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LABELING OF RED BLOOD CELLS WITH RADIOACTIVE DIISOPROPYLFLUOROPHOSPHATE (DFP^{32}): EVIDENCE FOR AN INITIAL RELEASE OF LABEL

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RADIOACTIVE diisopropylfluorophosphate (DFP^{32}) has been frequently used as a label for blood cells and proteins. It is a basic assumption that DFP^{32} is irreversibly bound to a protein site, no label being released until the protein breaks down. Contrary to this assumption, we observed that there is a definite and, sometimes, a marked release of the label from red cells during a short period after the labeling. Bove and Ebaugh[1] and van Putten[2] have recently reported similar findings.

The practical use of DFP^{32} as a red cell tag depends, to a considerable degree, on its behavior during the first few days after the labeling, and also on its suitability as an in vitro tag. Therefore, these two questions were investigated in the present study.

MATERIALS AND METHODS

In vivo experiments in man were carried out in male medical students without anemia. For animal experiments we used Sprague-Dawley rats weighing 300 to 400 grams, and either sex of white New Zealand rabbits weighing about 2,000 grams.

Nonradioactive DFP was synthesized by Dr. Fischer in the Department of Biochemistry, University of Washington. It was assayed by a modification of the method of Marsh and Neale[3] suggested to us by Dr. Dixon in the same department.† The result was checked against the total phosphorus content in the preparation, and the DFP by these measurements proved to be at least 95 per cent pure. It was dissolved in water-free propylene glycol to a concentration of 400 μg per ml.

Radioactive DFP^{32}‡ was diluted in water-free propylene glycol. The concentration of DFP^{32} and the total phosphorus content were determined in the same manner as were those of the nonradioactive DFP. The radioactivity was determined by comparison with standards of similar energy. These measurements were made 2 to 3 days after the synthesis. The purity in terms of phosphorus was found to be 70 per cent or better, and the specific activity was 150 to 200 μc per mg. The ampules were stored in the cold, and the DFP^{32} was used

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†A description of this technique can be obtained from us.

‡Radioactive DFP^{32} was supplied by Manning Research Laboratory, 27 Baldwin Road, Waltham 54, Mass.

immediately after the ampules had been opened. The experiment in Fig. 4 was made with one batch of DFP^{32} from The Netherlands.* Eleven days after synthesis the purity in terms of phosphorus was 70 per cent, and the specific activity was 130 μc per mg.

Radioactive Diisopropylphosphate (DIP^{32}).—DFP^{32} was hydrolyzed in 1:10 v/v of 1N NaOH for 30 minutes at room temperature. No DFP^{32} could then be detected, and the solution was adjusted to physiological pH and ionic strength before injection.

Radioactive chromium ($Na_2Cr^{51}O_4$)† had a specific activity of 30 to 40 μc per microgram Cr. It was dissolved in saline.

Labeling in vivo was done by intramuscular injection in rats and by intravenous injections in humans.

Labeling In Vitro.—Citrated blood (containing 1:10 v/v of 3.13 per cent sodium citrate dihydrate) or ACD blood was centrifuged at 1,500 r.p.m. (500 g) for 10 minutes at 4° C. The plasma was discarded, the red cells were washed once in a volume of saline equal to the plasma volume, and resuspended in saline to a hematocrit of about 60. After incubation with DFP^{32} in a stoppered vessel, the cells were washed twice in saline (unless otherwise stated). In some experiments Cr^{51} in a concentration of less than 0.10 μg per milliliter red cells was added before incubation. For transfusion, the red cells were resuspended in saline to the original volume of blood.

Collection of Blood Samples After Labeling.—Human blood was drawn by venipuncture into dried oxalate mixture, 2 mg. per milliliter blood.[4] Rabbit blood was collected from ear veins or by heart puncture; the same anticoagulant was used. Rat blood was collected from vena cava; 1:20 v/v of 5 per cent sodium versenate was used as anticoagulant.[5]

Processing of Blood Samples.—One milliliter whole blood was mixed with 9 ml. 0.9 per cent saline and centrifuged at 1,200 r.p.m. (340 g) for 5 minutes. The supernatant was discarded, the washing was repeated twice, and the red cells were suspended in saline to 2 to 10 ml. volume, depending on their radioactivity.

Aliquots of red cells were also precipitated with trichloroacetic acid (TCA). The washed red cells from 1 ml. blood were suspended in 1 ml. saline, and 2 ml. 20 per cent TCA was added. Fifteen minutes later, the precipitate was sedimented by centrifugation for 3 minutes at 1,000 r.p.m. (235 g), washed 2 times in 5 ml. portions of 10 per cent TCA, dissolved in 2 ml. 2N NaOH, and made up to 3 to 10 ml. volume with saline.

Hematocrit was measured in a Wintrobe tube[4] after centrifugation at 3,000 r.p.m. (2,110 g) for 30 minutes.

Measurement of the Radioactivity.—One to 2 ml. of the sample was pipetted into counting planchets, 0.2 to 0.5 ml. 0.1N NaOH was added,[6] and, a few minutes later, 1 ml. nonradioactive blood was added. The internal diameter of the planchets was 24 mm., and the dry weight of the samples was 300 to 600 mg. The samples were dried at room temperature and counted in an end window Geiger Muller counter. A total of 4,096 counts were compiled for each sample. The background radioactivity was 24 to 26 and 14 to 16 c.p.m. in the 2 counters used. The samples gave at least 50 net counts per minute. In some experiments the activity was lower, and 10 ml. liquid samples were then counted in a Veall tube.[7] The background radioactivity was here 9 to 14 c.p.m., and at least 2,000 counts were compiled for each sample. The Cr^{51}-labeled samples were pipetted into counting vials, 1 to 2 ml. in each, and counted in a scintillation well counter. The background radioactivity was 73 to 90 and 54 to 58 c.p.m. in the 2 counters that were used. At least 4,000 counts were compiled for each sample. Correction for physical decay was made on the basis of the decay of standards of DFP^{32} and Cr^{51} which were counted with the samples. In some experiments it was necessary to correct for cross-counting between the 2 isotopes: the Cr^{51} count had to be reduced with 6 per cent of the DFP^{32} count, and the DFP^{32} count with 0.2 per cent of the Cr^{51} count. When the activity was referred to red cells, the results were corrected for variations in the hematocrit.

*Supplied by Dr. J. A. Cohen, Medical Biological Laboratory, National Defense Council, Rijswijk, The Netherlands.

†Radioactive chromium was supplied by Abbott Laboratories, Oak Ridge, Tenn.

RESULTS

DFP^{32} Binding Capacity of Red Cells In Vitro.—DFP combines only with certain proteins,[6, 8] and red cells should, therefore, have a limited capacity for carrying DFP^{32} in an irreversible bond. To evaluate this capacity, aliquots of 1 ml. human red cells were incubated for 15 hours at 4° C. with varying concentrations of DFP^{32}. The red cells were then washed 3 times in 10 ml. saline, and aliquots were treated with TCA to eliminate nonprotein-bound DFP^{32}.

Fig. 1 shows the result of this experiment. There appears to be a relative saturation point at 0.14 μg DFP^{32} per milliliter packed red cells, at which point about 90 per cent of available DFP^{32} was bound. When the amount of DFP^{32} was increased above this point, the relative uptake decreased, and part of the total uptake was no longer bound to proteins. The protein-bound DFP^{32} continued to increase, and in a straight line fashion with increasing amounts of DFP^{32}, but the slope was now less steep, suggesting a different type protein-binding. Identical results were found when human red cells were incubated at 37° C. for 1 hour. Similar saturation levels and slopes were obtained with rabbit and rat cells.

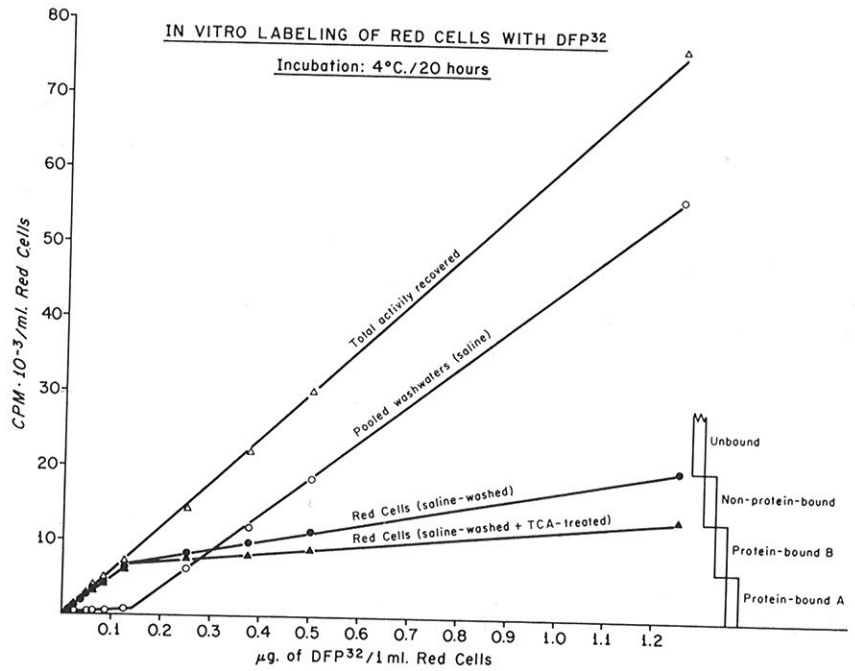

Fig. 1.—In vitro labeling of human red cells with DFP^{32}. The total activity recovered is the sum of the activity of the pooled washwaters and the activity of the red cells.

Evidence for Red Cell Saturation In Vivo.—One group of rats was prepared by 2 intramuscular injections of nonradioactive DFP (400 μg per kilogram) 13 and 7 days prior to injection of radioactive DFP^{32} (180 μg per kilogram). The red cell uptake of radioactivity by this loaded group was compared to a normal group (Fig. 2). Despite similar hematocrits in the 2 groups (46.0

and 46.6), the uptake was 5 to 6 times greater in the unloaded group. This indicates that the loaded group had only 17 to 20 per cent of the normally available binding sites. The rat's red cell life span is estimated to be 60 days,[2] so that 12 per cent new cells should have been formed after the last injection of nonradioactive DFP. Therefore, the nonradioactive DFP very nearly saturated red cell binding sites at the time of its injection.

Early Loss of Label After In Vivo Injection.—In both groups of rats (reported in previous paragraph) there was an initial drop of approximately 50 per cent in red cell radioactivity during the first few days after in vivo labeling. This drop could not be explained by red cell destruction, since the hematocrit was stable. Nor could it be explained by contamination with white cells or platelets, since platelets carry only about 0.3 per cent,[5] and white cells about 0.5 per cent,[9] of the total radioactivity in blood shortly after the injection of DFP^{32}. Therefore, the drop must be due to a loss of label from the red cells. Four fifths of this loss as determined by TCA precipitation was protein-bound DFP^{32}.

The early loss following intramuscular injection of large doses in rats lasted for about 5 days, and about 75 per cent of the 1 hour activity was lost during this period (Fig. 3).

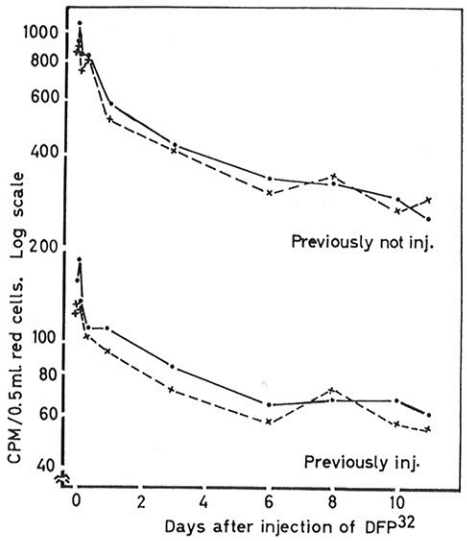

Fig. 2.—The effect of preliminary loading with nonradioactive DFP: red cell radioactivity after intramuscular injection of DFP^{32} in 2 groups of rats, one of which had been previously loaded with nonradioactive DFP. Each point represents 1 rat. Semilogarithmic paper is used to facilitate comparison of the 2 groups.
●——● = radioactivity of washed red cells.
×- - - - × = radioactivity of washed and TCA-treated red cells.

To determine whether this loss of label could be avoided by using a very small dose, 32 μg, representing about 5 μc DFP^{32}, was injected intravenously into 2 human subjects. Five minutes later, 16 and 17 per cent of the injected dose was in circulation, and 82 and 86 per cent of this was carried by the red cells. (These calculations are based on an assumed blood volume of 5,000 ml.)

Fig. 4 shows that there was a loss of about 20 per cent of the activity during the first 24 hours from washed red cells, and about 15 per cent from their precipitated proteins. Thus, the loss is smaller and lasts for a shorter time, but cannot be avoided.

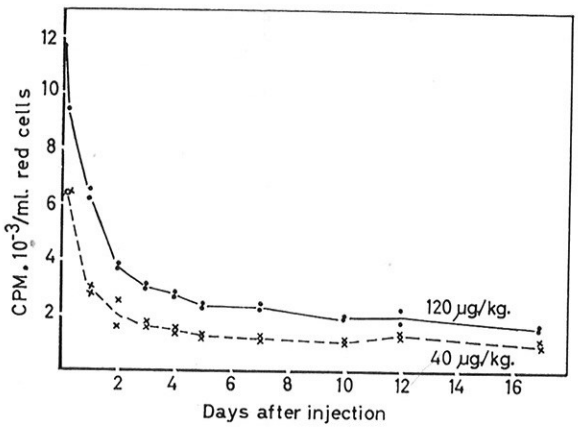

Fig. 3.—Radioactivity of washed red cells after intramuscular injection of DFP^{32} in rats. Each point represents 1 rat.

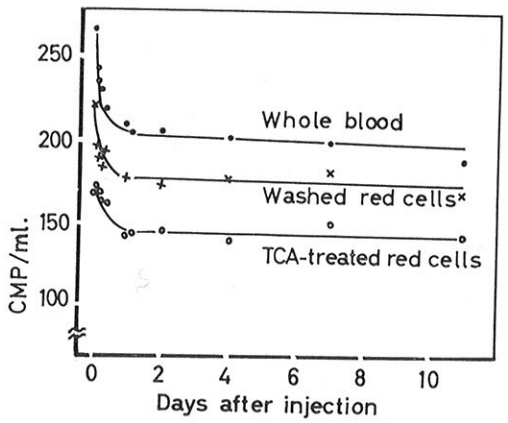

Fig. 4.—Intravenous injection of a small dose (32 µg or 5 µc) of DFP^{32} into a healthy volunteer.

Transfusion of Red Cells Tagged In Vitro With DFP^{32}.—Rabbit erythrocytes were incubated overnight at 4° C. with 10 times the saturation dose of DFP^{32}. The following morning, 90 per cent of the activity was removed by washing, so that the amount transfused corresponded approximately to the in vitro saturation level. The cells were also tagged with Cr^{51} before transfusion. During the first 21 hours after transfusion the Cr^{51} activity dropped to 80 per cent of the 5 minute value, whereas the DFP^{32} activity dropped to 52 per cent (Fig. 5). These studies indicate that there may be some red cell destruction after incubation with high levels of DFP^{32}, but the loss of DFP^{32} was far in excess of the loss of red cells as determined by the chromium tag.

A similar study was performed in man, using the smallest possible amount of DFP^{32} per milliliter red cells. The red cells from one unit (450 ml.) of blood were incubated for 1 hour with DFP^{32} in a concentration of 0.1 to 0.3 μg per milliliter red cells and with Cr^{51} in a concentration of less than 0.10 μg

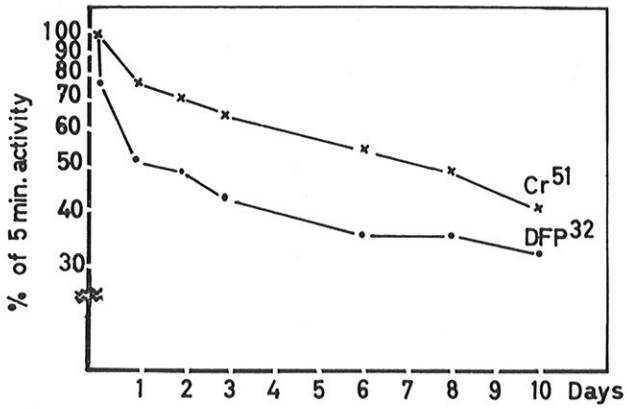

Fig. 5.—Transfusion of washed rabbit red cells labeled in vitro with 1.2 μg DFP^{32} and 0.08 μg Cr^{51} per milliliter packed cells. The curves represent the activity of washed red cells.

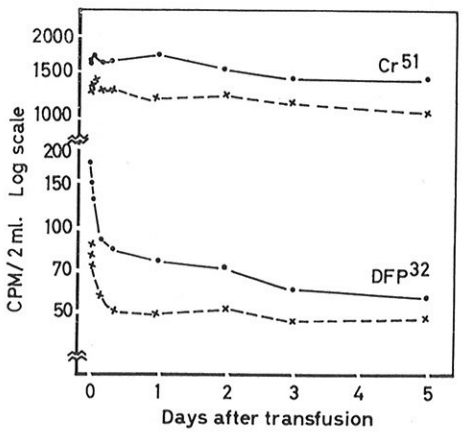

Fig. 6.—Transfusion of washed human red cells labeled in vitro with 0.30 μg DFP^{32} and 0.004 μg Cr^{51} per milliliter packed cells.
O——O = radioactivity of washed red cells.
×- - - -× = radioactivity of washed and TCA-treated red cells.

per milliliter red cells. About 30 to 60 per cent of the DFP^{32} and 5 per cent of the Cr^{51} activity were lost in the wash. The washed cells were transfused back into the donors, and the radioactivity of the red cells followed during 8 days. The transfused cells showed an early loss of DFP^{32} in excess of the Cr^{51} loss. Fig. 6 shows an experiment with 0.30 μg DFP^{32} per milliliter red cells. The cells lost 40 to 50 per cent of their DFP^{32} activity during the first day. There was no detectable loss of Cr^{51}. When only 0.10 μg DFP^{32} was used per milliliter red cells, the early loss in DFP^{32} was about 20 per cent.

Injection of Hydrolyzed DFP^{32} (DIP^{32}).—One possible explanation for the early loss of label would be that part of the DFP^{32} preparation had been

hydrolyzed to DIP^{32}. To test this possibility, DIP^{32} was injected intraperitoneally in rats in doses corresponding to 130 μg DFP^{32} per kilogram. The rats were killed at intervals, and their red cells were examined for radioactivity. As shown in Fig. 7, the saline-washed red cells contained detectable radioactivity for 8 hours after the injection, but the TCA-treated cells were free of activity. Similar results were found in rabbits. These experiments suggest that the protein-bound radioactivity in previous experiments was originally present in the preparation as DFP^{32}.

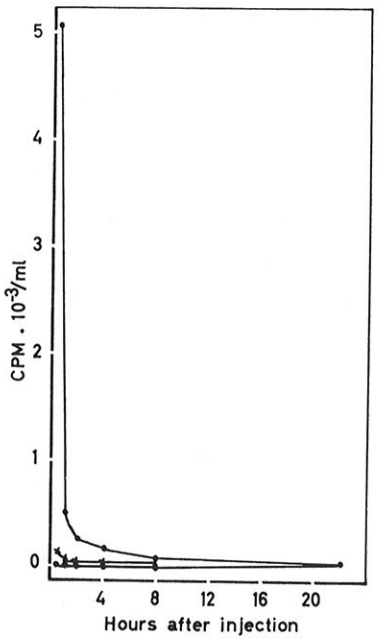

Fig. 7.—Injection of hydrolyzed DFP^{32} (DIP^{32}) into rats. The equivalent of 130 μg DFP^{32} was injected intraperitoneally.
●——● = radioactivity of whole blood.
×——× = radioactivity of washed red cells.
○——○ = radioactivity of washed and TCA-treated red cells.

DISCUSSION

DFP^{32} is considered to be irreversibly bound to proteins. Elution is, therefore, thought to be impossible,[8, 10-13] and several investigators found no indication for elution from red cells.[12, 14, 15] However, Bove and Ebaugh,[1] and van Putten,[2] have recently reported an unexplained early drop in the red cell life span curve. We have confirmed this, and have shown that the drop is due to a release of label from the cells, i.e., to elution.

Our in vitro experiments showed that red cells have a limited capacity for carrying DFP^{32}, and 0.14 μg per milliliter packed red cells appears to be the saturation dose. The uptake was about 90 per cent with this dose. The in vitro uptake depends on the dose of DFP^{32} and on the treatment of the cells before assay (Fig. 1). The different uptakes reported in the literature,[1, 14] are, therefore, not at variance with our findings.

Elution might be explained by at least 4 different mechanisms:

1. *Overloading.*—Since the in vitro experiments showed that red cells could be overloaded, we first thought that elution was always due to this mechanism. However, elution takes place even after extremely small doses of DFP^{32}, and the results with the TCA-treated cells confirmed that some of the protein-bound label was lost. Therefore, elution is not due to overloading only, and it cannot be avoided by using small doses. However, overloading leads to different types of binding and to increased elution. In agreement with this rule, Bove and Ebaugh[1] found that 90 per cent of the activity (of saline-washed red cells) disappeared in 24 hours after transfusion of red cells labeled in vitro with 2 μg DFP^{32} per milliliter blood. The risk of overloading is great when cells are labeled in vitro, when large doses are injected, and when preliminary blocking doses of nonradioactive DFP are used.

2. *Radioactive Contaminations.*—P^{32} was the only radioactive material present in the DFP^{32} preparation, since the decay curve corresponded exactly to the expected curve for P^{32}. Inorganic phosphorus was probably not present, since the last step in the production of DFP^{32} is a distillation. Several organic phosphorus compounds might be present, but this is not a likely explanation, since hydrolyzed DFP^{32} failed to label red cells (Fig. 7). We also observed elution after injections of small doses of DFP^{32} preparations in which the total phosphorus corresponded closely to the amount of phosphorus to be expected from the DFP^{32} content. Therefore, elution is probably not due to contamination with non-DFP^{32} radioactive compounds. However, the possibility of contamination calls for better standardization of the DFP^{32} preparations, and we have recently discussed this problem.[16] To avoid overloading, it is necessary to have well standardized preparations.

3. *Protein Absorption on Red Cells.*—The results could be explained by the hypothesis that DFP^{32} is in part attached to a protein on the red cell surface, which has a more rapid turnover than the red cell itself. This would not be true elution and would be compatible with the assumption that the binding of DFP to proteins is irreversible. From a practical point of view, however, this may be considered elution of label from the cells.

4. *Fluorophosphatase.*—The label might be split off by a specific mechanism, and the red cells do contain small amounts of this enzyme.[17] If this should be the mechanism of elution, it is difficult to see why the elution would stop after a few days.

We have not been able to elucidate the mechanism of elution, but the practical conclusion is that DFP^{32} does elute from the red cells during a short period after labeling. Because of this elution, DFP^{32} should not be used for studies of red cells with rapid turnover, since the results would then be grossly influenced by elution. For red cells with a more normal turnover, however, the early elution will not disturb the results, since there is probably no elution later on.[1, 2]

SUMMARY AND CONCLUSIONS

1. In vitro experiments show that red cells can carry only a limited amount of radioactive diisopropylfluorophosphate (DFP^{32}). When the dose exceeds 0.14

μg DFP^{32} per milliliter packed human red cells, the uptake is incomplete and there are apparently several types of binding.

2. After injection of DFP^{32} the red cell activity drops rapidly during an initial period, followed by a slow and constant disappearance. The early drop is caused by a loss of both protein-bound and nonprotein-bound label from the cells. This elution takes place even after very small doses of DFP^{32} (32 μg in humans).

3. Red cells were labeled in vitro with small amounts of DFP^{32} and Cr^{51} and were then transfused back into the donors. The early drop in DFP^{32} greatly exeeded the drop in Cr^{51}, an indication that DFP^{32} was lost by elution.

4. The mechanism of elution is unknown, but it is not due to radioactive contaminations. It is greater after large doses of DFP^{32}, probably because of overloading.

5. Because of elution, the results are erratic during the first 1 to 3 days, and DFP^{32} should not be used as a red cell tag when this period is important.

REFERENCES

1. Bove, J. R., and Ebaugh, F. G., Jr.: The Use of Diisopropylfluorophosphate^{32} for the Determination of in Vivo Red Cell Survival and Plasma Cholinesterase Turnover Rates, J. Lab. & Clin. Med. 51: 916, 1958.
2. van Putten, L. M.: The Life Span of Red Cells in the Rat and the Mouse as Determined by Labeling With DFP^{32} in Vivo, Blood 13: 789, 1958.
3. Marsh, D. J., and Neale, E.: A Colorimetric Method for the Detection and Determination of Certain Acid Halide and Acid Anhydride Compounds, Chem. & Ind. p. 494, June, 1956.
4. Wintrobe, M. M.: Clinical Hematology, ed. 4, Philadelphia, 1956, Lea & Febiger.
5. Hjort, P. F., and Paputchis, H.: Platelet Life Span in Normal, Splenectomized, and Hypersplenic Rats. In press.
6. Jandorf, B. J., and McNamara, P. D.: Distribution of Radiophosphorus in Rabbit Tissues After Injection of Phosphorus-Labeled Diisopropyl Fluorophosphate, J. Pharmacol. & Exper. Therap. 98: 77, 1950.
7. Veall, N.: A Geiger-Muller Counter for Measuring the Beta-Ray Activity of Liquids, and Its Application to Medical Tracer Experiments, Brit. J. Radiol. 21: 347, 1948.
8. Athens, J. W., Mauer, A. M., Aschenbrucker, H., Cartwright, G. E., and Wintrobe, M. M.: Leukokinetic Studies. I. A Method for Labeling Leukocytes With Diisopropylfluorophosphate (DFP^{32}). In press.
9. Athens, J. W.: Personal communication, 1958.
10. Grob, D., Lilienthal, J. L., Jr., Harvey, A. M., and Jones, B. F.: The Administration of Cholinesterase Activity by Diisopropyl Fluorophosphate, J. Biol. Chem. 163: 261, 217, 1947.
11. Mazur, A., and Bodansky, O.: The Mechanism of in Vitro and in Vivo Inhibition of Cholinesterase Activity by Diisopropyl fluorophosphate, J. Biol. Chem. 163: 261, 1946.
12. Cohen, J. A., and Warringa, M. G. P. J.: The Fate of P^{32} Labelled Diisopropylfluorophosphonate in the Human Body and Its Use as a Labelling Agent in the Study of the Turnover of Blood Plasma and Red Cells, J. Clin. Invest. 33: 459, 1954.
13. Leeksma, C. H. W., and Cohen, J. A.: Determination of the Life Span of Human Blood Platelets Using Labelled Diisopropylfluorophosphonate, J. Clin. Invest. 35: 964, 1956.
14. Mayer, K., and Ley, A. B.: The Measurement of Erythrocyte Survival With P^{32}-Tagged Diisopropylfluorophosphonate (DFP^{32}), Clin. Res. Proc. 4: 80, 1956.
15. Pollycove, M., Dal Santo, G., and Lawrence, J. H.: Simultaneous Measurement of Erythrocyte, Leukocyte, and Platelet Survival in Normal Subjects With Diisopropylfluorophosphate^{32} (DFP^{32}), Clin. Res. Proc. 6: 45, 1958.
16. Hjort, P. F.: Discussion in ad hoc Conference on DFP^{32}, Atlantic City, 1958, National Academy of Sciences.
17. Jandorf, B. J.: Discussion in ad hoc Conference on DFP^{32}, Atlantic City, 1958, National Academy of Sciences.