Radioactive Diisopropyl Fluorophosphate as a Platelet Label: An Evaluation of in Vitro and in Vivo Technics

By T. C. Bithell, J. W. Athens, G. E. Cartwright and M. M. Wintrobe

In spite of the availability of many radioisotopes and the applicability of a variety of technics, no entirely satisfactory method of labeling blood platelets has yet been developed. The central problem in all in vitro methods has been the fact that many platelets so labeled cannot be accounted for when returned to the circulation. In most instances, this initial survival problem has been attributed to platelet injury during collection and extracorporeal storage. Although with advancing knowledge some of the responsible factors have been defined, at best only 70 per cent of platelets labeled in vitro will circulate in vivo. Unless the initial recovery of labeled platelets approximates 100 per cent, or unless the inability to do so is otherwise explained, the possibility of extracorporeal injury cannot be excluded. The results of all in vitro labeling technics must be interpreted in this context.

An alternative explanation for the initial survival problem is the possibility that the labeled cells equilibrate with an extravascular or marginal platelet pool. In this case the failure to initially recover 100 per cent of the infused labeled platelets would be physiologic rather than artifactual.

In vivo platelet labeling circumvents the problem of extracorporeal injury, but provides only a definition of platelet lifespan. Consequently, physiologic measurements such as pool size and turnover rate, which are essential to a clear understanding of platelet production, distribution, and destruction, cannot be made by any presently available technic. Moreover, in spite of intensive study, the question as to whether the primary determinant of platelet lifespan is senescence or random destruction remains controversial.

Diisopropyl fluorophosphate (DFP) has been widely used in the form of DFP and H3-DFP as an in vivo platelet label, but except for a single preliminary report has not been used in vitro. This compound does not appear to elute significantly and is capable of labeling platelets in whole blood, thus...
avoiding possible platelet injury during the preparation of platelet concentrates. The studies reported here concern an attempt to develop an in vitro method of labeling platelets with DFP\(^{32}\). The results obtained are compared to in vivo labeling methods employing DFP\(^{32}\) and to current in vitro methods using chromium-51 (Cr\(^{51}\)). Additional studies pertinent to the problem of initial platelet survival are presented.

**Materials and Methods**

**Subjects**

Hematologically normal volunteers whose platelet counts (performed by the method of Brecher and Cronkite\(^{16}\)) were stable throughout the duration of the described experiments were used in these studies. The majority were healthy inmates of the Utah State Prison and were from 20 to 50 years of age. The remainder suffered from chronic nonhematologic disorders and were inpatients on the geriatric wards of the University of Utah Hospital. All subjects received a regular diet while under study.

**Platelet Labeling**

All procedures were carried out at room temperature, and sterile technic was employed throughout. Preliminary studies confirmed that initial platelet recovery was markedly reduced when ethylene-diamine-tetra acetic acid (EDTA) was used in the collection of blood. Consequently, blood for in vitro platelet labeling was collected in acid-citrate-dextrose, prepared as described by Aster and Jandl.\(^{1}\) by mixing approximately 500 ml. of venous blood with 90 ml. of anticoagulant in disposable plastic bags.\(^{4}\) The final pH of plasma so prepared was 6.5.

1. **In Vitro Platelet Labeling with DFP\(^{32}\).** Sufficient DFP\(^{32}\) was added to anticoagulated whole blood to give a final concentration of from 0.4 to 1.2 μg./ml. Preliminary experiments established that the labeling of platelets with DFP\(^{32}\) is not instantaneous, but the platelet radioactivity in the presence of DFP\(^{32}\) reaches a stable asymptote after 30 to 45 minutes. Accordingly, the mixture of blood, ACD and DFP\(^{32}\) was gently mixed every 10 to 15 minutes for 1 hour.

2. **In Vitro Platelet Labeling with Chromium\(^{51}\).** Platelet-rich plasma was prepared by centrifuging anticoagulated blood at 275 g., a procedure which yielded approximately 200 ml. of platelet-rich plasma containing an average of 450,000 platelets/mm\(^3\). Sufficient Cr\(^{51}\) was added to this plasma to give a final concentration of approximately 1.25 μc./ml. plasma. Gentle mixing was carried out for 15 minutes. Thirty mg. of ascorbic acid was then added.

3. **In Vitro Platelet Labeling with Both DFP\(^{32}\) and Cr\(^{51}\).** As in the in vitro DFP\(^{32}\) method, anticoagulated whole blood was periodically mixed for 1 hour with sufficient DFP\(^{32}\) to give a concentration of approximately 1.0 μg./ml. Platelet-rich plasma was then prepared and the platelets were labeled with Cr\(^{51}\) as previously described.

After labeling was complete in all of the aforementioned in vitro technics, bag aliquots for the determination of radioactivity were removed, and the remaining labeled blood or plasma was weighed and reinfused into the donor. Both of these operations
were carried out through a standard blood filter which did not remove significant numbers of platelets. Serial blood samples were obtained from the recipient beginning between 10 and 15 minutes after reinfusion of the labeled platelets (time zero or T0). Platelets were harvested from these blood specimens, and from bag aliquots by the same technic.

4. In Vivo Platelet Labeling with DFP22. From 0.8 to 1.9 mg. of DFP22 was diluted in from 50 to 100 ml of 0.15 M NaCl and injected intravenously over a period of from 5 to 10 minutes.

**PLATELET SEPARATION**

Silicone-coated glassware and pipettes were used throughout. Venous blood was drawn into disposable plastic syringes through 18-gauge needles, and mixed with sufficient EDTA to give a final concentration of 3 mg./ml. blood. Sufficient dextran was added to the blood sample to give a final concentration of 10 mg./ml. blood. After mixing, the blood was centrifuged at 250 g for 10 minutes, and the supernate transferred to clean tubes and recentrifuged at 750 g for 10 minutes. The resulting platelet-rich plasma, which contained a few residual erythrocytes and leukocytes, was then centrifuged at 1900 g for 30 minutes. The resulting platelet button was washed twice in 30 ml. of 0.07 M ammonium oxalate (1 per cent w/v) (NH4)2C2O4·H2O). In the DFP22 methods, two additional washes in 30 ml. of 0.15 M NaCl were used to remove contaminating nitrogen. The platelets were centrifuged at 1900 g after each wash. The final platelet button contained approximately 25 per cent of the platelets present in shed blood. Approximately 300 μg. of platelet nitrogen, or 109 platelets, could be harvested from 20 ml. of normal blood with this method.

**ISOTOPE COUNTING METHODS**

All platelet samples were counted twice for either 80 minutes or until 8000 counts were obtained. The standard error of counting for either Cr51 of P32 was below ±5 per cent throughout the course of the reported platelet survival studies. The net c.p.m. obtained for each sample was corrected back to the first day of the study for radioactive decay.

1. **DFP22**

Platelets were transferred to Mylar-covered Sintilon planchettes and the radioactivity of P32 was determined in a Nuclear Chicago liquid scintillation counter adapted for plastic scintillation counting (efficiency approximately 80 per cent). The Mylar "sandwich" containing the platelets was then quantitatively transferred to a Coleman nitrogen analyzer, and the nitrogen content of the platelets was determined. The standard error of the nitrogen determination was ±5 per cent. Specific activity (SA) was expressed as c.p.m. per mg. of

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* Obtained from National Radiac, Newark, New Jersey.

1A 0.27 M stock solution (10 per cent w/v) of EDTA was prepared by dissolving its di-sodium salt in distilled water. The pH was adjusted to 7.4 with 1N NaOH.

1The dextran employed was obtained from the R. K. Laros Company, Bethlehem, Pennsylvania. It had a mean molecular weight of 225,000 and was dissolved in 0.15 M NaCl in a concentration of 6 per cent (w/v).
PLATELET LABELING WITH DFP\textsuperscript{32}

platelet nitrogen. This averaged 10,000 c.p.m./mg. platelet nitrogen in bag samples. The coefficient of variation of replicate platelet buttons so labeled and handled was ±14 per cent.

2. Cr\textsuperscript{51}

Cr\textsuperscript{51} labeled platelets were suspended in exactly 10 ml. of 0.07 M ammonium oxalate and counted. The suspension was then centrifuged at 100,000 g for 10 minutes,\textsuperscript{*} a process which removes in excess of 99 per cent of the platelets. The radioactivity of the buttons was determined in a well-scintillation counter. Because of the difficulty in quantitatively transferring the platelets from the counting vials employed, nitrogen analysis was not done in this method. Rather, specific activity was expressed as c.p.m./10\textsuperscript{9} platelets. This averaged 6000 c.p.m./10\textsuperscript{9} platelets in bag samples. The coefficient of variation of replicate platelet buttons so labeled and handled was ±20 per cent.

3. DFP\textsuperscript{32} and Cr\textsuperscript{51} Together

In this method, the radioactivity of Cr\textsuperscript{51} was determined as described under the Cr\textsuperscript{51} method, after which the platelets were transferred to planchettes and the radioactivity of P\textsuperscript{32} and their nitrogen content were determined as described under the DFP\textsuperscript{32} method. The cross-counting of P\textsuperscript{32} in the well-scintillation system was negligible (<0.1 per cent), but 3 per cent of the net c.p.m. obtained by plastic scintillation was subtracted in order to correct for the cross-counting of Cr\textsuperscript{51} in this system.

CALCULATIONS

Blood volume of subjects was obtained from a standard nomogram,\textsuperscript{44,45} and the weight of blood or plasma infused was converted to volume by appropriate correction factors: i.e., weight of whole blood-ACD/1.055; weight of platelet rich plasma/1.028; weight of platelet rich plasma containing dextran/1.026. Throughout this paper, "platelet survival" refers to the disappearance of platelet radioactivity from the peripheral blood.

1. Calculation of Platelet Survival in the in Vitro Methods

Platelet survival at indicated time (T) was expressed as a percentage of the platelets infused and was calculated from equation I.

\[
\text{Equation I:} \quad \frac{\text{SA (bag)}}{\text{blood vol. (ml.)}} \times \frac{\text{plat. count (bag)}}{\text{recipent's plat. count (at } T_0)} \times \frac{\text{volume infused}}{\times \text{100 platelet survival (T)}}
\]

2. Calculation of Platelet Survival in the in Vivo DFP\textsuperscript{32} Method

In order to correlate data from different subjects, rectilinear regression lines were fitted to all points between 10 and 180 hours in each individual experi-

\textsuperscript{*}This step utilized a Spinco Model L preparative ultracentrifuge.
\textsuperscript{44,45}Calculated by this means, the blood volume of our subjects averaged 4923 ml., and ranged from 4315 ml. to 5494 ml.
Table 1.-Efficiency of Platelet Separation Method in Excluding Radioactivity from Erythrocytes and Leukocytes

<table>
<thead>
<tr>
<th>Cell</th>
<th>Isotope</th>
<th>Total c.p.m./mg. Added</th>
<th>Radioactivity Recovered in Platelet Buttons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Radioactivity Recovered in Platelet Buttons</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.p.m./mg. Added</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>DFP$^{52}$</td>
<td>24,300</td>
<td>36.3</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>DFP$^{52}$</td>
<td>13,500</td>
<td>8.0</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Cr$^{51}$</td>
<td>27,500</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Whole blood in ACD was labeled with 1 μg. DFP$^{52}$/ml or 0.5 μc. Cr$^{51}$/ml. Suspensions of erythrocytes and leukocytes essentially free of platelets were prepared. The radioactivity of an aliquot of these suspensions was determined, and the remainder mixed with 20 ml. of unlabeled autologous whole blood in ACD. The final concentration of labeled cells was $10^5$ leukocytes/ml unlabeled blood and $10^6$ erythrocytes/ml unlabeled blood. Platelet buttons were then harvested from this mixture and their specific activity determined. Each result tabulated represents the average of three separate experiments.

Platelet Separation

Since DFP$^{52}$ is known to label both erythrocytes and leukocytes, a scrupulous platelet separation method was required. The ammonium oxalate washing employed lysed all residual erythrocytes, and the leukocytes remaining in the platelet button did not exceed 1 per 5000 platelets and averaged 1 per 16,000 platelets. Experiments in which platelet buttons were harvested from blood to which large numbers of DFP$^{52}$ or Cr$^{51}$-labeled leukocytes or erythrocytes had been added are summarized in Table 1. The specific activity of platelet buttons harvested from blood containing DFP$^{52}$-labeled leukocytes (Table 1,A) was less than 5 per cent of the lowest $T_0$ platelet specific activity obtained after the infusion of platelets labeled in vitro with DFP$^{52}$. Even less

Statistical Operations

Standard statistical operations were employed in the evaluation of the data. Lines were fitted to experimental points by the method of least squares employing both rectilinear and semilogarithmic-estimating equations. From these equations the respective standard error of estimate ($S_{Y,X}$ and $S_{Y,X}$) and the coefficients of determination ($r^2$) were computed. The standard error of estimate is a measure of the general reliability of the fit, and 95 per cent of the fitted points should fall within 2 standard errors of the fitted line if their distribution around this equation is normal. The coefficient of determination defines that proportion of the variation present in the dependent variable which is predictable by the estimating equation. Values of $p$ refer to the probability of the difference in question being due to chance alone.

Results

Platelet Separation

Since DFP$^{52}$ is known to label both erythrocytes and leukocytes, a scrupulous platelet separation method was required. The ammonium oxalate washing employed lysed all residual erythrocytes, and the leukocytes remaining in the platelet button did not exceed 1 per 5000 platelets and averaged 1 per 16,000 platelets. Experiments in which platelet buttons were harvested from blood to which large numbers of DFP$^{52}$ or Cr$^{51}$-labeled leukocytes or erythrocytes had been added are summarized in Table 1. The specific activity of platelet buttons harvested from blood containing DFP$^{52}$-labeled leukocytes (Table 1,A) was less than 5 per cent of the lowest $T_0$ platelet specific activity obtained after the infusion of platelets labeled in vitro with DFP$^{52}$. Even less
In Vitro Platelet Labeling with DFP$^{32}$

The results of studies on 12 subjects are summarized in Figure 1. The mean recovery of platelets at $T_0$ (initial survival) was 79 per cent and ranged from 50 to 109 per cent. In no case did platelet specific activity rise significantly above the $T_0$ value on subsequent samples. The survival curve appears to consist of three phases. These are arbitrarily defined as: phase I (between $T_0$ and 5 hours), phase II (between 5 hours and 180 hours), and phase III (after 180 hours). During phase I, a rapid initial decrease in platelet specific activity was evident and most points fell above the range of $2\ S_{x,y}$ of the illustrated regression line fitted to the points of phase II (the midportion of the curve). In phase II, platelet radioactivity declined in a rectilinear manner, with a half disappearance time ($T_{1/2}$) of...
Table 2.—*In Vivo* Platelet Labeling by Residual DFP<sup>32</sup> in Infused Plasma

<table>
<thead>
<tr>
<th>DFP Concentration (μg./ml. infused plasma)</th>
<th>Maximum Platelet Radioactivity (c.p.m./mg. Platelet Nitrogen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>42.3</td>
</tr>
<tr>
<td>1.9</td>
<td>28.5</td>
</tr>
<tr>
<td>1.2</td>
<td>3.7</td>
</tr>
<tr>
<td>0.8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Maximum radioactivity in harvested platelets was recovered 1 hour after the plasma infusion. Each tabulated result represents the average of two experiments on two different subjects.

95 hours (range 60 to 115). When the illustrated regression line was extrapolated to T<sub>n</sub>, it intersected the ordinate at a value of 52 per cent survival.

Significant "tailing" of the curve was observed (phase III), as evidenced by the fact that beyond 180 hours most points fell above the range of 2 S<sub>n</sub> of the phase II estimating equation. During phase III platelet radioactivity ranged from 5–15 per cent of that present at T<sub>n</sub> and detectable radioactivity was present at 280 hours.

Since DFP<sup>32</sup> is capable of labeling platelets in vivo as well as in vitro, experiments were designed to evaluate the possibility that when in vitro labeled blood is infused residual unbound DFP<sup>32</sup> may label additional platelets in vivo. Blood was removed from 8 subjects and platelet-rich plasma was prepared as previously described. DFP<sup>32</sup> was added in the concentrations shown in Table 2. The mixture was gently mixed for 1 hour. Approximately 95 per cent of the platelets were then removed by centrifugation at 1900 g for 30 minutes, and the resulting 200–300 ml. of platelet-poor plasma was infused into the donor. Platelets were harvested at intervals for the next 7 days. It can be seen from Table 2 that DFP<sup>32</sup> concentrations below 1.2 μg./ml. plasma produced no significant in vivo labeling. Above 1.9 μg. DFP<sup>32</sup>/ml. plasma, significant in vivo labeling was observed. The specific activity recovered was approximately two per cent of the lowest T<sub>n</sub> samples obtained after the infusion of in vitro labeled platelets. However, in the in vitro studies described above (Fig. 1), the DFP<sup>32</sup> concentration did not exceed 1.2 μg./ml. of whole blood. It is thus apparent that in vivo labeling by unbound DFP<sup>32</sup> does not contribute significantly to the results obtained with the in vitro DFP<sup>32</sup> labeling technic.

**In Vivo Platelet Labeling with DFP<sup>32</sup>**

In order to compare the results obtained with in vitro DFP<sup>32</sup> labeling to those obtained when DFP<sup>32</sup> was given in vivo, 14 subjects were studied with the latter technic. The results obtained are illustrated in Figure 2. The decline in platelet radioactivity observed after DFP<sup>32</sup> was injected intravenously did not follow a simple linear relationship, and appeared to consist of the same three phases observed in the curve obtained with the in vitro technic. At 1 hour, the determined specific activity correlated well with the value obtained by extrapolating the phase II regression line to T<sub>n</sub>. However, between 1 and 5 hours platelet specific activity fell rapidly, 75 per cent of the points falling below the range of 2 S<sub>n</sub> of the phase II.
PLATELET LABELING WITH DFP$^{32}$

Fig. 2.—Survival of platelets labeled in vivo with DFP$^{32}$. Samples were obtained at 10 to 30 minute intervals throughout the first 5 hours, and at 12–24 hour intervals thereafter. The dashed line (phase I) was fitted by eye to points between $T_s$ and 5 hours, while the solid line (phase II) was fitted to points between 5 and 180 hours by means of a rectilinear estimating equation. The shaded area encompasses the range of ±2 standard errors of this estimate ($2 S_{xx}$). For the data illustrated (14 studies), these values were:

$$Y \text{(survival—%) } = (100 - 0.43 X \{\text{time—hrs.}\} \pm 10$$

regression line. This was a transient phenomenon, since after 5 hours observed points fell within 2 $S_{xx}$ of the fitted phase II regression line and platelet specific activity decreased in a rectilinear fashion thereafter. The $T_{1/2}$ during phase II averaged 120 hours and ranged from 91 to 164 hours in individual experiments. Most points beyond 200 hours fell above the range of 2 $S_{xx}$ of the phase II estimating equation, and as in the in vitro curve, this "tailing" persisted at a level of about 5–15 per cent of the $T_o$ radioactivity for at least 320 hours.

To ascertain whether or not di-isopropyl phosphate$^{32}$ (DIP$^{32}$), the primary degradation product of DFP$^{32}$, is recycled into human platelets, the following experiment was performed. Two mg. of DFP$^{32}$ containing 800 $\mu$C. of P$^{32}$ was hydrolyzed overnight in sterile 0.5 N NaOH. This hydrolysate was free of DFP as determined by colorimetric assay and was capable of labeling platelets in vitro to the extent of <5 per cent of unhydrolyzed DFP$^{32}$. After correction of the pH and ionic strength to physiologic ranges with sterile HCl and H$_2$O, the hydrolysate was administered intravenously to a volunteer, and platelets were isolated at intervals during the next 10 days. No significant radioactivity was detected in the platelet buttons.
Fig. 3.—Survival of platelets labeled in vitro with CR$^{51}$. Samples were obtained at 30–60 minute intervals throughout the first 5 hours, and at 12–24 hour intervals thereafter. The solid line was fitted to illustrated points between 5 and 180 hours by means of a rectilinear estimating equation. The shaded area encompasses the range of $\pm 2$ standard errors of this estimate ($2 S_{r.x}$). For the data illustrated (6 studies), these values were:

$$Y \text{ (survival—%)} = (79 - .38 \times \text{[time—hrs.]}) \pm 22$$

In Vitro Platelet Labeling with CR$^{51}$

In order to compare the results of the aforementioned technics which employ DFP$^{32}$ to those obtained with CR$^{51}$, 6 patients were studied by means of the in vitro CR$^{51}$ labeling technic. The results are summarized in Figure 3. The mean platelet recovery at $T_0$ was 81 per cent (range 48–111), and no curves were noted wherein platelet specific activity rose significantly above the $T_0$ value on subsequent samples. The survival curves obtained with this technic did not show the significant initial deviation from linearity noted in both of the DFP$^{32}$ methods, and were essentially rectilinear between $T_0$ and 200 hours. The mean $T^{1/2}$ was 100 hours (range 75 to 130). These experiments were not followed beyond 200 hours.

In Vitro Platelet Labeling with Both DFP$^{32}$ and CR$^{51}$

To evaluate the possibility that the rapid initial loss of platelet radioactivity noted in the in vitro DFP$^{32}$ survival curves is due to elution of the label, platelets were labeled simultaneously with DFP$^{32}$ and CR$^{51}$, and the resulting
Fig. 4.—Survival of platelets labeled in vitro with both DFP\(^{32}\) and Cr\(^{51}\). Samples were obtained at 30 minute intervals throughout the first 5 hours, and at 12-24 hour intervals thereafter. Points obtained with DFP\(^{32}\) are illustrated as circles, while those obtained with Cr\(^{51}\) are illustrated as squares. The dashed line (phase I) was fitted by eye to points between \(T_0\) and 5 hours, while the solid line (phase II) was fitted to all illustrated points between 5 and 140 hours by means of a rectilinear estimating equation. The shaded area encompasses the range of ±2 standard errors of this estimate (\(2 S_n\)). For the data illustrated (4 studies), these values were:

\[ Y (\text{survival—}T) = (42 - .22 \times \text{time—hrs.}) \pm 12 \]

survival curves compared to those obtained with Cr\(^{51}\) and DFP\(^{32}\) alone. The survival curve illustrated in Figure 4 is similar to, if not identical with, that obtained with the in vitro DFP\(^{32}\) method (Fig. 1). When evaluated separately, both the curve obtained with the Cr\(^{51}\) points and that obtained with the DFP\(^{32}\) points demonstrated a rapid initial fall in specific activity during the first 5 hours. The difference between the determined survival at \(T_0\) and the survival value obtained when the line fitted to the points of phase II was extrapolated to \(T_0\) was large (with Cr\(^{51}\), extrapolated \(T_0\) survival = 50 per cent, determined \(T_0\) survival = 71 per cent; with DFP\(^{32}\),
extrapolated \( T_0 \) survival = 40 per cent, determined \( T_0 \) survival = 64 per cent). No curves were obtained wherein the platelet specific activity rose significantly above the \( T_0 \) value on subsequent samples. After the first 5 hours, platelet specific activity appeared to fall rectilinearly with a \( T_{\frac{1}{2}} \) of 95 hours, and 95 per cent of the points, whether determined by DFP\(^{32}\) or Cr\(^{51}\), fell within the range of 2 \( S_{\alpha} \), of the same rectilinear estimating equation. The value of \( X \) in the estimating equations (slope of the fitted line) was virtually identical for the line fitted to the Cr\(^{51}\) points and that fitted to the DFP\(^{32}\) points (\( X = .24 \) and .20, respectively).

**The Effects of Dextran**

To evaluate the possibility that agents which coat platelets might alter initial platelet survival, the effects of dextran on the survival of platelets labeled in vitro with DFP\(^{32}\) were evaluated. Whether added to the ACD in the blood bag prior to the collection and labeling of the platelets (four subjects), or infused into the recipient prior to the reinfusion of the labeled platelets (one subject), 100 ml. of 6 per cent dextran* did not increase initial platelet recovery. In seven additional subjects, blood was collected and labeled with DFP\(^{32}\) in the usual manner. One hundred ml. of 6 per cent dextran were then added and the bag spun at 250 g for 10 minutes, in the hope that clumped nonviable platelets would be preferentially removed by slow centrifugation. This maneuver likewise failed to increase initial platelet recovery. In all instances, the \( T_{\frac{1}{2}} \) and the contour of the platelet survival curves were unaffected by dextran.

**The Effects of Autonomic Drugs**

To evaluate the possibility that failure to initially recover 100 per cent of labeled platelets reflects the equilibration of infused labeled platelets with an extravascular or a marginal pool, attempts were made to mobilize such marginated platelets by the administration of autonomic drugs. For such studies the mean of at least three different samples (12 separate chambers and pipettes) was used for the baseline platelet count (CV = ± 10%).

Nine subjects were given epinephrine intravenously in doses of 0.2 to 0.4 mg. over a period of from 5 to 10 minutes. This produced marked sympathomimetic symptoms and a significant rise in the leukocyte count, but did not change the platelet count significantly. In four of these subjects the epinephrine infusion was carried out on the second or third day after the infusion of platelets labeled in vitro with DFP\(^{32}\). The platelet specific activity was unchanged by the epinephrine infusion.

Acetyl-beta-methylcholine (Mecholyl), a potent cholinergic drug, was administered subcutaneously in 30 mg. doses to two subjects. This produced marked parasympathomimetic symptoms, but no significant change in the

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*The molecular weight of this product, obtained from Cutter Laboratories, Berkeley, California, ranged from 25,000 to 125,000.
platelet count or in the specific activity of labeled circulating platelets. Finally, two subjects were immersed in hot water (40–43°C) for 30 minutes in order to produce maximal sustained cutaneous vasodilation. This procedure did not change either the platelet count or the specific activity of labeled circulating platelets.

**Discussion**

1. The Definition of and Determinants of Platelet Survival

The question as to whether the primary determinant of platelet survival is senescence or random destruction remains controversial, and the answer devolves mainly on the interpretation of the contour of platelet survival curves. Several workers have found rectilinear platelet survival curves and have interpreted this as indicating that platelet lifespan is determined primarily by senescence. Others have found platelet survival to be a semilogarithmic function, an observation which suggests that random destruction is the primary determinant of platelet survival.

Recycling of radioactivity can make a rectilinear survival curve appear semilogarithmic, and since it has been demonstrated that P32 and C14-labeled serotonin24 recycle to a significant degree, this phenomenon probably explains the results obtained with these isotopes.23 However, the semilogarithmic survival curves which have been obtained with other isotopes are not so readily explained, and in all instances the difficulty is compounded by the fact that the difference between a rectilinear and a semilogarithmic plot is often not clear-cut. This is due to the wide scatter of points, inevitable in cell survival studies, and to the complexity of platelet survival curves obtained with most current technics.

These problems are well illustrated by the present data. The platelet survival curves obtained with DFP using both the in vitro and the in vivo technics (Figs. 1 and 2) and triphasic, and when analyzed statistically in their entirety (i.e., phases I, II, III), fit both a rectilinear and a semilogarithmic plot with equal accuracy. The deviations from rectilinearity which occur during phase I and the pronounced "tailing" of the survival curves noted in phase III can be excluded from the calculations by fitting the curve to points obtained after 5 hours and before 180 hours (i.e., points of phase II). When the in vitro DFP survival curve (Fig. 1) is analyzed in this manner, the data fit a rectilinear estimating equation with greater accuracy than a semilogarithmic estimating equation. A similar analysis of the data obtained with the in vivo DFP2 technic (Fig. 2) produced identical results. This suggests that during phase II, platelet survival is mainly a rectilinear function and is determined primarily by senescence. It must be emphasized that, due to the grossness of the technics employed, a component of random destruction cannot be excluded. Some indirect evidence suggests that some platelets are destroyed by random processes, possibly by being consumed.

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*rectilinear $S_{y,x} = \pm 15\%$, $r^2 = .785$; semi-logarithmic $S_{\log y,x} = \pm 16\%$, $r^2 = .729$.

†rectilinear $S_{y,x} = \pm 8\%$, $r^2 = .855$; semi-logarithmic $S_{\log y,x} = \pm 14\%$, $r^2 = .705$. 
during physiologic intravascular coagulation. It is possible, as others have suggested, that both senescence and random destruction are involved in the physiologic fate of platelets, the former predominating under normal circumstances.

Due to the complexity of platelet survival curves, it is difficult to define platelet lifespan exactly. In the present data, the figures obtained for the T½ of phase II or the rectilinear phase of platelet survival, and the approximate lifespan obtained by extrapolating the regression lines of phase II to zero survival, were the same with all four technics employed—i.e., 92 to 120 hours for the T½, and 8 to 10 days for total lifespan. These approximations compare closely to data previously obtained using other methods and isotopes.

2. The Initial Survival Problem

The inability to initially account for approximately 100 per cent of the labeled platelets remains a central and unsolved problem in all in vitro labeling methods. Whether this is due to extracorporeal injury or to the equilibration of labeled platelets with an extravascular or marginal pool cannot be answered by presently available data.

In the present experiment, platelets were labeled in whole blood or in platelet-rich plasma in an attempt to minimize platelet injury during the preparation, washing, and resuspension of platelet concentrates. In the in vitro DFP method (Fig. 1), the time elapsed between phlebotomy and the injection of the labeled platelets ranged from 90 minutes—the absolute minimum for in vitro labeling with DFP—to 210 minutes, but within this range storage time did not significantly affect initial survival. The Cr survival studies reported here (Fig. 3) were performed primarily as controls, but employed some modifications of the technic described by Aster and Jandl, in order to ascertain whether or not the inability of these workers to initially recover 100 per cent of the labeled platelets is the result of mechanical damage produced during the preparation of platelet concentrates. Platelets were labeled in plasma to avoid centrifugation and manual resuspension of the platelet button. The resulting platelet specific activity was much lower than that obtained when platelet concentrates were labeled in a smaller volume, but the determined initial survival was 81 per cent, which is slightly greater than the 65 per cent initial recovery reported by Aster and Jandl. The scatter of points was somewhat greater than in the comparable in vitro DFP technic, as evidenced by the larger S, an observation which is probably due to the necessity of including a platelet count, with its high error, in the calculation of specific activity. Otherwise, the results obtained did not differ significantly from those of Aster and Jandl. The initial survival problem thus does not appear to be the result of mechanical platelet injury. A more subtle injury inherent in extracorporeal storage may be the explanation.

Dextran has been found to coat platelets and vascular endothelium and in larger doses to prolong the bleeding time and retard the formation of experimental thrombi in animals. The effects of this compound on platelet
survival were assessed, but in the amounts utilized and under the various circumstances tested, dextran did not affect either the initial recovery of labeled platelets or the contour of the platelet survival curve.

Were the initial survival problem due to the equilibration of the infused labeled platelets with an extravascular or marginal pool, drugs or physiologic stimuli which affect small vessels might mobilize such margined platelets. The possibility that platelets are margined in skin vessels is attractive teleologically, but parasympathomimetic agents and prolonged cutaneous vasodilatation by heat failed to alter the platelet count. Epinephrine, which is capable of mobilizing the marginal granulocyte pool in humans,17 in our hands did not significantly affect the platelet count in normal subjects. However, Aster34 was able to produce a consistent rise in the platelet count of normal subjects by infusing larger amounts of epinephrine over a longer period of time. This suggests that our negative results may be attributable to the smaller doses of epinephrine administered or to the injection technic employed. It is thus possible that a physiologic platelet pool in the spleen, as hypothesized by Aster34 and others,35,36 may explain the initial survival problem.

3. DFP2 as a Platelet Label

Whether employed in an in vitro technic or given in vivo, DFP2 provides an approximation of platelet lifespan which compares closely to most other methods. However, the deviations from rectilinearity noted during phase I and phase III remain unexplained.

During phase I of the in vitro survival curve (Fig. 1), a rapid fall in platelet specific activity was the most striking feature. This “sag” can be measured roughly by the difference between the determined T0 survival and the extrapolated T0 survival. A comparable “sag” was not observed when platelets were labeled in vitro with the Cr51 technic (Fig. 3) which employs the same platelet manipulation, anticoagulant, and storage time. This suggests that the initial “sag” in the in vitro DFP2 survival curve is an artifact due either to the elution of DFP2 or to the removal of a population of platelets injured by DFP2.

Elution of DFP2 has not been considered a problem in the labeling of either erythrocytes or leukocytes, and in vitro studies have established that DFP2 is a firm platelet label37 when employed in low concentrations (0.1 to 0.5 μg./ml. blood), but concentration-dependent elution from platelets labeled with the higher concentrations employed in the in vitro platelet labeling method (0.4 to 1.2 μg./ml. blood) could explain the “sagging” curve of phase I. However, the experiments in which platelets were labeled simultaneously with both Cr51 and DFP2 (Fig. 4) appear to exclude significant elution of DFP2. Were initial elution responsible for the phase I “sag,” one would expect a dissociation of the Cr51 points from the DFP2 points, resulting in a rectilinear survival curve computed with the Cr51 points and a “sagging” curve computed with the DFP2 points. Such was not the case. The survival curves of Figure 4, whether plotted with Cr51 points,
DFP$^{32}$ points, or both together, evidence a significant initial “sag,” an identical slope, and are in most respects identical with the in vitro DFP$^{32}$ survival curves (Fig. 1).

The alternative conclusion seems likely—i.e., that DFP$^{32}$ in some manner injures some of the platelets. In the in vitro technics this effect appears to be irreversible, since in no case did platelet specific activity rise toward or above the T$_0$ value. This contrasts to the toxic effects of EDTA, which produces a low initial survival and a subsequent rise in platelet specific activity, due presumably to temporary hepatic sequestration of reversibly injured platelets.$^{1,2}$ The results further suggest that DFP$^{32}$ affects only a portion of the labeled platelet population and that the remainder survives normally, since in the data obtained with the in vitro DFP$^{32}$ technic, there was no correlation between either the initial recovery of platelets or the magnitude of the phase I “sag,” and the T$_{1/2}$ of phase II or the extrapolated total lifespan.

In phase I of the in vivo curve (Fig. 2), the initial deviation from rectilinearity appears as a reversible “sag.” This phenomenon has been observed by others$^5$ and is reproducible. The aforementioned results obtained with the in vitro DFP$^{32}$ labeling technic suggest that DFP$^{32}$ is, in some manner, injurious to platelets. Consequently, it is possible that phase I of the in vivo curve may represent temporary sequestration of a portion of the labeled platelets due to a reversible injury produced by low concentrations of DFP$^{32}$, whereas phase I of the in vitro curve may represent permanent sequestration produced by irreversible platelet damage due to the higher concentrations of DFP$^{32}$ required in this technic. This hypothesis implies that the labeling of the circulating platelet population with DFP is not entirely uniform. Presently available data cannot explain phase I of the in vivo curve with certainty.

The “tail” or phase III of the survival curves obtained with both technics using DFP$^{32}$ (Figs. 1 and 2) has been observed by several workers.$^6,8,13$ This also cannot be interpreted with certainty on the basis of presently available data. One possibility is that this phenomenon could be due to the recycling of $P^{32}$. The present data suggest that radioactivity from DIP$^{32}$, the main degradation product of DFP$^{32}$, does not significantly recycle into human platelets, but animal studies have demonstrated pathways by which $P^{32}$ could recycle.$^6$ and some recent preliminary data$^{13}$ suggest that recycled radioactivity from DFP$^{32}$ can be detected in human platelets beginning 4 days after the administration of the isotope. It nevertheless remains uncertain whether recycling alone can explain the tailing curve observed in phase III. There are two reasons for this statement. First, the survival curves obtained with in vitro Cr$^{51}$ technics$^1$ have a comparable tail,$^{1,21}$ and there are no data which suggest that this isotope recycles. Second, the tailing of phase III of the in vitro DFP$^{32}$ curve is equivalent in magnitude to that found in the in vivo studies, in spite of the fact that the total DFP$^{32}$ administered in the latter method is 4-5 times greater than in the in vitro method. This argues against recycling as the sole cause of the tailing curve, since one would expect
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recycling to be proportional to the total amount of isotope administered. Since high concentrations of H\textsuperscript{3}-DFP have been shown autoradiographically to label megakaryocytes \textsuperscript{38} in animals, a further possibility is that DFP\textsuperscript{32} could label the megakaryocyte and thereby introduce an additional cohort population.\textsuperscript{2} Finally, since the circulating platelet population is apparently heterogenous,\textsuperscript{39} the tailing curve of phase III could represent the persistence of a long-lived population of labeled platelets.\textsuperscript{1}

CONCLUSIONS

A technic for the in vitro labeling of human platelets with DFP\textsuperscript{32} is presented, critically evaluated, and compared to in vivo methods employing DFP\textsuperscript{32} and to in vitro methods using Cr\textsuperscript{51}. The initial recovery of platelets labeled in vitro with DFP\textsuperscript{32} averaged 79 per cent, but the survival curve was characterized by an irreversible initial loss of platelet radioactivity. Experiments in which platelets were simultaneously labeled in vitro with both DFP\textsuperscript{32} and Cr\textsuperscript{51} suggest that this is not due to elution of DFP\textsuperscript{32}. The survival curve of platelets labeled in vivo with DFP\textsuperscript{32} shows an initial transient reduction in platelet radioactivity. It is suggested that both of these aberrations in initial survival are the result of platelet injury by DFP\textsuperscript{32}. Significant “tailing” was observed in the survival curves obtained with DFP\textsuperscript{32}, and possible explanations of this phenomenon are discussed. DFP\textsuperscript{32}-labeled platelets circulating after 5 hours apparently survive normally and disappear from the circulation as a rectilinear function over the next 6–8 days. Although both in vitro and in vivo labeling methods employing DFP\textsuperscript{32} provide a meaningful approximation of platelet lifespan, the initial and terminal aberrations of the survival curves greatly complicate further interpretation. Dextran had no detectable effect on platelet survival, and epinephrine, Mecholyl, and cutaneous vasodilatation did not alter the platelet count or the specific activity of circulating labeling platelets in human subjects. The problem of initial platelet survival and the question of an extravascular or marginal platelet pool is discussed in the light of these data.

SUMMARIO IN INTERLINGUA

Un technica pro le marcage in vitro de plachettas human con fluorophosphato diisopropylic a phosphoro radioactive (DFP\textsuperscript{32}) es presentate, evaluatate criticamente, e comparaate con metodos in vivo empleante DFP\textsuperscript{32} e con metodos in vitro empleante Cr\textsuperscript{51}. Le recovrage initial de plachettas marcate in vitro con DFP\textsuperscript{32} esseva al media 79 pro cento, sed le curva de superviventia esseva characterisate per un irreversibile perdita initial de radioactivitate plachettal. Experimentos in le quales plachettas esseva marcate in vitro simultaneemente con DFP\textsuperscript{32} e Cr\textsuperscript{51} suggestione que isto non es efecto de un elision de DFP\textsuperscript{32}. Le curva de superviventia de plachettas marcate in vivo con DFP\textsuperscript{32} monstra inicialmente un reduction transiente in le radioactivitate plachettal. Es suggestione que ambe iste aberrationes in le superviventia initial es le resultato de un traumatismo del plachettas per DFP\textsuperscript{32}. Un significative
phase "caudal" esseva observate in le curvas de supervivencia obtenite con FDP². Explicationes possibile de iste phenomeno es presentate. Plachettas circulante post 5 horas supervive apparentemente de maniera normal e dispare ab le circulation in function rectilineari in le curso del proxime 6 a 8 dies. Ben que tanto le methodos a marcage in vitro como etiam illos a marcare in vivo que emplea FDP² provide resultatos que representa un significative approximation al longevitate de plachettas, le aberrationes initial e terminal del curvas de supervivencia introduce un grande complication pro le interpretation ulterior. Dextrano exerceva nulle detegibile effecto super le supervivencia plachettal, e epinephrina, Mecholyl, e vasodilatation cutanee non alterava le numeration plachettal o le activitate specific de circulante plachettas marcate in subjectos human. Le problems del supervivencia initial de plachettas e le question de un pool de plachettas extravascular o marginal es discutite in le lumine de iste datos.

ACKNOWLEDGMENTS

The cooperation of Dr. William Knott, Warden John Turner, and the officers of the Utah State Prison is gratefully acknowledged. The authors are also indebted to the inmates who volunteered for these studies, and to Miss Vreni Oberholzer and Miss Helen Ashenbrucker who provided valuable technical assistance.

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Radioactive Diisopropyl Fluorophosphate as a Platelet Label: An Evaluation of in Vitro and in Vivo Technics

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