hr; less than 0.1% of the initial anticholinesterase activity remained at this time. Denatured enzyme was prepared by heating the enzyme solution to 80° C for 20 min to complete inactivation; 0.45% concentrations of protein were used in all cases. Dialyses were carried out in a rotating rack against 15 successive changes, at 8-hr intervals of 0.3% NaCl (30 ml per 4 ml enzyme solution each time); no C^{14} could be detected in any dialyzates after the tenth change. Precipitation of the protein from the dialyzed solutions was performed by the addition of five volumes of acetone to one of enzyme solution, after preliminary trial had shown that results obtained by this procedure did not differ from those obtained under the much more elaborate conditions required to obtain a dry precipitate of native protein. For counting, samples weighing 2.8–3.1 mg were collected on filter paper disks of 3.9 cm^2, dried in a vacuum desiccator, and counted directly (2.0 mg/cm^2 end window counter). Since all samples fell within the same narrow and low range of mass thickness, no self-absorption correction was applied. A standard was prepared by combustion of a known weight of HETP, collection of the CO_2 formed as $BaCO_3$, dilution of the activity with a known proportion of $BaCO_3$ in a homogenizer, and counting of a 3.0-mg sample on filter paper.

Three experiments carried out by these procedures yielded results in close agreement. Table 1 shows the averages of the values obtained. Three points are immediately evident from inspection of the figures. There is a definite, measurable uptake of C^{14} by the enzyme on reaction with active inhibitor. There is much less, though yet measurable, protein fixation of C^{14} under conditions which do not result in enzymologically detectable reactions between protein and phosphorus compounds. Only a small fraction of the HETP required to inhibit the enzyme is actually bound by the protein.

From the figures for C^{14} uptake and for the specific activity of the sample of HETP employed, molecular relations can be calculated if the molecular weight of the protein is known. Taking 3×10^5 as, at present, the most likely value for the mean molecular weight of this protein, a figure of 3.45×10^{-2} mol of HETP bound per mol of protein is obtained.

Assuming that inhibition of 1 mol of enzyme involves the firm fixation of 1 mol of inhibitor, these data suggest that the protein preparation employed actually contained about 3% active enzyme. This estimate is in good accord with values derived from other considerations.

The protein-to-inhibitor ratio derived from the fixation of C^{14} is also in good accord with the ratio calculated for TEPP from data on enzyme inhibition. In this case, 50% inhibition of the present enzyme preparation occurs at a ratio of 1.41×10^{-2}M TEPP/M protein, or a calculated uptake for complete inhibition (in view of the linear concentration-inhibition curve [*2*] of 2.82×10^{-2}M TEPP/M protein). This figure is in fair agreement with that calculated above if correction is made for C^{14} uptake that does not appear related to the enzyme-inhibitor reaction.

The data presented indicate the formation of a stable compound between plasma cholinesterase and a small fraction of the hexaethyl tetrapolyphosphate required to inhibit the enzyme. From 82 to 88% of the C^{14} fixation thus obtained appears to be related to the enzyme inhibition, the rest being accounted for by a much less specific reaction. Calculations have been presented indicating that the amount of C^{14} taken up in connection with the specific reaction is close to the amount of tetraethyl pyrophosphate required for complete inhibition of the enzyme, again suggesting that the anticholinesterase activity may reside in this or closely similar compounds of this group. Finally, an estimate of the molecular relations between HETP and the enzyme has been presented; a reasonable value (about 3%) for the purity of the enzyme preparation employed could be arrived at on the assumption of a mean molecular weight of 3×10^5 for the protein, and of a reaction involving 1 mol of HETP and 1 mol of protein.

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Iron Metabolism.[1] Heme Synthesis *in Vitro* by Immature Erythrocytes

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Studies with radioiron have indicated that there is no exchange of iron between the mature erythrocyte and surrounding plasma (*3, 4*). The following data, however, indicate that reticulocytes will assimilate iron and synthesize heme *in vitro*, and that this uptake of radioiron may be used as an indicator of the rate of hemoglobin synthesis.

Fe^{59} and Fe^{55} with specific activity of about 20 and 200 μc/mg iron respectively were prepared at the Massachusetts Institute of Technology cyclotron. Blood with an increased content of reticulocytes from patients with pernicious anemia during a response to liver and from patients with iron deficiency, and bone marrow from rats and humans were used. *In vitro* studies were performed in rocking boats at 37° C in a gas mixture of 95% oxygen and 5% CO_2, as previously described by Geiman and his associates (*2*), over a period from 4 to 24 hr. Those experiments extending beyond 6 hr were carried out with sterile precautions.

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While blood containing less than 1% reticulocytes took up no measureable quantity of radioiron, uptake of radioactivity was demonstrated repeatedly in blood with a high reticulocyte content. That the radioactivity was localized in the immature cell population was shown by correlation of the reticulocyte count and radioactivity

TABLE 1

	Reticulocytes	Cpm/ml red cells
Top		
(low cell specific gravity)	53%	500
Bottom		
(high cell specific gravity)	5%	35

in various fractions of this blood separated by the albumin flotation technique (7).

Table 1 shows such a separation of whole blood previously incubated for 4 hr with radioiron, washed five times in ten times its volume of 1% saline, and then partitioned by albumin flotation.

Various types of blood were studied, including iron-deficiency anemia, acquired hemolytic anemia, sickle cell anemia, and pernicious anemia. The uptake of iron in all instances was attributable to the presence of reticulocytes. It would further appear that in pernicious anemia at least the rate of uptake is also related to the type of reticulocyte present. The early reticulocytes after liver therapy contain more reticular material and pick up more radioactivity, as shown in Fig. 1.

Conditions necessary for the uptake of radioiron have been studied to a limited degree. Ferrous iron added to a saline suspension of erythrocytes containing an increased number of reticulocytes is taken up perhaps twice as rapidly as ferric iron. Iron carried by the iron-binding protein of the serum (5) is assimilated by the reticulocyte but less rapidly than the inorganic ferrous and ferric iron. The iron uptake is impaired by lack of glucose in the media and by low temperature. In an effort to determine whether the uptake of iron indicated hemoglobin formation, red cells have been fractionated after incubation with radioiron. The largest amount of activity in the reticulocyte portion was found in the stroma of the hemolyzed cells. However, significant amounts of radioiron were also demonstrated in recrystallized heme from these cells. These observations indicate that the physiological process of the assimilation of iron by the developing red cell is, first, the attachment of iron to acceptors in the red cell stroma capable of removing iron from the serum and second, the synthesis of heme. These studies also indicate that reticulocytes are still capable of completing the process of hemoglobinization in the peripheral blood, in keeping with observations that tagged reticulocytes live a normal life span (1).

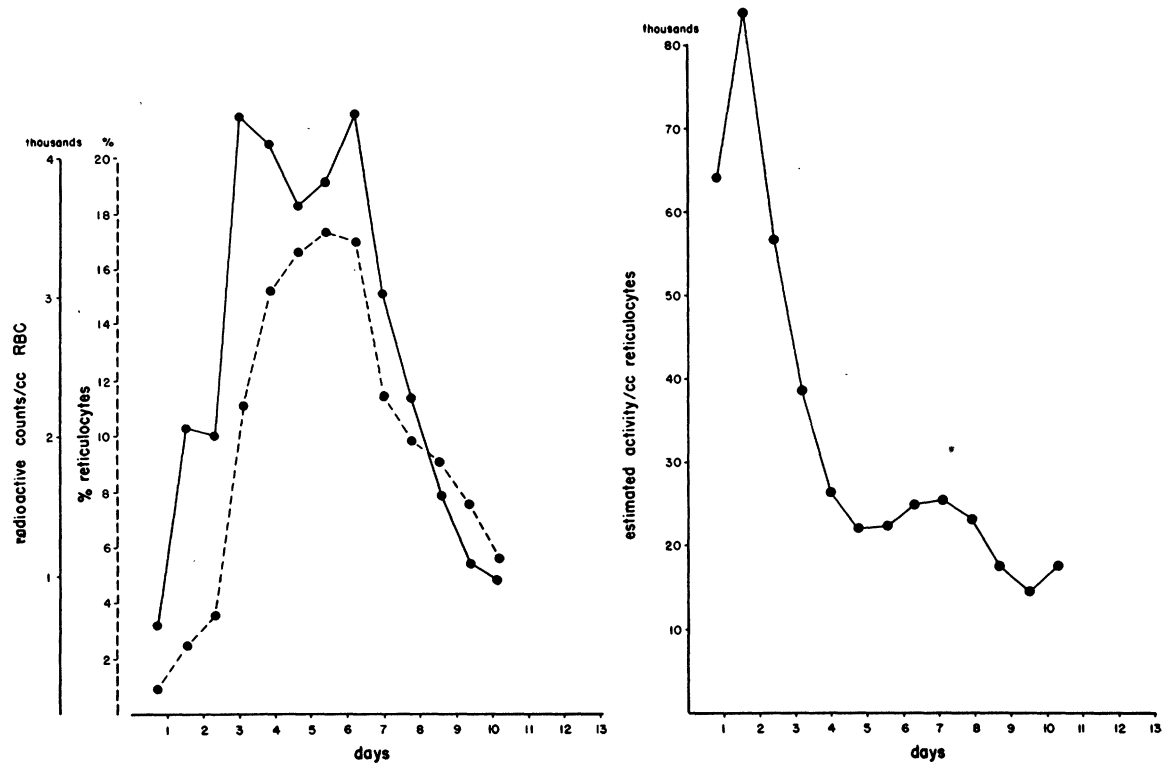

FIG. 1. Incubation of blood with radioiron *in vitro*. The patient studied had pernicious anemia and was given 60 units of liver concentrate on 0 day. On each subsequent day 20 ml of blood was drawn and incubated with radioiron over a period of 6 hr. The % reticulocytes and uptake of radioiron per ml of blood and per ml reticulocytes are shown above. It is assumed in the calculation of activity per ml reticulocytes that only reticulocytes take up radioiron.

The dotted line in the figure indicates % reticulocytes; the solid line indicates radioactive counts.

Similar incubation studies have been conducted on bone marrow aspirations. As might be expected from the spectrophotometric studies of Thorell relating to hemoglobin synthesis (*6*), the uptake of radioiron by normoblasts is much greater than that observed in reticulocytes. Again, radioactive heme could be demonstrated after incubation of radioiron with these marrow cells.

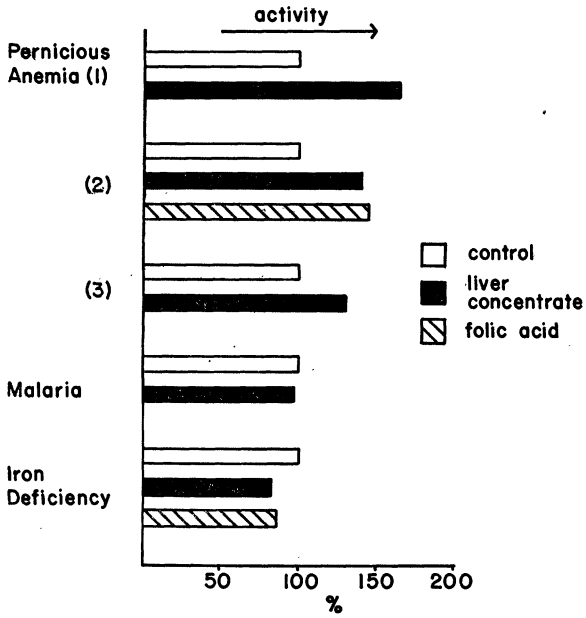

FIG. 2. Marrow incubation. Effect of folic acid and liver. Marrow cells obtained by sternal aspiration from patients with untreated pernicious anemia showed an increase in radioiron uptake of 66%, 44%, and 28% after addition of liver (1×10^{-5} unit) or folic acid (5 μg). This was in contrast to slight decreases found in other conditions not involving a deficiency in these substances.

It is possible to use radioiron as an indicator of altered hematopoiesis, as shown in Fig. 2. Suspensions of marrow from patients with untreated pernicious anemia have repeatedly shown an acceleration of the rate of iron uptake after the addition of liver or folic acid as compared with control studies. This has not occurred in conditions other than those characterized by specific deficiencies of the substances used. These observations on the marrow cells in untreated pernicious anemia indicate that these effective therapeutic agents act directly on the immature erythrocytes.

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Freezing of Whole Blood

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It is a generally accepted hypothesis that freezing and thawing of red cells results in hemolysis. Alternate freezing and thawing is a procedure commonly employed in the laboratory for the purpose of obtaining hemolysis. Preliminary experiments carried out in 1942 showed that the hypothesis is not correct under all conditions, i.e., it is possible to freeze whole blood in a solid mass and thaw it without appreciable hemolysis. "Freezing of blood" is here intended to mean solidification of blood by means of temperatures well below the freezing point of blood.

Early in our experiments, evidence showed that the type of water crystallization resulting from freezing was not an essential factor in the behavior of the red cells. Thus, equally good preservation of red cells was obtained by slow freezing at −3° C or by very rapid freezing at −60° C. In the case of slow freezing, the mass of frozen blood showed formation of large crystals; whereas with fast freezing, the mass of frozen blood appeared very uniform. Fast freezing was carried out as follows: 1 ml of whole human blood collected in an acid-citrate-dextrose mixture was placed in a glass test tube. The blood was frozen by manually rotating the tube in cracked CO_2 ice. Freezing occurred in a few seconds, and the tube containing the solid blood was removed instantly upon solidification and placed in the water bath at 37° C to thaw with the aid of agitation. The hematocrit before freezing was 39.37; after freezing and thawing, 39.07; the supernatant fluid showed no appreciable discoloration from hemoglobin. However, if frozen blood was allowed to remain in contact with CO_2 ice for even a few seconds after freezing, massive hemolysis resulted upon thawing.

Experiments on freezing of whole blood were resumed about a year ago. More than 150 specimens of blood have been frozen and thawed under varying conditions of temperature, heat dissipation (affecting the time of freezing), concentration of electrolytes, pH, concentration of diffusible and nondiffusible sugars (affecting the size of the erythrocytes), etc. In a series of a little over 100 specimens of blood, freezing and thawing were accomplished with a resulting hemolysis of less than 1% of the cells. Most of these specimens were collected in an acid citrate solution, with and without glucose. In these experiments the hematocrit was determined with the use of an air turbine (*2*) and the osmotic fragility by the method proposed by Parpart (*3*). The concentration of hemoglobin in the supernatant plasma was measured by the method of Karr and Chornock (*1*).

It is of practical importance to note that even when freezing and thawing of red cells at −3° C results in

[1] With the technical assistance of Miss Margaret M. Dolan and Miss Louise Colwell.