 50%. The precipitate was dissolved in 0.05 M Tris-0.1 M NaCl-0.001 M EDTA, pH 7.4, and dialyzed overnight against the same buffer. The soluble fraction from the homogenized platelets and the precipitated protein fraction were examined for tritium activity in the same way as previously described for PRP and PPP samples.

The two fractions were then electrophoresed on 7% SDS-polyacrylamide gels under reducing conditions.<sup>16</sup> The gels were stained with Coomassie brilliant blue, and the relative molecular weights of the proteins were estimated. After destaining, the gels were sliced into 1-mm sections and incubated in 0.5 ml of 30% H<sub>2</sub>O<sub>2</sub> for 12 hr at 50°C.<sup>17</sup> Once the gel slices were dissolved, 10 ml of toluene-based scintillation fluid were added to each vial and the samples were counted in a liquid scintillation spectrometer. Several gel sections containing tritium activity corresponded to Coomassie-stained protein bands. In control experiments, platelets were harvested from an untreated animal and prepared in the same way as labeled platelets. Control samples were electrophoresed, and the gels were solubilized and counted. The background radioactivity of these samples was subtracted from the radioactivity of the test samples.

#### Identification of <sup>3</sup>H-Labeled Platelet Factor XIII

Samples of PRP and PPP, the soluble fraction of the homogenized platelets, and the precipitated protein fraction were assayed for factor XIII activity with the <sup>14</sup>C-putrescine incorporation assay of Lorand et al.<sup>18</sup> The PRP was frozen and thawed three times to disrupt the platelets before assaying. Platelet factor XIII activity was determined by subtracting the factor XIII activity in the PPP from the factor XIII activity in the PRP. Additionally, the soluble fraction was incubated with rabbit antisera to factor XIII  $\alpha$  subunit (Anti-A from Behring). After incubation overnight at 4°C, the mixture was centrifuged, and the supernatant was electrophoresed on 7% SDS-polyacrylamide gels under reducing conditions as previously described. This supernatant was also assayed for factor XIII activity.

## RESULTS

### Platelets Labeled With <sup>3</sup>H-Arginine

The amount of tritium activity present in the platelets of the dogs receiving <sup>3</sup>H-arginine was monitored daily. The amount of radioactivity present in platelet-poor plasma was subtracted from the amount of radioactivity present in platelet-rich plasma. Also, the washed platelet pellet from 0.7 ml platelet-rich plasma was counted for tritium activity. The platelet pellets contained 72%–92% of the radioactivity associated with the platelet component in PRP. The amount of tritium activity present in the total platelets of each animal was calculated as dpm/10<sup>9</sup> platelets  $\times$  *n* platelets/ml blood  $\times$  weight (g)  $\times$  blood vol/g. In this study, the amount of tritium activity present per 10<sup>9</sup> platelets was about 430 dpm for the animal that received 100  $\mu$ Ci and 18,000 dpm for the animals that received 2 mCi of <sup>3</sup>H-arginine.

Two doses of <sup>3</sup>H-arginine were tested. One-hundred

microcuries of <sup>3</sup>H-arginine were injected into one hip of a normal, healthy dog; and it was determined that 0.5  $\times$  10<sup>6</sup> dpm were present in the animal's platelets 3 days following injection. There were three experiments in which 2 mCi of <sup>3</sup>H-arginine were injected, 1 mCi into the bone marrow of each iliac crest. Three days following injection, the average amount of tritium activity present in the platelets of these animals was 9.6  $\times$  10<sup>6</sup> dpm. As shown in Fig. 1, tritium activity in the platelets of the animals varied in proportion to the amount of <sup>3</sup>H-arginine that was administered. The maximum platelet incorporation of <sup>3</sup>H-arginine in the animals that received 2 mCi was 19 times greater than the maximum incorporation of <sup>3</sup>H-arginine into the platelets of the dog that received 100  $\mu$ Ci.

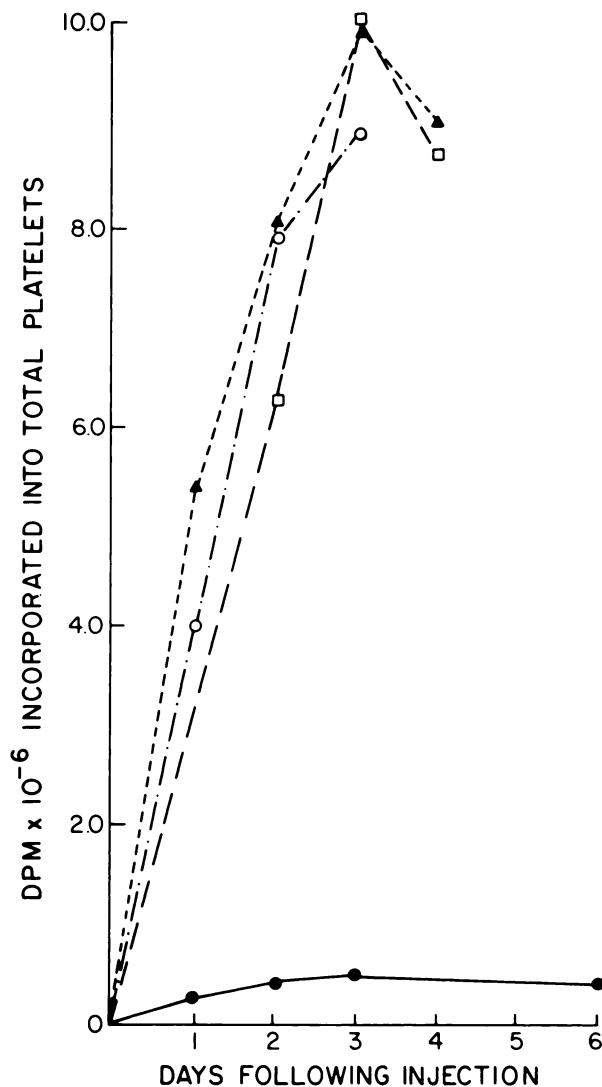


Fig. 1. Amount of radioactivity incorporated into the platelets of dogs receiving <sup>3</sup>H-arginine. (●) 100  $\mu$ Ci injected into the bone marrow of the left iliac crest. (□, ○, △) 2 mCi injected, 1 mCi into the bone marrow of each iliac crest.

The percentage uptake of <sup>3</sup>H-arginine by the total number of platelets was calculated as: (the total dpm in the platelets - background) × 100/total dpm injected. The maximum percentage of <sup>3</sup>H-arginine taken up by the total platelets of the animals ranged between 6% and 7% of the amount of <sup>3</sup>H-arginine that was administered (Fig. 2). Although the dose of the radiolabeled amino acid differed by a factor of 20 and the counts present in the platelets differed by a factor of 19, the maximum percentage incorporation of <sup>3</sup>H-arginine was constant and independent of the amount of labeled amino acid that was injected.

<sup>3</sup>H-Labeled Platelet Proteins

After the <sup>3</sup>H-labeled platelets were separated from canine blood, the platelets were washed extensively. The number of platelets obtained from the animals ranged from 15.5 × 10<sup>9</sup> to 98.4 × 10<sup>9</sup>. The platelets were homogenized, and the soluble protein fraction was separated by centrifugation. The insoluble fraction that was precipitated contained 70%–75% of the tritium activity as compared to the intact platelets. This fraction was not analyzed further. It was not assayed for factor XIII activity because it has been demonstrated that platelet factor XIII is localized in the platelet-soluble compartment.<sup>19</sup> The crude soluble fraction was electrophoresed on 7% SDS-polyacrylamide gels under reducing conditions. Several

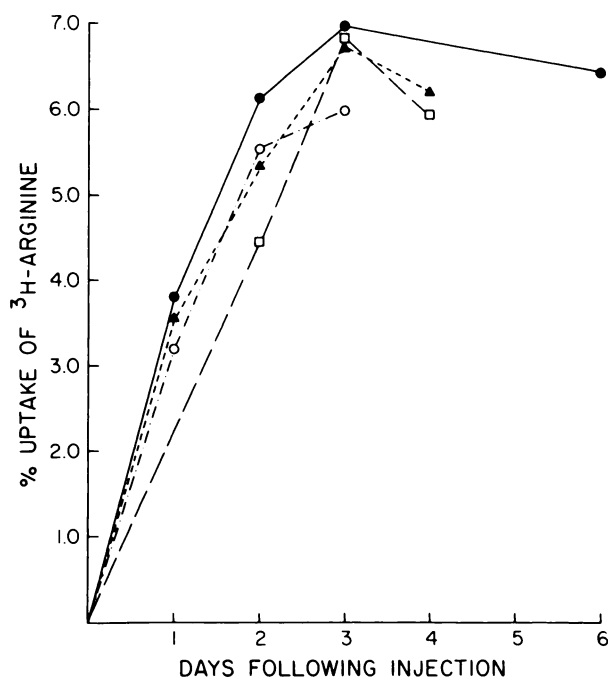


Fig. 2. Percent uptake of <sup>3</sup>H-arginine by total platelets during in vivo synthesis. (●) 100 μCi injected into the bone marrow of the left iliac crest. (□, ○, △) 2 mCi injected, 1 mCi into the bone marrow of each iliac crest.

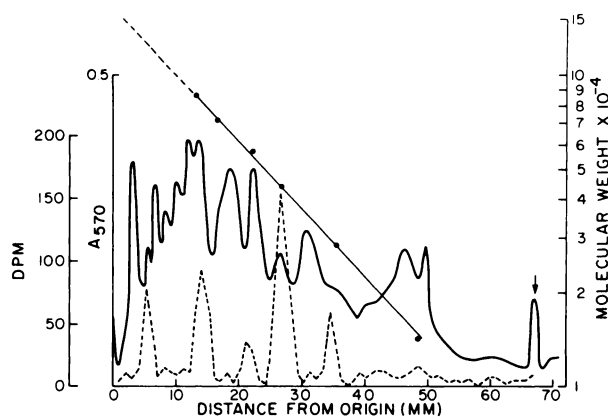


Fig. 3. Scan of 7% SDS-polyacrylamide gel of the soluble fraction obtained from homogenized canine platelets of a <sup>3</sup>H-arginine injected dog. Absorbance at 570 nm (—); tritium activity in each one mm slice of gel, with average background activity (52 dpm) subtracted (---). Arrow indicates bottom of gel. Points on the diagonal line show the migration of the BDH molecular weight markers: 14,300, 28,600, 42,900, 57,200, 71,500, and 85,800.

Coomassie-blue-stained protein bands appeared as shown in the electrophoretogram in Fig. 3. After the gels were sliced into 1-mm sections and solubilized in 30% H<sub>2</sub>O<sub>2</sub>, 5 peaks of radioactivity were obtained from the gels of the crude soluble fraction; these peaks corresponded to proteins with molecular weights of 132,000, 81,000, 60,000, 42,000, and 29,000.

Once it was determined that <sup>3</sup>H-labeled proteins were present in the crude soluble fraction following homogenization and centrifugation of the <sup>3</sup>H-labeled platelets, some of the <sup>3</sup>H-containing proteins were precipitated from the soluble fraction by adding saturated ammonium sulfate to a final concentration of 50%. This precipitated protein fraction was also electrophoresed on 7% SDS-polyacrylamide gels under

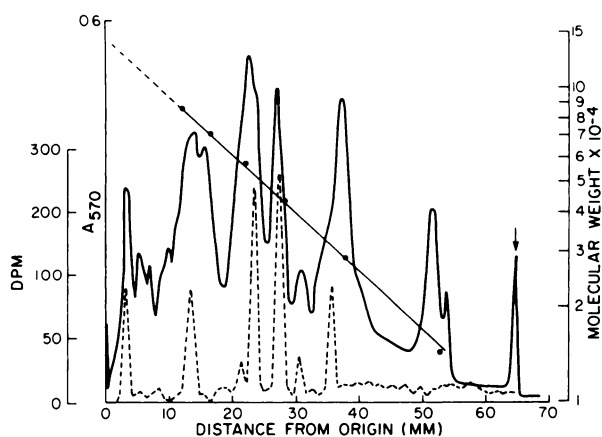


Fig. 4. Scan of 7% SDS-polyacrylamide gel of platelet proteins, obtained from a 50% ammonium sulfate precipitation of platelet soluble fraction, from a <sup>3</sup>H-arginine injected dog. Absorbance at 570 nm (—); tritium activity in each 1-mm slice of gel (---). Other details are given in Fig. 3.

reducing conditions. As shown in the electrophoretogram in Fig. 4, many Coomassie-blue-stained protein bands were present. Seven peaks of radioactivity were obtained from these gels; these peaks corresponded to proteins with molecular weights of 130,000, 81,000, 58,000, 53,000, 42,000, 39,000, and 31,000. Two of these peaks, with molecular weights 81,000 and 42,000, directly corresponded to two peaks present in the crude soluble fraction. Additionally, the tritium peaks with apparent molecular weights of 130,000, 58,000, and 31,000, which were obtained from the gels of the precipitated fraction, were similar to three peaks of radioactivity that were obtained from the gels of the crude soluble fraction. The two additional radioactive peaks in this fraction (mol wt 53,000 and 39,000) may have resulted from a concentrating effect of the ammonium sulfate precipitation or from degradation of other labeled proteins.

### <sup>3</sup>H-Labeled Platelet Factor XIII

Throughout the study various samples from the dogs receiving injections of <sup>3</sup>H-arginine were assayed for factor XIII activity. The platelet-rich plasma and the platelet-poor plasma contained <sup>14</sup>C-putrescine incorporating activity, and it was determined that approximately 44% of the factor XIII activity found in canine platelet-rich plasma is derived from platelets. The homogenized platelets and the soluble fraction obtained from them were also assayed for factor XIII activity. Both of these samples contained substantial factor XIII activity as compared to canine plasma samples. It appeared that the <sup>3</sup>H-containing protein with a molecular weight of 81,000 was platelet factor XIII based on its relative mobility on 7% SDS-polyacrylamide gels and on the presence of factor XIII enzymatic activity in the crude soluble fraction. This was directly confirmed immunochemically. It has been demonstrated by several investigators that platelet factor XIII is enzymatically and immunologically identical to plasma factor XIII *a* subunit.<sup>20-23</sup> Since canine factor XIII cross-reacts with antisera to human factor XIII subunits,<sup>24</sup> an aliquot of the crude soluble fraction, obtained from homogenized platelets that had been labeled with <sup>3</sup>H-arginine, was incubated with rabbit antiserum to human factor XIII *a* subunit. Following centrifugation of this mixture, the factor XIII activity in this fraction was reduced to 11.4% of the original activity that was present before the addition of the rabbit antiserum. In addition, the tritium activity in the section of gel corresponding to platelet factor XIII (mol wt 81,000) was reduced to 15% of the original amount of radioactivity present in that band prior to incubation with the antiserum to factor XIII *a* subunit (Fig. 5). In one additional band, the radioac-

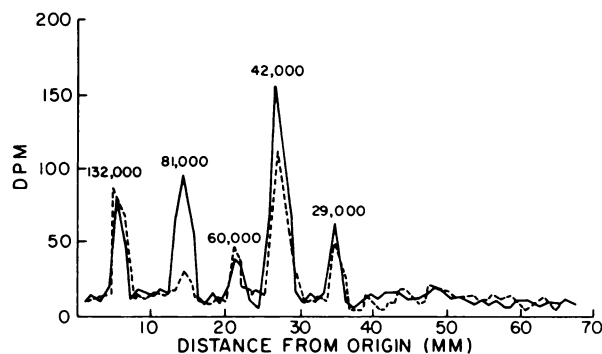


Fig. 5. Tritium activity in each 1-mm slice of a 7% SDS-polyacrylamide gel containing soluble platelet proteins in an extract from canine platelets. Tritium activity in the whole extract (—); tritium activity in the extract after incubation with rabbit antisera to factor XIII *a* subunit (---). Radioactive peaks correspond to Coomassie-stained bands of molecular weights 132,000, 81,000, 60,000, 42,000, and 29,000.

tivity was decreased to 80% of the original value, and in the other bands, the radioactivity remaining after incubation with antiserum to *a* subunit was about 90%.

### DISCUSSION

Previous reports have shown that following intravenous injection of <sup>75</sup>Se-methionine, the maximum uptake of label into platelets during synthesis was 0.12%. These studies were done with rabbits, mice, rats, and humans.<sup>7-12</sup> Since platelets and platelet proteins are synthesized by the megakaryocyte in the bone marrow, in this study, <sup>3</sup>H-arginine was injected directly into the bone marrow of the iliac crest of dogs. This proved to be a more direct and more effective way of labeling platelets and platelet proteins *in vivo*. With this method, 76%–98% of the injected tritium activity was recovered in the platelet-rich plasma of the animals, and 6%–7% of the original radioactivity was incorporated into the platelets of the dogs 3 days following injection. This improved uptake of label into platelets may partially be explained by the fact that erythrocytic and granulocytic cells have low levels of intracellular arginine and require less free arginine than megakaryocytes.<sup>25</sup> The maximum incorporation of <sup>3</sup>H-arginine into canine platelets represents a 50–60-fold increase in maximum uptake of a radiolabeled amino acid by platelets. This leads to the possibility that the percentage of radiolabel incorporated into the total number of platelets might be increased further if several additional bone marrow sites, such as the femur and the sternum, were injected with a radioactive amino acid.

In addition to obtaining a substantial number of <sup>3</sup>H-labeled, viable platelets with this method, it was also possible to separate labeled proteins from the

platelets. One tritium-containing protein was clearly identified, enzymatically and immunologically, as platelet factor XIII. It seems likely that other  $^3\text{H}$ -labeled proteins could also be identified using enzymatic and immunologic assays. However, in this study, the identification of the other labeled proteins was not a major consideration. The protein with an apparent molecular weight of 42,000 and significant tritium activity probably corresponds to platelet actin that has been labeled *in vitro* with  $^3\text{H}$ -leucine and identified immunologically by Nachman et al.<sup>14</sup> Some of the other  $^3\text{H}$ -labeled protein bands may correspond to cathepsin E (mol wt 60,000), elastase (mol wt 28,000),

and tubulin ( $\alpha$ -monomer, mol wt 52,000 and  $\beta$ -monomer, mol wt 55,000).<sup>26,27</sup>

The extended application of this method of labeling platelets and platelet proteins are numerous. This technique could be useful in obtaining quantitative data for binding studies, for turnover studies, for synthesis, and for platelet protein function studies.

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