Life Span of Thrombocytes and Erythrocytes in Normal and Thrombocytopenic Calves

By N. S. Mizuno, V. Perman, F. W. Bates, J. H. Sautter and M. O. Schultze

Severe hypoplasia of the bone marrow,1,2 including any desired degree of thrombocytopenia,3 can be induced in the bovine by feeding of appropriate amounts of trichloroethylene-extracted soybean oil meal (TCESOM). This affords a unique opportunity for analytic study of thrombocytes from thrombocytopenic animals in which this condition might be the result of decreased rate of formation or a decreased life span of the thrombocytes or of both factors. The life span of human erythrocytes4 and thrombocytes5 has been estimated recently by labeling them in vivo with radioactive diisopropylphosphorofluoridate (DFP32). This compound combines with esterase enzymes, the presence of which has been demonstrated in bovine erythrocytes6 and thrombocytes.7,8

This study was undertaken to determine whether bovine thrombocytes and erythrocytes could be labeled in vivo with DFP32, and to establish whether the life span of these cells was changed in calves with hypoplastic bone marrow.

METHODS

Studies with Nonradioactive DFP

Effect of DFP on the function and survival of thrombocytes.—Preliminary studies with nonradioactive DFP7 showed that calves tolerated an intramuscular injection of from 1.4 to 5.6 mg. of DFP per 100 Kg. of body weight without signs of physical distress. With thrombocytes from calves thus treated, studies were made of clot retraction by the method of Minor and Burnett,5 and of thromboplastin generation by the method of Miale and Wilson,9 with the modification that in both procedures the number of thrombocytes in the concentrates was adjusted to 300,000 per cu.mm.

Survival time of thrombocytes was determined by transfusion of thrombocyte concentrates prepared from bovine slaughterhouse blood into calves with severe thrombocytopenia (induced8 by feeding 198 Gm. TCESOM per day per 100 Kg.) and noting the time required for blood thrombocyte counts to return to pre-transfusion levels. To determine the effect of DFP on the survival time, some of the calves were injected intramuscularly with 1.4 or 2.8 mg. of DFP per 100 Kg. of body weight immediately before the transfusion.
In Vitro Studies with DFP

DFP combines with purified cholinesterase. To determine whether P of cells which have reacted with DFP is firmly bound, DFP (1 mg. in 0.5 ml. of isopropanol, specific activity: 3 × 10⁶ CPM per mg.) was added to washed bovine erythrocytes suspended in 50 ml. of 0.9 per cent sodium chloride. When the activity of acetylcholinesterase was completely inhibited, the cells were washed by repeated centrifugation and suspension in 0.9 per cent sodium chloride until the washings were free of radioactivity. The erythrocytes were suspended in bovine blood plasma and kept at room temperature. Aliquots were removed at intervals, the plasma was separated from the erythrocytes by centrifugation, and the radioactivity was determined in each fraction.

In Vivo Studies with DFP

Treatment of animals.—Young female Holstein calves were used. Three control calves were fed alfalfa hay, milk and hexane-extracted soybean oil meal. In lieu of the latter, three other calves were fed 50 Gm. of TCESOM per 100 Kg. per day, an amount which was known to produce thrombocytopenia within 40 days. On about the 40th day of feeding, when the TCESOM-treated calves had thrombocyte counts of 450,000, 420,000 and 430,000 per cu.mm. (control calves: 790,000, 840,000 and 750,000), all calves were given an intramuscular injection of 5 mg. of DFP in propylene glycol (specific activity: 200 μg. per mg.) per 100 Kg. of body weight. Blood was withdrawn from a jugular vein at intervals for measurement of radioactivity of erythrocytes and thrombocyte concentrates. One normal and one thrombocytopenic calf were placed in metabolism cages, and the urine was collected daily for 27 days following the administration of DFP.

Preparation of thrombocyte concentrates.—All glassware used was coated with silicone. To 100 ml. of blood was added an equal volume of anticoagulant solution and the mixture was centrifuged for 5 minutes at 1000 × g. The turbid supernatant was removed, and the process of suspending the cells in an equal volume of anticoagulant solution, centrifugation and removal of supernatant was repeated twice. The supernatants were pooled and centrifuged at 2000 × g for 20 minutes. The supernatant was discarded, the sediment was suspended in anticoagulant solution, and the erythrocytes were preferentially removed by centrifugation at 150 × g for a few minutes and by transfer of the turbid supernatant to another tube until the sediment contained no visible erythrocytes. This thrombocyte preparation was centrifuged at 2000 × g for 20 minutes, the supernatant was discarded, and the residue diluted to 3 ml. with 0.9 per cent sodium chloride. Thrombocyte and erythrocyte counts and radioactivity measurements were then made.

Separation of erythrocytes.—Erythrocytes were obtained from the cell layer which remained after removal of the thrombocytes. From the bottom of this layer, 3 to 5 ml. of erythrocyte suspension were removed, washed with 3 to 4 volumes of 0.9 per cent sodium chloride and centrifuged at 2000 × g for 20 minutes. The procedure of removing cells from the bottom of the cell layer, suspending them in 0.9 per cent sodium chloride, centrifuging and discarding of the supernatant was repeated twice more. Finally, a suspension was made of equal volumes of erythrocytes and 0.9 per cent sodium chloride. Cell counts and radioactivity measurements were made on this final preparation.

Determination of radioactivity.—All of the thrombocyte concentrate, duplicate specimens of 2 ml. of urine or of 0.5 ml. of erythrocytes were placed into stainless steel cups (25 mm. OD), thoroughly mixed with 0.6 ml. of a solution of 0.1 M barium chloride and 0.02 M sodium hydroxide and dried under an infrared lamp. The alkali prevented loss of volatile radioactive compounds from solutions containing DFP. The dried cups were counted with a thin window gas flow counter to an accuracy of ±2 per cent. The counts

*Purchased from Manning Research Laboratories, Waltham, Mass.
†CPM = counts per minute.
‡TCESOM-6, of known toxicity, previously described.
§25 Gm. sodium citrate, 9 Gm. sodium chloride, 1000 ml. distilled water.
were corrected for background, decay of the radioactivity and for self absorption, which was determined by ashing of the samples as well as by addition of radioactive, aqueous DFP to the same quantities of nonradioactive urine or erythrocyte suspension that was used for counting. The radioactivity was calculated as counts per minute per 10^9 erythrocytes or thrombocytes, or per ml. of urine.

**Plasma volume determinations.**—To correct for changes in total erythrocyte mass which occurred in growing calves during the progress of the experiment, the plasma volume was determined by the method of Reynolds. The dye T-1824 was purified chromatographically on an alumina column, and 5 ml. of sterile aqueous solution containing 50 mg. of dye was injected intravenously into the calves. The concentration of the dye was determined in the plasma from blood removed 10, 20, 40 and 60 minutes after the injection. Red cell mass was calculated from plasma T-1824 levels at zero time, obtained by extrapolation, and from the hematocrit.

**Urinary P excretion.**—Phosphorus compounds were concentrated by adding a few drops of concentrated NH₄OH, and aqueous 0.1 M AgNO₃ to 100 ml. of urine until precipitation was complete. The precipitate was collected by centrifugation, dissolved in 1 M NaOH, and the solution was saturated with H₂S. After centrifugation, the clear solution was chromatographed by placing 40 microliters on Whatman #4 filter paper and developing with 1-butanol-acetic acid - H₂O solvent (40:11:50 v/v). Aqueous solutions of DFP and diisopropylyphosphinous acid, the latter prepared by treating isopropanol with PCl₅, served as standards. Phosphorus compounds were detected on the chromatograms with the reagent of Hanes and Isherwood. Autoradiograms were made from the dried chromatograms by placing them in direct contact with Kodak Medical X-Ray Film for seven days.

**RESULTS AND DISCUSSION**

**The use of DFP labeling for life-span studies.**—Ideally, cells which are tagged should not be altered except for the attachment of the label. As shown in table 1, the administration of DFP to calves did not subsequently impair the ability of the thrombocytes to induce clot retraction. The ability of thrombocytes to generate thromboplastin in a coagulating system, in which the thrombocytes were the only variable factor, was likewise not changed after DFP administration. The clotting time was 20 to 25 seconds, and maximal activity was reached in 2 to 3 minutes with thrombocytes isolated from blood before and 1.5 hours after intramuscular injection of DFP (1.4 mg. per 100 Kg. body weight).

The survival time of bovine thrombocytes transfused into severely thrombocytopenic calves was 3 to 5 days, and it was not changed by intramuscular injection of DFP just prior to the transfusion. The average results are summarized in table 2, and the course of two typical experiments is represented in figure 1.

Judged by these limited criteria, the function and viability of thrombocytes were not impaired by the labeling procedure.

**Stability of P bound to erythrocytes after reaction with DFP.**—As shown in table 3, the radioactivity of non-labeled blood plasma equilibrated for periods up to 24 hours with DFP-labeled erythrocytes remained very low. While the conditions in vivo are different from those in vitro, the results indicate that the tracer was firmly bound to the erythrocytes. Widespread distribution of alkylfluorophosphatases in animal blood and tissues, as well as the rapid hydrolysis of DFP in aqueous media, would preclude the formation of
TABLE 1.—Effect of DFP on Clot Retraction Induced by Thrombocytes

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Saline control</th>
<th>ml. serum expressed</th>
<th>Thrombocytes* added Before DFP</th>
<th>After DFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.45</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>0.40</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

*Thrombocytes were isolated from blood collected before and 1.5 hours after an intramuscular injection of DFP (1.4 mg. per 100 Kg. body weight).

TABLE 2.—Effect of DFP on Survival Time of Thrombocytes Transfused into Thrombocytopenic Calves

<table>
<thead>
<tr>
<th>DFP</th>
<th>No. of transfusions</th>
<th>Mean survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>4</td>
<td>2.8 ± 0.6*</td>
</tr>
<tr>
<td>1.4–2.8 mg. per 100 Kg. body wt.</td>
<td>5</td>
<td>3.3 ± 0.4*</td>
</tr>
</tbody>
</table>

*Standard error of mean.

a significant DFP$^{32}$ depot in the body. The lack of affinity of the hydrolytic product, diisopropylphosphinous acid, for enzymes, and its rapid excretion from the body provide for the quick elimination of unbound $^{32}P$. These considerations have justified the use of DFP$^{32}$ in these studies.

Life span of bovine thrombocytes.—During the 24 days of this experiment the thrombocyte counts remained essentially constant. Cell counts performed on thrombocyte concentrations showed that these were, on the average, contaminated with 1 erythrocyte per 5000 thrombocytes, and with less than 1 leukocyte per 10,000 thrombocytes. The radioactivity of the thrombocytes was therefore not due to contamination with significant numbers of other cells. In figure 2 the corrected counts per minute (CPM) per $10^9$ thrombocytes are plotted against time. From the first through the eighth day the values fell essentially along a linear decay curve, and the life span was calculated by dividing the CPM per $10^9$ thrombocytes at zero time (obtained by extrapolation) by the numerical value of the slope of the linear portion of the curve. It should be noted that a low radioactivity persisted in the thrombocytes after the 10th day. All of this residual activity was extractable with ethanol-ether (3/1: v/v), and was therefore not firmly bound to protein. The compounds which are responsible for this residual radioactivity are not known. One of them might be O-serine phosphate-$^{32}P$, which has been isolated after enzymatic and acid hydrolysis of DFP$^{32}$-treated cholinesterase of the eel.$^{20}$ During the turnover of proteins which have reacted with DFP$^{32}$, such as the plasma esterases,$^{21}$ serine phosphate-$^{32}P$ may be liberated and become available for incorporation into phosphatidyl serine. This would account for the presence of $^{32}P$ in the lipid fraction of the thrombocytes. The amount of lipid-soluble radioactivity was small and appeared to be constant, introducing an uncertainty of about one day in the estimation of the life span of the thrombocytes.

Calculated from the data summarized in figure 2 the life span of the throm-
Standard error of the mean, 

Fig. 1.—Changes in thromocyte counts of calf after intravenous injection of thrombocyte concentrates. First injection, $2.4 \times 10^7$ thrombocytes, without prior intramuscular injection of DFP. Second injection, 13 days later, immediately after intramuscular administration of 2.8 mg. of DFP per 100 Kg., $2.0 \times 10^7$ thrombocytes.

Thrombocytes was $9.7 \pm 0.1^*$ days in the normal calves and $10.9 \pm 0.4^*$ days in the thrombocytopenic calves. Taking the uncertainty due to the presence of lipid-soluble radioactivity into account, the difference between the two groups is not statistically significant and therefore the cause of thrombocytopenia in the calves fed TCESOM must reside in factors other than peripheral destruction.

The survival time of transfused thrombocytes (3 to 5 days) shown in figure 1 was shorter than the life span measured by the disappearance of labeled thrombocytes, a divergence which is probably due to loss of viability of the transfused thrombocytes during the preparation of concentrates.

Transfusion of thrombocytes into human subjects with thrombocytopenia, attributed to various causes, resulted in survival times of 0 to 6 days. When thrombocytes were labeled in vitro with radioactive compounds ($\text{P}^{32}\text{-orthophosphate, P}^{31}\text{-iodide, }^{29}\text{Cr}^{51}\text{-chromic chloride, }^{86}\text{Cr}^{51}\text{-chromate}$) and injected into different animals, the survival time was relatively short (2 hours to 4 days). There is no assurance that the life span of thrombocytes subjected to physical manipulations remains unimpaired. The life span of rat thrombo-

*Standard error of the mean.
TABLE 3.—Partition of P32, Originally Present in Erythrocytes, between Plasma and Erythrocytes

<table>
<thead>
<tr>
<th>Time</th>
<th>CPM* per ml plasma</th>
<th>CPM per ml erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 minutes</td>
<td>0.0042</td>
<td></td>
</tr>
<tr>
<td>30 minutes</td>
<td>0.0050</td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td>0.0063</td>
<td></td>
</tr>
<tr>
<td>2 hour</td>
<td>0.0059</td>
<td></td>
</tr>
<tr>
<td>6 hour</td>
<td>0.0107</td>
<td></td>
</tr>
<tr>
<td>24 hour</td>
<td>0.02351</td>
<td></td>
</tr>
</tbody>
</table>

*Counts per minute.
(Slight hemolysis in this sample.)

Fig. 2.—Radioactivity of thrombocytes isolated from blood of calves treated with DFP on day 0. The mean thrombocyte counts for the normal and thrombocytopenic calves are given in parentheses following the numbers of the animals.

cytes has been estimated to be 3 to 4 days by measuring the rise and fall of radioactivity of thrombocytes labeled in vivo with C14-formate and S35-sulfate. In these procedures, the metabolic stability of the label in the thrombocytes remains to be demonstrated. Recently, survival studies made with thrombocytes injected after labeling in vivo with P32 gave half-life
estimates of 1.5 to 3 days in humans and dogs. From the rate of disappearance of thrombocytes labeled in vivo with DFP on day 0, a life span of 8 to 9 days was calculated, a value which corresponds more closely to the mean life span of about 10 days obtained in the present study.

Life span of erythrocytes.—Two general technics have been used to estimate the survival time of erythrocytes: transfusion of erythrocytes which can be differentiated from the recipient's cells by agglutination and tagging either in vivo or in vitro with tracers (N, Cr, P). Depending on the technic, different types of survival curves have been obtained. In this study, the curves obtained by plotting CPM per 10 erythrocytes against time (fig. 3) began with an inflection and then followed a linear course from about the 15th to the 55th day. The relatively short physical half-life of P did not permit the extension of the measurements beyond the 55th day. The curvature at the beginning may be a consequence of an increased destruction or of contamination of the erythrocytes with plasma proteins which were concomitantly labeled. Application of Jeffrey's K-ratio as a criterion for linearity of
the curve between 15 to 55 days, gave \( K > 1 \), which indicated that a straight line calculated by the method of least squares is a satisfactory fit of the curve to the data. The life span calculated from the linear portion of the curve gave mean values of 107 ± 8* days for normal calves and 121 ± 2* days for the thrombocytopenic calves. The difference is not statistically significant. The mean plasma volume of these calves was 53.3 ± 4.7* ml. per Kg. of body weight, a figure which is in accord with others recently published. This, with the hematocrit, was used to correct the CPM of the erythrocytes for increase in red cell mass which occurred during the experiment with these growing calves. Less than 1 per cent of the radioactivity could be extracted from the erythrocytes with ethanol-ether (3/1: v/v); therefore the error due to non-protein-bound \( {P}^{32} \) was insignificant.

Attempts have been made to resolve differences in the survival curves obtained by various technics by the application of mathematical interpretations. With the differential agglutination technic, the curve is linear except for inflections at the beginning and the end. This has been explained by assuming that the life of the cell reflects the sum of the aging time of the cell, which yields a linear curve, and the average life of aged cells undergoing random destruction, which yields an exponential curve. The curvature in the beginning has been explained by the differences in the cellular environment between the donor and the recipient. This technic yielded estimates of 100 to 110 days for the aging time and 15 to 40 days for the average survival of the aged human red cells. When \( {N}^{15} \)-glycine was fed to human subjects, the \( {N}^{15} \) concentration of heme of the erythrocytes reached a maximum level which remained constant for about 70 days, then decreased along an S-shaped curve. Only the newly formed erythrocytes were labeled by this procedure, and the results indicated a determinate life span for the erythrocytes of about 127 days. With the \( {C}^{51} \) technic, the survival curves are usually exponential, and the half-life is around 35 days. The discrepancy between the curvilinear plot of the \( {C}^{51} \) technic and the linear plot of the differential agglutination method has been attributed to elution or leaking of \( {C}^{51} \) from the erythrocytes. After the administration of \( {D}^{32} \) to human subjects, the disappearance of \( {P}^{32} \)-labeled erythrocytes followed a linear course, indicating a life span of 116 to 128 days. In the present study, after about the 15th day the \( {P}^{32} \) activity of the erythrocytes decreased linearly with time, and the mean life span calculated from this straight-line portion of the curves was 107 days for normal calves and 121 days for thrombocytopenic calves. There was no significant difference between the two groups; and, as with the thrombocytes, there was no evidence for increased peripheral destruction of erythrocytes in moderate bone marrow hypoplasia induced by TCESOM.

**Urinary excretion of \( {P}^{31} \) compounds.**—During the first day, the control calf (no. 1703) excreted 28 per cent, and the thrombocytopenic calf (no. 1704) excreted 46 per cent of the administered dose. These values increased by slow increments to 51 and 66 per cent, respectively, up to the 27th day when the

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*Standard error of the mean.*
FIG. 4.—Radioactivity of urine of calves treated on day 0 with 5.0 mg. DFP$^{32}$ per 100 Kg. body weight.

**TABLE 4.—Urinary Phosphorus Compounds After DFP$^{32}$ Administration**

<table>
<thead>
<tr>
<th>Material resolved</th>
<th>Phosphorus Compounds</th>
<th>Radioactive Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>urine, calf 1703</td>
<td>0.28</td>
<td>0.85</td>
</tr>
<tr>
<td>urine, calf 1704</td>
<td>0.26</td>
<td>0.88</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.24</td>
<td>—</td>
</tr>
<tr>
<td>DIP$^{*}$</td>
<td>0.84</td>
<td>—</td>
</tr>
<tr>
<td>DFP$^{32}$, hydrolyzed$^\dagger$</td>
<td>—</td>
<td>0.85</td>
</tr>
</tbody>
</table>

$^*$Synthetic diisopropylphosphinous acid.
$^\dagger$Hydrolyzed by prolonged standing in H$_2$O at room temperature.

collection of urine was discontinued. Examination of the urine by chromatography and autoradiography of phosphorus compounds excreted during the first 24 hours (table 4) revealed that the compound giving the phosphate color reaction corresponds to orthophosphate, and that the radioactive compound corresponds to diisopropylphosphinous acid. This is in accord with the results obtained in the human.$^4$

**SUMMARY**

1. Bovine thrombocytes and erythrocytes became labeled with P$^{32}$ following an intramuscular injection of DFP.$^{32}$
2. The life span of bovine thrombocytes calculated from survival curves of DFP$^{32}$-labeled thrombocytes was about 10 days.
3. The life span of bovine erythrocytes calculated from the linear portion of the survival curve was about 110 to 120 days.

4. There was no significant difference between the life span of the thrombocytes and erythrocytes from normal calves and those from calves with thrombocytopenia induced by the feeding of trichloroethylene-extracted soybean oil meal.

**SUMMARIO IN INTERLINGUA**

1. Post un injection intramuscular de diisopropylphosphorofluoridato radioactive (DFP\textsuperscript{32}) thrombocytos e erythrocytos bovin deveniva marcate con \textsuperscript{32}P.

2. Le supervivencia de thrombocytos bovin calculate ab le curvas de supervivencia de thrombocytos marcate con DFP\textsuperscript{32} esseva approximativamente 10 dies.

3. Le supervivencia de erythrocytos bovin calculate ab le portion linear del curva de supervivencia esseva approximativamente 110 o 120 dies.

4. Nulle differentia significative esseva constatate inter le supervivencia de thrombocytos e erythrocytos ab vitellos normal, e illos ab vitellos con thrombocytopenia induce per un alimentation con torta molite de soja, a extraction trichloroethylenic.

**REFERENCES**


13. Reynolds, M.: Plasma and blood volume in the cow using the T-1824 hemato-


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