Hemoglobin, of the iron-containing compounds, has received the most attention because of its quantitative importance and ease of isolation. In contrast, tracer studies with radioiron of ferritin, cytochrome c, and myoglobin, which are more difficult to isolate, have been less numerous. The uptake of C\textsuperscript{14} by these proteins has received almost no study.

Of particular interest in relation to the present work is the comparative study of iron metabolism in organs of the rat by Copp and Greenberg (1). It was desired to extend these studies, based on tissues, to an investigation of the iron uptake by well defined iron-containing proteins and to measure the comparative uptake of C\textsuperscript{14} of acetate, which is a precursor of heme (2), by these proteins. The present experiments were designed to measure the comparative incorporation of C\textsuperscript{14} of methyl-labeled acetate and radioiron in normal guinea pigs and of radioiron in guinea pigs stimulated to hemapoiesis by cobalt and by blood loss. Proteins that were studied are hemoglobin, plasma proteins, cytochrome c of skeletal muscle, myoglobin of skeletal muscle, liver ferritin, and bone marrow.

**EXPERIMENTAL.**

*Animals*—Guinea pigs weighing 400 to 1000 gm. were chosen because of their adequate muscle mass for the isolation of myoglobin, which was of special interest in these studies. Stock animals were maintained on a diet of rabbit pellets\textsuperscript{1} supplemented by fresh lettuce twice weekly.

Polycythemia was induced by the intraperitoneal injection of 0.5 mg. of cobalt as cobalt acetate in isotonic solution on alternate days for 2 weeks. Anemia was produced by withdrawal of 30 per cent of the blood volume by heart puncture from the animal anesthetized with ether. Radioactive materials were injected intraperitoneally in isotonic solution. Animals

\* Aided by a research grant from the Christine Breon Fund of the University of California School of Medicine.

† Condensed from a thesis submitted by Harold L. Helwig to the Graduate Division of the University of California for the degree of Doctor of Philosophy, January, 1952. Present address, Radiation Laboratory, University of California, Berkeley, California.

\textsuperscript{1} Supplied by the California Milling and Grain Company, San Francisco.
anesthetized with Nembutal for withdrawal of blood by heart puncture were sacrificed by bleeding from the jugular vein.

Isolation and Purification of Proteins—Heparinized blood was taken by heart puncture and centrifuged to separate cells and plasma. Hemoglobin in solution from washed and lysed erythrocytes was chilled at 0° to permit crystallization. The crystalline hemoglobin was collected by centrifugation and was washed with 95 per cent ethanol, absolute ethanol, and ethyl ether. Plasma protein was precipitated by addition of 95 per cent ethanol and was washed successively with absolute ethanol and ethyl ether.

Cytochrome c was isolated from skeletal muscle according to the method of Keilin and Hartree (3). Twice crystallized ferritin was obtained from liver according to the method of Granick (4).

Bone marrow was collected by cutting off the ends of the long bones and forcing out the marrow by a blast of air through a hypodermic needle attached to an air pressure line. The marrow was washed successively with 95 per cent and absolute ethanol and ethyl ether.

The isolation and purification of myoglobin from skeletal muscle of the guinea pig have been described (5).

Wet Ashing of Proteins—Dry proteins were ashed in 50 ml. Kjeldahl flasks on a sand bath with the minimum amount of nitric acid. Ashing was completed with a few drops of Superoxol and heating to dryness. The ash was dissolved in hydrochloric acid and heated to near dryness to convert the iron to its chloride.

Preparation of Radioactive Samples—Because the radiations of Fe⁶⁵ are x-rays of about 0.007 m.e.v. energy and soft conversion electrons, it is essential that self-absorption be minimized. This is best accomplished by electroplating the iron in a thin metallic layer on copper. The method described by Vosburgh and coworkers (6), with slight modifications, was used. Self-absorption was standardized by addition of 3.75 mg. of carrier iron to each sample. The iron was deposited on an area of 4.91 sq. cm. by a current of 300 ma. at 8 volts d.c. in a period of 6 hours.

For the method of determining total iron see Helwig and Greenberg (5).

Samples of proteins labeled with C¹⁴ were prepared by transferring a suspension of dry alcohol-ether-washed protein in ethyl ether to a glass cylinder clamped against a weighed aluminum plate. A few ml. of petroleum ether were added and the solvents were allowed to evaporate. The deposited protein layer was dried at 100° for 1 hour in an oven and the plate was reweighed.

For the method of counting the radioactivity see Helwig and Greenberg (5). Self-absorption of radiations of C¹⁴ was corrected by factors calculated from measurement of multiple samples of a labeled plasma protein preparation.
Radioactive Materials—Fe$^{55}$ was obtained from the Isotopes Division, United States Atomic Energy Commission, Oak Ridge, Tennessee. The target material was enriched Fe$^{64}$. The preparation was 99 per cent radiochemically pure exclusive of Fe$^{59}$, which represented less than 10 per cent of the activity. Radioiron solution for injection contained 10 μg of iron with an activity of $3.84 \times 10^5$ c.p.m. per ml. in isotonic saline. The acetate solution for injection contained 1 mg of sodium acetate-2-C$^{14}$ with an activity of $2 \times 10^6$ c.p.m. per ml. in isotonic saline.

Doses of 1 mg. of the labeled sodium acetate per kilo were administered on 5 successive days. Radioiron was administered in a single dose of 20 μg of iron, with an activity of $7.68 \times 10^5$ c.p.m., per kilo. The results are expressed as counts per minute of C$^{14}$ per mg. of protein and counts per minute of Fe$^*$ per microgram Fe.$^2$

RESULTS AND DISCUSSION

Uptake of C$^{14}$ of Methyl-Labeled Acetate and Radioiron in Normal Guinea Pigs—In Fig. 1 are shown the curves of specific activities against time of five iron-containing proteins of normal guinea pigs given methyl-labeled acetate. Not included in Fig. 1 are the data for bone marrow protein,

$^2$ Fe$^*$ is used to designate radioiron.
which was nearly identical in specific activity to plasma. Of the proteins isolated, plasma and marrow were the first to reach a peak of specific activity. Hemoglobin, myoglobin, cytochrome c, and ferritin reached their maximum specific activity between the 10th and 15th days. Peak uptake of C^{14} was greatest in ferritin, followed, in decreasing order, by plasma, marrow, hemoglobin, myoglobin, and cytochrome. The rates of decrease of specific activity of hemoglobin and myoglobin were strikingly similar. That liver ferritin is a relatively labile protein was demonstrated by its rapid turnover of C^{14}.

The uptake of radioiron in proteins of normal guinea pigs is shown in Fig. 2. Maxima of specific activity in marrow, ferritin, and plasma occurred before the 9th day. Maximum incorporation of iron in hemoglobin and myoglobin was attained at 20 days. The specific activity of marrow iron initially exceeded that of the other proteins and approximated that of hemoglobin iron after the 20th day. That the specific activity of ferritin iron was lower than that of marrow iron indicated that the injected...
dose was preferentially deposited in marrow. Of the heme proteins, hemoglobin was most active in incorporating iron, followed by myoglobin and cytochrome, the iron metabolism of the latter being very sluggish indeed.

In a recent study of the distribution of radioiron in normal guinea pigs by Theorell and coworkers (7), the specific activity of iron of liver ferritin, red blood cells, and cytochrome c varied with time in a pattern qualitatively similar to our results. However, they found insignificant specific activity of myoglobin iron before the 30th day in myoglobin frac-

![Graph](http://www.jbc.org/)

**Fig. 3.** Disappearance of C$^{14}$ and radioiron from hemoglobin and myoglobin of normal guinea pigs.

tionated with (NH$_4$)$_2$SO$_4$ and corrected for hemoglobin contamination. It was our experience with this method and comparable muscle samples, but with Fe of higher specific activity, that the correction for specific activity of hemoglobin was unfavorably high in the precipitates at 70 to 90 percent saturated (NH$_4$)$_2$SO$_4$ and that the small yields of myoglobin in fractions at higher salt concentration gave counting rates of low accuracy. Our finding of significant specific activity of myoglobin iron in 7 days appears to result from our use of iron of higher specific activity and preparation of myoglobin which was free of measurable hemoglobin.

Comparison of the data of Figs. 1 and 2 shows that the turnover rates of C$^{14}$ and Fe$^+$ in plasma protein, bone marrow, and ferritin were greater than in the heme proteins.

Semilog plots of the specific activity of C$^{14}$ and radioiron of hemoglobin
and myoglobin against time are shown in Fig. 3 for normal animals sacrificed over a period of 7 to 62 days. It should be emphasized that these data cannot be used to determine the biological life of the proteins because the activity was not rapidly introduced into the proteins and because iron may be reutilized in heme synthesis. However, several valid comparisons may be made. It is apparent that the maximum in specific activity of C¹⁴ in the two proteins occurred before the maximum in iron,

![Graph showing specific activity of iron and carbon over time](image)

indicating that the heme or protein moieties may be synthesized as much as 5 days prior to the incorporation of iron into the molecules.

The values of the half times of the disappearance of labeled carbon were approximately 30 days for hemoglobin and 34 days for myoglobin and, for labeled iron, the values were 33 days for hemoglobin and 38 days for myoglobin. Within the error of estimation, it can be concluded that hemoglobin iron and carbon labels disappeared at the same rate, which is consistent with our knowledge of the destruction and renewal of hemoglobin. These data also indicate that myoglobin metabolism followed a similar pattern and that the rates of turnover of hemoglobin and myoglobin were approximately the same.

*Uptake of Radioiron in Guinea Pigs Treated with Cobalt*—For investiga-
tion of the effect of cobalt on the uptake of iron in iron-containing proteins, male guinea pigs were given 0.5 mg. of cobalt as cobalt acetate in isotonic solution on alternate days for 2 weeks. On the day following the last injection of cobalt, radioiron was administered. Hematocrit values of 48 to 50 per cent and hemoglobin values of 16 to 18 gm. per 100 ml. of blood at the end of cobalt treatment declined rapidly to normal values of 40 to 44 per cent and 12 to 14 gm. respectively. Data for the uptake of iron during recovery from cobalt stimulation of hematopoiesis are shown in Fig. 4, a and b.

Although the blood picture of the cobalt-treated animals did not differ significantly from normal at the time of sacrifice, the uptake of iron by the proteins studied showed marked changes from that of the normal animals. Increased activity of iron transport by plasma was indicated by specific activity values 2 to 3 times normal. Instead of reaching a maximum at 20 days, as in the normal animal, the specific activity of hemoglobin iron continued to increase over the entire experimental period. The depressed utilization of iron for hemoglobin synthesis was reflected by a maximum specific activity of marrow iron equivalent to one-third the normal value and a decreased turnover rate of marrow iron during the first 20 days.
That the specific activity of ferritin iron was higher than normal and exceeded that of marrow iron was indicative of increased storage of iron in the cobalt-treated group. The specific activity of myoglobin iron approximated the normal maximum value, but remained nearly constant, while normal values declined after 20 days. The uptake of iron by cytochrome was significantly increased in the cobalt-stimulated animals.

**Fig. 5.** Uptake of radioiron by proteins of bled guinea pigs. 30 per cent of the blood volume was withdrawn by heart puncture. 3 days later, radioiron was administered in a single intraperitoneal dose of 20 g of Fe (7.68 X 10⁶ c.p.m.) per kilo.

*Uptake of Radioiron in Guinea Pigs after Hemorrhage*—Male guinea pigs were anesthetized with ether and 30 per cent of the blood, calculated from a blood volume equivalent to 5 per cent of body weight, was withdrawn by heart puncture. 3 days later, radioiron was administered intraperitoneally. Normal values for the hematocrits of these animals at sacrifice showed a rapid recovery from hemorrhage. The hemoglobin levels of 9.0 to 12.5 gm. were lower than the normal values of 12.0 to 14.0 gm. per 100 ml. of blood. Curves of the specific activity values of iron of the proteins at the time of sacrifice are shown in Fig. 5.

In the animals recovering from anemia of hemorrhage, the specific activity of plasma iron increased rapidly and was nearly twice the normal
value throughout the experimental period. The plasma iron concentration was 50 per cent above normal. The elevated specific activity and concentration of plasma iron indicated an increase of iron transport by plasma in response to bleeding. In the hemorrhagic animals the specific activity of hemoglobin iron increased rapidly during the first 10 days and thereafter increased at a slower rate throughout the experimental period. It attained only half the normal maximum value at the 15th day. A compensatory shift of unlabeled storage iron from ferritin to bone marrow after bleeding and prior to radioiron administration was indicated by the values of specific activity of iron in these proteins. During the first 15 days, the specific activity of ferritin iron was only slightly less than normal and that of marrow iron was one-third of normal, in contrast to decreased iron storage in ferritin and increased uptake of iron in bone marrow expected in chronically iron-deficient animals, unsupplemented with iron. The turnover rate of marrow iron was greater than normal after the 20th day. Whereas in the normal animal the specific activity of myoglobin reached a maximum at 20 days and declined thereafter, in the bled animals the value increased throughout the period of 50 days. The rates of uptake of iron by hemoglobin and myoglobin were nearly equal and suggested that the stimulus of blood loss had a similar effect on iron metabolism of both of these proteins. The specific activity of cytochrome iron of bled animals was significantly higher than in normal animals. Hemorrhage appeared to stimulate the turnover of iron in this pigment.

SUMMARY

A comparative study has been made of the uptake of intraperitoneally administered C\textsuperscript{14} of methyl-labeled acetate and radioiron by hemoglobin, plasma protein, myoglobin, liver ferritin, cytochrome c, and bone marrow of guinea pigs. In normal animals the uptake of C\textsuperscript{14} per gm. of protein was greatest in ferritin, followed by plasma, marrow, hemoglobin, myoglobin, and cytochrome. Incorporation of iron per gm. of protein was greatest in marrow, followed by plasma, ferritin, hemoglobin, myoglobin, and cytochrome. Turnover rates of C\textsuperscript{14} and iron in ferritin, plasma, and marrow were greater than in the heme proteins. The lability of liver ferritin was indicated by its rapid turnover of C\textsuperscript{14}. The half times of the disappearance of C\textsuperscript{14} and radioiron of hemoglobin and myoglobin were approximately the same. Treatment of guinea pigs with polycythemic doses of cobalt increased the uptake of iron by plasma and ferritin. During recovery from polycythemia, the uptake of iron by hemoglobin and marrow was depressed. In the posthemorrhagic state, radioiron was incorporated into hemoglobin and myoglobin at similar rates. The uptake of iron by cytochrome was increased and the turnover of marrow iron was accelerated in bled animals.
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