The Use of $^{32}$Di-Isopropylfluorophosphate ($^{32}$DFP)
As A Platelet Label: Evidence for Reutilization
of This Isotope In Man

By D. P. Cooney, B. A. Smith and D. E. Fawley

Many isotopes have been used to label human blood platelets, but two
have been used extensively: sodium chromate$^{5}$ ($^{51}$Cr) and $^{32}$DFP.$^{1,2,3,4,5}$
The former requires in vitro manipulation of platelets with possible damage to
these cytoplasmic fragments. Moreover, when EDTA is used as the anticoagu-
lant, only one third of the injected radioactivity circulates in platelets, and the
peak in vivo activity is delayed six to twenty-four hours after infusion of the
labeled platelets. With the use of “acid citrate,” Aster and Jandl$^{6}$ have devel-
oped a method of in vitro labeling that eliminates the early sequestration and
allows two thirds of the platelets to circulate in normal individuals. Platelet
damage during the labeling process is minimal with their method.

Because of early sequestration when EDTA is used, and because of a “tail-
ing” of the curve after day 8 regardless of the anticoagulant (see discussion),
it has been difficult to determine the nature of the platelet decay curve in man.
Most investigators agree that it is linear, but statistical support of this concept
is lacking. Another problem with $^{51}$Cr labeling is that when marked thrombo-
cytopenia is present, one must use isologous platelets. In this situation, the
problem of an undetected isoantibody against platelets arises.$^{7}$

$^{32}$DFP can be used as an in vivo platelet label that avoids in vitro manipu-
lation. Furthermore, the compound has been reported to have some of the
attributes of an ideal label in that it binds by covalent bonds to proteins and that
its major metabolic product $^{32}$di-isopropylphosphosphate ($^{32}$DIP), does not label
cells nor is it reutilized. For these reasons we chose this isotope for determining
the nature of the platelet decay curve in normal man. Another purpose of this
study was to develop a sensitive enough counting technic so that patients with
low platelet counts could be evaluated with autologous in vivo labeled plate-
lets. During the course of this investigation, several problems arose with the
use of $^{32}$DFP; the solution of these problems led us to a study of some aspects
of the metabolism of this compound in man.

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MATERIALS AND METHODS

The Isotope

The $^{32}$DFP used in these experiments was dissolved in propylene glycol (1 mg./ml.) and has a stated specific activity of from 100 to 355 ftc/mg. (New England Nuclear Corp.). We assayed this material in the following manner:

1. Measurements of inorganic phosphate (Pi) and total phosphate were made.8
2. DFP content was measured by the methods of Marsh and Neale9 based on a standard curve determined with pure DFP (kindly supplied by Merck and Co.).
3. Thin-layer chromatography (TLC) was performed with cellulose powder (Macherey, Nagel & Co., MN-300) and water:redistilled n-butanol:acetic acid (50:40:11) as the developing solvent. These two phases were thoroughly mixed and development obtained with the n-butanol phase in an atmosphere saturated with the water phase held in small beakers. The method of Hanes and Isherwood10 was used for detection of the phosphate compounds. This chromatographic technic separates Pi, DFP, and DIP with Rf values of .31, .60, and .92, respectively. The DIP standard was prepared from the pure DFP by hydrolysis in water for several days at room temperature. Mixtures of two or three of these compounds were readily separated, and as little as 1 per cent Pi contamination of the DFP could be detected. Unfortunately, the propylene glycol used as a diluent for the $^{32}$DFP and the isotope’s dilute state made detection difficult, and a $^{32}$DFP spot was rarely seen. However, mixtures of $^{32}$DFP with standard Pi and DIP could be separated and a 1 per cent Pi contamination detected. $^{32}$DIP present in samples of $^{32}$DFP that had been kept several months at room temperature could be detected with TLC.

Subjects

Normal subjects were volunteer students with normal blood counts and several hematologically normal individuals drawn from the wards and clinics of Palo Alto-Stanford Hospital. Certain data from patients with hypersplenic thrombocytopenia before and after splenectomy and from patients with polycythemia vera are also reported. Platelet counts were obtained before injection and every third day of the study and were stable during the lifespan determination.

Injection

Just before intravenous administration, the $^{32}$DFP was diluted in 5 ml. of normal saline and injected at 1 ml./min. No pharmacologic side effects were noted in normal controls with doses of 0.69 to 2.2 mg. (100 to 242 μc). In three individuals, we confirmed the previously reported11,12 urinary excretion of radioactivity, 50 per cent in five days with most of this occurring in the first day. Considering the physical T½ of $^{32}$P and the biologic T½ of $^{32}$DFP, and assuming distribution in the total body water, one can estimate that the total body dose is 0.3 rad./100 μc $^{32}$DFP in a 70 Kg. man. However, animal data indicate that the kidneys and liver tend to concentrate radioactivity three to ten times the amount in plasma.13 Our calculations are not directly comparable to Carby’s14 but appear to be of the same order of magnitude.

Platelet Separation

Samples were routinely obtained at three hours after injection and then daily for nine to fourteen days. During the latter days of study, double samples were obtained in order to maintain counting efficiency.

Fifteen ml. of whole blood were collected by free flow into 3 ml. of 1 per cent versene in saline and 7 ml. of a high-molecular-weight dextran (m.w. 460,000, kindly supplied by Pharmuchem Corp.). Siliconized glassware was used throughout. A platelet-rich plasma (PRP) was obtained by allowing the red cells to sediment for one and a half hours at room temperature. Virtually all of the platelets in the original 15 ml. of whole blood were present in the PRP. Contaminating red and white cells were then removed by several 5 min., 100 g. centrifugations at 25 C. in an International PR-2 centrifuge. The final PRP contained one-
half to three-fourths of the original platelets and had a red and white cell contamination of less than one cell per five thousand platelets. It is possible that the centrifugation selects a less dense platelet population for radioactive counting. The total number of platelets and the contamination were determined by chamber counts and volume of the PRP. The latter was centrifuged at 1,000 g. at 25 C. for 20 min., and the platelet button washed twice in versene-saline. Platelet losses during the later stages of preparation were less than 0.1 per cent, and the second wash contained no detectable radioactivity. The number of platelets harvested varied from day to day and from subject to subject, but a minimum of .7 billion was obtained.

**Platelet Fractions**

Platelet lipids were obtained by extraction with redistilled chloroform:methanol (2:1) of whole washed platelets frozen and thawed twice in distilled water or of a trichloroacetic acid (TCA) precipitate of the disrupted platelets. There was no difference between these two starting materials in the yield of lipid phosphorus or radioactivity. The lipid fraction was chromatographed by a modification of Skipski's technic that employed silica gel H (E. Merck AC) and a developing solvent of redistilled chloroform: redistilled methanol: water:ammonium hydroxide (75:25:4:1). Iodine vapor was used for detection. Standards were obtained through the courtesy of Dr. John Farquhar. The lipid fractions were eluted from the gel with chloroform:methanol (1:1) and counted by dissolving them in the toluene scintillating fluid.

The TCA-soluble fraction was obtained by adding 2 volumes of 28 per cent TCA to the platelets which were frozen and thawed twice in distilled water; the TCA was removed by extraction with 4 volumes of ether. In this step there were some losses of radioactivity because the TCA-soluble radioactivity determined by direct counting was usually less than the indirectly calculated activity (whole-platelet-specific activity minus TCA-precipitate-specific activity).

For the more detailed evaluation of the TCA-soluble activity, platelets were harvested from 4 units of PRP (Fenwal double-plasmapheresis units) on days 1 and 16 after $^{32}$DFP injection. The TCA-soluble fraction was dialyzed in two changes (25:1) of distilled water at 4 C. for a total of eighteen hours. The outer dialysate was concentrated to 30 ml. by flash evaporation (Buchler Instruments), made alkaline (pH 8-10) with 1 to 2 drops of ammonium hydroxide, and chromatographed by modification of Bartlett's technic. A 1 by 7 cm. column of an anion exchange resin (Cal. Biochem. AG 1-x8 as the chloride form, 100 to 200 mesh) was rinsed with 100 ml. 1N HCl and the dialyzable material applied. The column was eluted with 25 ml. .003N HCl (tube 1), 125 ml. .01 N HCl (tubes 2 to 6), and 100 ml. .02 N HCl (tubes 7 to 10). Further elution with .02 N HCl or .50 N ammonium chloride failed to yield radioactive material. Unfortunately, radioactivity was low, which required the pooling of tubes (1 + 2, 3 + 4, etc.) in order to have enough activity for accurate counting. The 50 ml. aliquots from two tubes were concentrated, one-half counted and the other half chromatographed on cellulose powder. The fractions were eluted with water from the cellulose and counted as outlined below.

**Determination of Radioactivity**

Whole platelet buttons (original method), TCA precipitates solubilized with sodium hydroxide (revised method), or water soluble fractions were placed on rectangles of #2 Whatman filter paper and dried. A wash of the centrifuge tube was dried on a second paper, and both were put in a siliconized counting vial containing 10 ml. of scintillating fluid (4 Gm. PPO, 50 mg. POPOP in 1 L. of toluene). Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer and the results expressed as counts per minute (CPM) per 10^9 platelets (whole platelet or TCA precipitate). All points have been corrected for background and isotope decay. More recently, we have taken 10 l aliquot of the TCA precipitate in sodium hydroxide solution for determination of total phosphate and counted 100 l of this solution. These results are expressed as CPM/µg. Pi. Background varied from 8 to 11 CPM, and all sample counts were at least twice background with a counting error of ±5 per cent.
Evaluation of this technic indicated that the paper position—folded, crushed, held against the side of the vial—made no difference in the CPM of standard papers. A second or third blank paper in the vial did not interfere with a standard paper, and two standard papers gave additive results. There was no quenching when a standard amount of radioactivity was added to a "cold" platelet button, and there was very little elution of radioactivity into the toluene scintillating fluid. This technic is 76 per cent efficient when a standard phosphate solution is used (Nuclear Chicago). Each batch of NaDFP was standardized and the efficiency varied from 62 to 72 per cent (mean of 66 per cent). These data indicate a NaDFP concentration of .87 mg./ml. which fits well with the DFP and phosphate assays (see results).

Statistics

The platelet decay curve was determined for the fourteen individuals studied by the original (whole platelet) technic, and for seven individuals studied by the revised (TCA precipitate) technic. The raw data (CPM/10⁶ platelets) from days 1, 2, 3 and 7, 8 and 9 ("plot data") were used to draw a line by the method of least squares, and the day 0 and 4, 5, 6 and 10 data ("test data") were used to test the hypothesis with the assistance of a computer. All data from each individual were plotted separately with no assumption about the lifespan or the extrapolated zero-time activity. We are indebted to Dr. Rupert Miller and his staff for these statistical analyses.

If a linear function is plotted with the "plot data," and the curve is an arithmetic function, then the "test points" should fall randomly about the line. If, however, the curve is an exponential function, then the early and late "test points" would fall above the line and the middle points below it. Conversely, a line can be drawn with the logs of the "plot data." If the line is an exponential, then the logs of the "test points" should fall randomly about the line. However, if the line is arithmetic, then the logs of the "test data" would fall below the line early and late, and above the line in the middle. Each of the above steps was performed, and the deviations of the "test data" from the lines was determined. A t value was obtained, and the p of the hypothesis determined for each test point.

The duration of the platelet lifespan of each person was determined by the method of least squares from all data obtained during the first nine days of each study. The point at which the line crossed the x-axis was taken as the lifespan.

Other Methods

Peripheral blood counts were done by routine hematologic technics. All platelet counts were performed by the phase-contrast method with a counting error of ±5 per cent (standard error of the mean). Megakaryocytes were concentrated and autoradiography performed by methods previously described. Platelet adhesion was determined by adding 0.1 ul. of 10⁻⁴ M adenosine diphosphate to 1 ml. PRP in citrate and observing for gross clumps at 37 C. The bleeding times were performed by Borchgrevink's modification of Ivy's method. Clot retraction was estimated grossly by observing a 1 ml. sample of whole blood incubated one hour at 37 C.

Results

The results of the studies on all batches of NaDFP indicated that no Pi was present (TLC and direct determination); this agrees with data furnished by the manufacturers and with the fact that the NaDFP is separated from its reaction mixture by distillation. The total phosphate and DFP assays were usually in good agreement, but indicated that the concentration of DFP was less than the reported 1 mg./ml. (mean .832 mg./ml. and .886 mg./ml., respectively). On several occasions the DFP assay showed far less NaDFP than the phosphate assay would indicate. Presumably this meant hydrolysis of the NaDFP to NaDIP...
Table 1.—Per Cent of Whole Platelet Radioactivity in Two Platelet Fractions after ³²DFP Injection

<table>
<thead>
<tr>
<th>Days After ³²DFP</th>
<th>TCA soluble</th>
<th>Chloroform-methanol soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Samples</td>
<td>Mean Range</td>
<td>Number of Samples</td>
</tr>
<tr>
<td>0–4</td>
<td>9 15</td>
<td>12–21</td>
</tr>
<tr>
<td>5–9</td>
<td>9 15</td>
<td>1–28</td>
</tr>
<tr>
<td>10–23</td>
<td>7 40</td>
<td>24–59</td>
</tr>
</tbody>
</table>

Per cent of whole platelet specific activity.

which we were able to confirm by TLC. The hydrolyzed ³²DFP gave lower than expected specific activity, but otherwise gave satisfactory results.

Platelet Injury

Injection of ³²DFP did not cause a change in the clot retraction, adenosine diphosphate aggregation of platelets, platelet count, or the bleeding time. When ³²DFP was added to whole blood or to PRP in vitro, there was no effect on clot retraction or platelet aggregation.

Megakaryocyte Labeling

On two occasions bone marrow was sampled twenty-four hours after injection of 150 μc. of ³²DFP, and no grains above background could be seen in the megakaryocytes. Weakly labeled myeloid forms could be detected. In addition, as much as 4 mc. of tritiated DFP was given to rabbits by aortic injection. Bone marrow sampled from the tibia showed no megakaryocyte activity in the autoradiograms.

Isotope Elution

Only one of our twenty-one normal curves has shown a decline between three and twenty-four hours greater than that expected due to platelet turnover during that interval combined with the error of any one point. During three studies, we obtained points at one, three, six, twelve and twenty-four hours, and no evidence of elution could be detected. Platelets harvested at one, three, and twenty-four hours were suspended in "cold" autologous plasma, and there was no loss of radioactivity into the plasma after one hour incubation at room temperature.

Isotope Reutilization

Some of the platelet lifespan curves plotted on linear paper tended to flatten out after day 8 (top line, Fig. 1); initially, we interpreted this to mean that the lifespan was an exponential function. However, when nine samples were obtained on six individuals between days 13 and 23, we found that this "late" activity did not continue to decline but remained constant.

Table 1 shows the results of five studies in which the whole platelet activity on each day was fractionated into TAC-soluble and chloroform-methanol-soluble portions. The TCA-soluble portion remains constant at about 15 per cent
The numbers refer to the per cent of whole platelet radioactivity in the three platelet fractions.

of the whole platelet specific activity for nine days and then rises to a mean of 40 per cent and a high of 59 per cent. The lipid fraction contains no activity for four days, gains some from day 5 to 9, and thereafter rises to a mean of 30 per cent and a high of 47 per cent of the whole platelet activity.

A representative experiment is shown in Figure 1 which depicts the day 5 to 16 part of a lifespan curve. In this figure, the top line represents the whole platelet activity, the middle line the TCA-precipitable activity, and the lower line the “residual” activity or TCA-precipitable minus the chloroform-methanol-soluble activity. The TCA-soluble portion rises from 9 to 21 per cent to 40 to 59 per cent while the chloroform-methanol soluble rises from zero to 12 to 37 per cent of the whole platelet activity. The residual activity declines from 85 per cent to 10 to 23 per cent of the whole-platelet-specific activity. This latter specific activity persisted at a constant level until day 21 and is probably a phosphorus-containing protein that is not soluble in lipid solvents.

Chromatography of the chloroform-methanol fractions from three individuals on day 16 showed that activity could be found in phosphatidyl choline,
phosphatidyl ethanolamine, and sphingomyelin, but none was clearly detectable in phosphatidyl serine or phosphatidyl inositol.

To study the TCA-soluble fractions in more detail, three normal individuals were injected with $^{32}$DFP, and 4 units of PRP were harvested on days 1 and 16. Their platelet counts did not change during the course of the study. The platelets ($3 \to 5 \times 10^9$) were separated, washed, and a TCA precipitate made. The latter was extracted with chloroform-methanol, and our earlier results with smaller numbers of platelets were confirmed; i.e., no activity on day 1 and 45 per cent on day 16. The TCA-soluble fraction was extracted with ether and dialyzed against distilled water. All of the TCA-soluble radioactivity on both days 1 and 16 passed the dialysis membrane, which means that these $^{32}$P-containing compounds were not bound to the glycoproteins known to be present in TCA supernatants or were hydrolyzed from these proteins.

The concentrated outer dialysate was then subjected to anion-exchange column chromatography. Figure 2 shows the results of one of the three experiments, all of which gave similar results, and also shows the elution of standard $^{32}$DIP from the column. Both on days 1 and 16 the peak activity is in tubes 5 and 6, though there is much more spread of the activity on day 16 (yields 110 to 130 per cent). The $^{32}$DIP elutes in tubes 7, 8, 9, and 10 (yield 82 per cent). Hence, little of the TCA-soluble activity is $^{32}$DIP.

Tubes 5 and 6 from both days were examined by TLC on cellulose powder. Three phosphorus compounds could be detected with Rf values of .39, .58,
Table 2.—Thin-Layer Chromatographic Separation of Phosphate-Containing Compounds in Tubes 5 and 6 of the Column Eluates

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th></th>
<th>Day 16</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts Per Minute</td>
<td>Per Cent</td>
<td>Counts Per Minute</td>
<td>Per Cent</td>
</tr>
<tr>
<td>Slow-moving fraction</td>
<td>8.6</td>
<td>17</td>
<td>11.3</td>
<td>22</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>20.9</td>
<td>40</td>
<td>33.1</td>
<td>64</td>
</tr>
<tr>
<td>Fast moving fraction</td>
<td>16.4</td>
<td>32</td>
<td>1.7</td>
<td>3</td>
</tr>
<tr>
<td>Remainder of plate</td>
<td>6.0</td>
<td>11</td>
<td>5.8</td>
<td>11</td>
</tr>
<tr>
<td>Per cent recovery</td>
<td>70</td>
<td></td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.—Comparison of Radioactivity in Several Platelet Fractions after \(^{32}\)DFP and Inorganic \(^{32}\)Phosphate Administration to Patients with Polycythemia Vera

<table>
<thead>
<tr>
<th></th>
<th>23 Days After 442 mc. (^{32})DFP</th>
<th>8 Days After 50 mc. (^{32})Pi</th>
<th>2 Mos. After 5 mc. (^{32})Pi</th>
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<tbody>
<tr>
<td></td>
<td>CPM/10(^{6}) Platelets</td>
<td>%</td>
<td>CPM/10(^{6}) Platelets</td>
</tr>
<tr>
<td>Whole platelet</td>
<td>37.0</td>
<td>100</td>
<td>40.6</td>
</tr>
<tr>
<td>TCA precipitate</td>
<td>22.0</td>
<td>59</td>
<td>18.5</td>
</tr>
<tr>
<td>TCA soluble*</td>
<td>15.0*</td>
<td>41</td>
<td>22.1*</td>
</tr>
<tr>
<td>Chloroform-methanol soluble</td>
<td>17.9</td>
<td>48</td>
<td>20.4</td>
</tr>
<tr>
<td>Sum of 3 and 4</td>
<td>32.9</td>
<td>89</td>
<td>42.5</td>
</tr>
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*By difference (1 minus 2).

and .74. When directly compared to Pi, DIP, and DFP, only one spot could be identified, namely, Pi with an RF of .58. This RF value is different from Pi when migrated alone (RF .31). However, we believe this fraction is inorganic phosphate or a low-molecular-weight compound that binds phosphorus for three reasons: first, about half the total phosphate in tubes 5 and 6 was inorganic by direct analysis. Second, when the plates were sprayed with the detecting solution, Pi spots turned yellow immediately and became dark blue on heating, while organic phosphates do not become yellow and require hydrogen sulfide exposure for development of a light blue color; third, mixtures of Pi with the platelet fraction present in tubes 5 and 6 from the column showed that the spot at RF .58 became much darker, and no spot at RF .31 was seen. Mixtures of DIP and DFP with this platelet fraction showed no change in the migration of these two compounds.

Table 2 compares the TLC results on the migration of the \(^{32}\)phosphate-containing compounds in tubes 5 and 6 on days 1 and 16. Activity is present in all three spots on day 1, but on day 16 the Pi spot increases at the expense of the fast-moving fraction. Thus, while the exact identification of the TCA-soluble compounds cannot be made, it does seem clear that they are dialyzable and contain inorganic phosphate. It is possible that the \(^{32}\)Pi is liberated during processing of the TCA-soluble fraction, since acid hydrolysis of DFP is known to liberate Pi from DFP. While there are minor differences between days 1 and 16, there is no clear change in the distribution of radioactivity early and late after \(^{32}\)DFP injection.
Fig. 3.—Comparison of the mean lifespan curves on normal subjects by the whole platelet method and the corrected TCA precipitate method (see text). Differences in specific activity occur after day 6 and are due to isotope reincorporation.

The data on phospholipid activity and, less convincingly, the results on the TCA-soluble fraction indicate that the platelet radioactivity has changed its locus during a platelet lifespan study. These data are consistent with the hypothesis that $^{32}$Pi is liberated after $^{32}$DFP injection. Table 3 compares platelet fraction radioactivity twenty-three days after 442 $\mu$c. $^{32}$DFP into one patient and after $^{32}$Pi injection into two patients, all three afflicted with polycythemia vera. Since the specific activities (corrected for $^{32}$P decay) of the platelet fractions in the first two patients are similar, one can estimate that about 50 $\mu$c. or one ninth of the $^{32}$DFP was liberated and entered the phosphate pool.

Platelet Lifespan in Normal Individuals

Figure 3 shows the linear plots of our platelet lifespan studies in normal individuals. The zero time or 100 per cent values were obtained for each curve from analysis by the method of least squares. The upper curve is a plot
Table 4.—Statistical Analysis

<table>
<thead>
<tr>
<th>Time</th>
<th>Linear Data</th>
<th>Log of Data</th>
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<tbody>
<tr>
<td></td>
<td>t</td>
<td>P-value</td>
</tr>
<tr>
<td>0</td>
<td>1.93</td>
<td>.79</td>
</tr>
<tr>
<td>4</td>
<td>-6.4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>.12</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-9.2</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>3.06</td>
<td>.01</td>
</tr>
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Revised Method (N = 7)

<table>
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<th>Log of Data</th>
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<tr>
<td></td>
<td>t</td>
<td>P-value</td>
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<tr>
<td>0</td>
<td>6.29*</td>
<td>.01*</td>
</tr>
<tr>
<td>4</td>
<td>-2.25*</td>
<td>.05*</td>
</tr>
<tr>
<td>5</td>
<td>.88</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1.74</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>3.30</td>
<td>.01</td>
</tr>
</tbody>
</table>

*Variation produced by one value that was greatly different from the other six.

†Not significant at 5 per cent level.

of whole platelet activity and shows the mean data from fourteen studies on thirteen people who had a mean platelet count of 234,000/mm.³ By this technic the mean lifespan is 11.8 days with a standard deviation of 1.2 days and a range of 9.7 to 13.9 days.

Seven individuals with a mean platelet count of 264,000/mm.³ were studied by the revised technic in which the specific activity in a TCA platelet precipitate was corrected for chloroform-methanol-soluble activity (lower curve Fig. 3). The mean lifespan is 10.9 ± 1.0 days with a range of 9.4 to 12.4 days. The combined data on twenty individuals gives a mean platelet lifespan of 11.5 ± 1.2 days.

Reproducibility

Duplicate lifespans were done on five individuals. The mean variation was 0.9 days with a maximum of 2.2 days. Sixteen duplicate points have been taken early and late during several platelet lifespans performed by the whole platelet method. These duplicates varied by ± 11 per cent (standard error of the mean). Ten duplicate points obtained with the revised technic have varied ± 15 per cent.

Statistical Analysis

Statistical analysis of these data is presented in Table 4. By the original method, the linear data are randomly distributed except for day 10 when isotope reincorporation artificially increases the platelet-specific activity. The logs of the "test points" are below at days 0 and 10, but not significantly so; however, they are significantly above the line on days 4, 5 and 6. This is what one would expect if an arithmetic function were being plotted on semilog paper.
By the revised platelet lifespan method, the variations noted in the linear data on days 0 and 4 were caused by only one of the seven values with the other six randomly distributed. This individual is the one referred to in the isotope-elution section. The variation on day 10 has been commented on above. The log data is consistent with the hypothesis of a linear function's being plotted on semilog paper: this is true despite the one high value on day 0 and isotope re-incorporation on day 10.

We feel that the weight of the evidence supports the hypothesis that the platelet decay curves are a regular arithmetic process and that platelets die a senescent rather than a random death.

**DISCUSSION**

³²DFP can be used to label platelets of all ages in the peripheral circulation without in vitro manipulation. We have shown that it does not interfere with several platelet functions in vitro or in vivo. Moreover, it does not appear to bind to megakaryocytes during the period of initial labeling. Our data on this point confirm that of Kurth et al. However, Mustard and his associates report that an occasional labeled megakaryocyte can be detected after ³²DFP administration. This labeling would presumably occur only during the first few hours after ³²DFP injection, and these megakaryocytes would release labeled platelets during their turnover time of three days, thereby causing the platelet decay curve to be flat during the first few days. We have not observed this type of curve in normal individuals. Hence, even though there may be some labeling of megakaryocytes, the magnitude of this is probably not great enough to influence the platelet decay curve.

Several investigators have shown that ³²DFP elutes from red cells of rats, rabbits, and man, but not from the red cells of mice. The elution from human red cells is limited to the first few days after labeling and occurs even though small doses (5 µc. in 32 µg.) are used. Athens, et al. also noted a dialyzable fraction in the supernatant of red and white cells and platelets labeled in vitro. Although they were unable to eliminate the possibility of elution from white cells, it seemed unlikely from their data.

Even though ³²DFP has been shown to elute from human red cells, the evidence for elution from platelets is less certain. Bithell, et al. reported that their lifespan curves did suggest early elution, especially after in vitro labeling. Gardner and Cohen interpret one of Mustard's curves to support the idea of elution. Only one of our twenty-one normal curves declined more rapidly than expected during the first twenty-four hours; these and other data reported earlier do not support the concept of DFP elution from platelets. This apparent lack of elution may result from the fact that only 0.04 per cent of the injected dose is bound to platelets in the normal human. It may be that saturation of platelets by ³²DFP does not occur. Moreover, it is possible that the platelet-binding sites are different from and more specific than those of the red cell.

Our data indicate that about 10 per cent of the injected ³²DFP or one of its metabolites is reutilized in man (Table 3). The presence of the new ³²P-containing compounds can be detected in platelets eight days after ³²DFP injection.
and reaches a maximum of 85 per cent of the whole platelet activity by days 14 to 16. If one allows a three day turnover time for megakaryocytes, then at least five days must elapse before a detectable amount of the $^{32}$P presents itself to these cells. This time lag makes it likely that the source of this late activity is from the catabolism of a $^{32}$DIP-protein complex. Since $^{32}$DFP is rapidly hydrolyzed, and since $^{32}$DIP does not bind to cells or tissues and is rapidly excreted, the direct metabolism of these two compounds is unlikely. The site of $^{32}$DFP binding to enzymes has been extensively studied and shown to be a serine moiety of the protein. Hence, it is likely that $^{32}$DIP-serine is released after metabolism of a protein linked with $^{32}$DIP, and once the two diisopropyl groups were split off the molecule, phosphorylserine could enter all of the phospholipids in platelets as well as the inorganic phosphate pool. All of the necessary steps for this reutilization are documented except for the splitting of the two isopropyl groups from DIP-serine or DIP-enzyme complex.

Mizuno and his colleagues were the first to consider the possibility of reutilization of $^{32}$DFP, and this possibility has been discussed in a review of granulocyte kinetics by Cartwright, et al. In vivo labeling with $^{32}$DFP has been widely used to study granulocyte and red cell kinetics, but for the most part the effect of $^{32}$P reincorporation has not been considered. It may be that some of these data have been influenced by the metabolism of $^{32}$DFP described above.

The mode of platelet disappearance remains an unsettled point. In two recent reviews, Gardner and Cohen support the concept of linear decay, while Mustard et al. suggest the possibility of exponential decay. Over two hundred normal individuals have been studied. Some authors favor a linear decay while other investigators feel that the decay is exponential or report their data both ways. Most of these opinions are derived from inspection of the lifespan curves; however, when statistical analyses have been employed, conflicting results emerge. Part of the difficulty in such analyses probably resides in the fact that even in arithmetic biologic phenomena, such as red cell turnover, there remains a certain variation in the time of cell death. Hence, the difference between senescent and random decay is not completely "either-or." Another difficulty may reside in the fact that there is a "tailing" of the $^{51}$Cr decay curve after day 8. This phenomenon has been interpreted several ways; it is clearly different from the residual activity we have observed with $^{32}$DFP. The former reaches base line by day 14 while the latter persists for over two weeks.

Statistical analysis of our data supports the concept of a senescent decay for platelets. However, acceptance of the statistical analysis is dependent upon accepting our data for isotope reutilization. The nature of the decay curve is of interest in itself as a basic biologic phenomenon. However, there are implications in other areas. For instance, if platelet death is indeed a function of age, then such concepts as day to day systemic blood coagulation or the function of platelets in vascular integrity in normal man would appear to be less likely since these concepts imply random platelet death.

Our data on the duration of the platelet lifespan agree with those of others.
who have used $^{32}$DFP, indicating a mean turnover time of about eleven days. The mean time with $^{51}$Cr is about nine days. There is no certain explanation of this difference, but platelet damage during in vitro labeling seems reasonable. Bithell et al. have shown that in vitro labeling with $^{32}$DFP resulted in a triphasic lifespan curve. The linear phase of this curve gave a mean lifespan of eight days. In their laboratory, in vitro $^{51}$Cr labeling gave a mean platelet lifespan of eight and a half days, while in vivo $^{32}$DFP labeling resulted in a mean lifespan of ten days. The longer time with in vivo $^{32}$DFP may be closer to the true time since no platelet manipulation is involved. Megakaryocyte labeling by $^{32}$DFP does not explain the longer lifespan. As mentioned earlier, this effect would be reflected by a flat curve during the first three days rather than a steady eleven day prolongation.

**Summary**

Certain aspects of $^{32}$DFP metabolism in man have been studied. It is probable that about 10 per cent of an injected dose is metabolized and reappears in the platelet. This affects the platelet decay curve after day 8 and may influence data on red and white cell kinetics obtained with this isotope.

Considering the effects of reincorporation of this compound, we have studied twenty normal individuals and have found a mean platelet lifespan of 11.5 days. Statistical analysis supports the concept that the platelet decay curve is linear: platelets are removed from the circulation because of senescence rather than in a random fashion.

The literature on human platelet lifespans is reviewed. Most authors support the concept of a linear decay curve, although the evidence is conflicting and opinions are divided.

**Summario in Interlingua**

Esseva studiate certe aspectos del metabolismo de $^{32}$DFP in le homine. Il es probable que approximativemente 10 pro cento del dose injicite es metabolisate e re-appare in le plachettas. Isto affice le curva del degradation plachettal post le octave die e exercite, possibilmente, un influentia super le datos de cinetica erythrocytic e leucocytic obtenite per medio del mentionate isotopo.

In consideration del effectos de un reincorporation del composito, nos ha studiate vinti subjectos normal e ha trovate un longevitate medie del plachettas de 11.5 dies. Le analyse statistic supporta le conception que le curva del degradation de plachettas es lineari. Isto significarea que plachettas es eliminate ab le circulation a causa de lor senescentia plus tosto que de manera aleatori.

Es presentate un revista del litteratura concernite con le longevitate de plachettas human. Le majoritate del autores supporta le conclusion que le curva de degradation plachettal es lineari, sed il existe dissidentes. Le evidentia es conflictive, e le opiniones es dividite.

**References**


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The Use of $^{32}$Di-Isopropylfluorophosphate ($^{32}$DFP) As A Platelet Label: Evidence for Reutilization of This Isotope In Man

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