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Early Labeled Heme Synthesis from Delta-Aminolevulinic Acid-4-[^{14}C] in Rats: Comparison with Glycine-2-[^{14}C][1] (40227)

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Studies with glycine-2-[^{14}C] have recently demonstrated two well-defined early peaks of labeled heme synthesis in both liver and young erythroid cells (1, 2). This bimodal configuration has not been reported in studies with other labeled precursors such as delta-aminolevulinic acid (ALA) (3–7). ALA is commonly used in investigations of hepatic heme metabolism because it is a relatively specific precursor, by-passing the rate-limiting step in heme biosynthesis (8), and is incorporated into heme in the liver to a far greater extent than in erythropoietic cells (9–11). These features confer a quantitative advantage on the use of ALA but may lead to somewhat unphysiologic results, as in studies of the sources of bile pigment formation (10–12). Labeled glycine has freer access to the multiple sites of heme synthesis and gives a more physiological representation of heme degradation and bile pigment production (11, 12), although it is incorporated into heme to only a small extent. The early phases of heme labeling were therefore measured with ALA-[^{14}C], exactly as done previously with glycine-[^{14}C] (1, 2), to test the comparability of these two substrates for studies of heme metabolism.

Materials and methods. A total of 28 male Sprague–Dawley rats (Charles River Laboratories, Wilmington, Mass.), weighing 200–250 g, received single intravenous injections of ALA-4-[^{14}C] (25.4 Ci/mM, New England Nuclear Corp.), 2 μCi per 100 g body wt. At frequent intervals (see Figures) groups of four animals were exsanguinated by cardiac puncture and the livers were perfused with 50 ml cold physiologic saline and excised. Liver was homogenized in cold 0.25 *M* sucrose and a portion of the homogenate used to prepare microsomal and mitochondrial fractions (2). A measured amount of normal rat hemoglobin was added as carrier to the liver samples and hemin was crystallized (13) for radioassay after addition of 1% deoxycholate as solubilizer (3). Incorporation of ALA-[^{14}C] into heme in total liver, microsomes and mitochondria was calculated from the measured hemin specific activity, the amount of nonradioactive carrier heme added initially in the form of hemoglobin and the fraction of the total liver homogenate and of the total microsomal and mitochondrial preparations that was used for extraction of hemin-[^{14}C]. These methods yielded similar recoveries of hemin-[^{14}C] from whole liver and microsomal and mitochondrial fractions, ranging from 65–70% (2). Reproducibility of hemin-^{14}C extraction was within 1% with all liver fractions. Assays of NADPH-cytochrome c reductase and cytochrome oxidase activities indicated 57.2% (SE 5.2%) and 38.7% (SE 3.0%) recoveries of microsomes and mitochondria respectively from the total liver homogenate, with minimal cross-contamination between these two fractions. Studies with ^{51}Cr-labeled red cells demonstrated negligible contamination of liver fractions with peripheral blood.

Hemin was crystallized from the cardiac blood samples by the same methods used for extraction of liver heme except that no carrier hemoglobin was added. Incorporation of ALA-[^{14}C] into total hemoglobin heme was calculated from the specific activity of the isolated hemin-[^{14}C] and the total amount of heme present in the peripheral blood, based on the hemoglobin concentration and the blood volume estimated from body wt (14).

Results. Figure 1 shows the measured incorporation of ALA-[^{14}C] into total liver hemes. In whole liver, heme labeling rose very rapidly to a peak value at 1 hr. This was followed by a transient fall, an increase to a second more moderate peak at 2.5 hr, and then a gradual decline over the remaining

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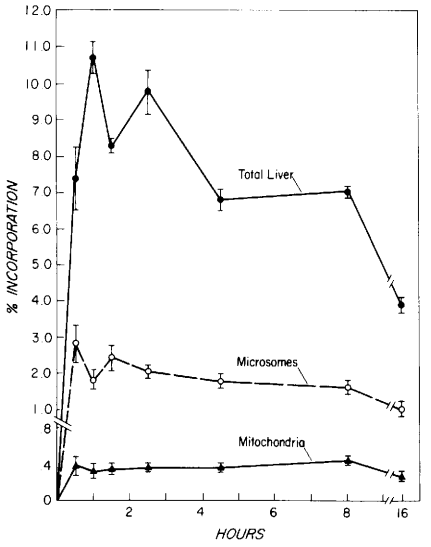

FIG. 1. Measured incorporation of ALA-[^{14}C] into total liver heme and total liver microsomes and mitochondria. Each value represents mean ± SE for four rats.

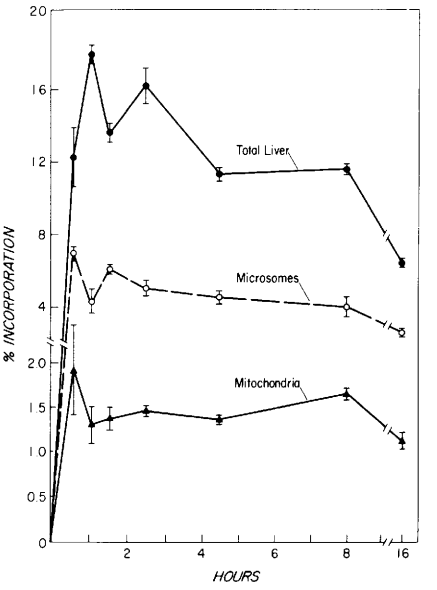

FIG. 2. Incorporation of ALA-[^{14}C] into total liver heme (from Fig. 1) corrected for the efficiency of heme extraction from the different liver fractions and for the recovery of microsomes and mitochondria from the whole liver homogenate (see text).

period of observation. Similar patterns were observed for the microsomal and mitochondrial fractions except for more rapid labeling of the heme peaks. In both subcellular fractions the initial peak occurred at 0.5 hr followed by a trough at 1 hr, as compared to 1 and 1.5 hr respectively with whole liver. In the microsomes the second heme peak also occurred earlier than in whole liver, 1.5 as compared to 2.5 hr. Possibly because of the low level of isotope incorporation, a second peak was not found in the mitochondrial fraction.

Figure 2 shows these same curves after correction for the efficiency of heme extraction and recovery of microsomes and mitochondria from whole liver. This provides an estimate of the true magnitude of ALA-[^{14}C] incorporation into liver heme, but is based on the assumption that the heme and the subcellular fractions that were isolated were fully representative of those present in situ. With this assumption, 18% of the injected ALA was recovered in heme in whole liver at 1 hr. The corresponding values for the initial peaks in microsomes and mitochondria were 7.0 and 1.9% respectively. Heme labeling in the microsomes exceeded that in the mitochondria throughout the duration of study. In addition, heme labeling in whole liver always exceeded that in both subcellular fractions combined. As shown in Fig. 3, about 73% of the total heme labeling could be accounted for by these two fractions at 0.5 hr, when there was already peak labeling in the microsomes and mitochondria but when label incorporation into whole liver heme was still increasing. This value fell to 31% at 1 hr when heme labeling had fallen in the two subcellular fractions but had reached maximal levels in whole liver. Thereafter this percentage varied between 49 and 54% except for a value of 39% at 2.5 hr, the time of the second peak in whole liver.

Figure 4 shows the incorporation of ALA into heme in peripheral blood erythroid cells. As with the liver preparations, there was a very rapid increase in heme labeling during the first 0.5 hr, reaching maximal levels at 1.0–1.5 hr. However, this was not followed by the distinct second peak that was observed in the studies with glycine-[^{14}C] (1, 2). The apparent increase at 4.5 hr is of doubtful significance because of the large standard error; if this value is disregarded, there was only a gradual, irregular increase in red cell heme labeling after 1 hr.

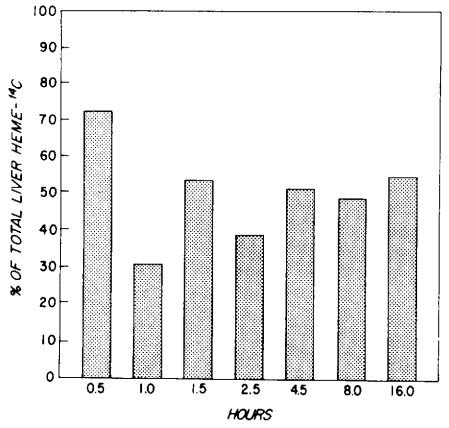

FIG. 3. Percent of total hepatic heme labeling present in both microsomes and mitochondria. Values have been corrected for the efficiency of recovery of heme and the subcellular fractions, as in Fig. 2.

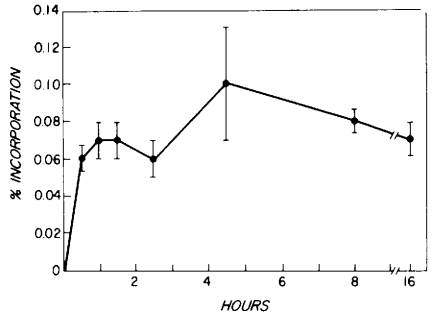

FIG. 4. Incorporation of ALA-[^{14}C] into total hemoglobin heme in the peripheral blood. Each value is mean ± SE for four rats. Incorporation at 48 hr (not shown) was 0.075%.

Discussion. The pattern of hepatic heme labeling with ALA-[^{14}C] differs in several respects from that observed with glycine-[^{14}C] under identical experimental conditions (1, 2). The bimodal configuration of the labeling curve is much less clear with ALA, because of a less marked trough between the 2 early peaks and because these peaks are less separated in time; the second peak occurs at 2.5 hr with ALA as compared to 4.5 hr with glycine. It is easy to see how such a brief fall in labeling may have been missed in studies in which less frequent measurements were made. In addition, the heme peaks in the microsomal and mitochondrial fractions occur earlier than the corresponding peaks in whole liver; with glycine-[^{14}C], by contrast, the kinetics of heme labeling are similar in microsomes, mitochondria and whole liver. In these and in earlier studies with ALA (6, 7), the bulk of heme labeling is found in the microsomal fraction; with glycine, heme labeling in mitochondria is almost equivalent to that in microsomes. The percentage of the total heme labeling that can be attributed to these 2 subcellular fractions (Fig. 3) is roughly comparable with ALA and glycine except at the earliest time point, 0.5 hr; this value is 73% with ALA as compared to 35% with glycine, a discrepancy which may be explained by the more rapid incorporation of ALA into the initial heme peak in both microsomes and mitochondria than in whole liver.

It is likely that the curves obtained with ALA are a less valid representation of hepatic heme synthesis than those obtained with glycine. There are several assumptions and limitations inherent in pulse-labeling studies such as these (2), whether ALA or glycine is used as precursor. Moreover, hemes labeled with either of these precursors may not be equally well isolated from hemoproteins, heme loosely attached to microsomal membranes and "free" heme. Similarly, all portions of the endoplasmic reticulum may not be equally susceptible to extraction. Thus, calculation of the total magnitude of hepatic heme labeling based on the overall efficiency of heme isolation and on the recoveries of microsomes and mitochondria from whole liver (Fig. 2) is best viewed as an approximation. Use of ALA as a precursor has the additional disadvantage that it enters the pathway of heme biosynthesis beyond the normal rate-limiting step (8). Moreover, the factors that cause ALA to label heme in hepatic cells to a much greater extent than in erythroid cells (9–11) (Figs. 2 and 4) might also lead to unphysiologic labeling of certain of the heme pools within the liver itself. Glycine, on the other hand, provides a more or less physiologic assessment of heme turnover and bile pigment production (11, 12).

It is not surprising that the peaks of heme labeling often occur earlier with ALA than with glycine, since substrate incorporation into heme is not retarded at the level of ALA-synthetase. However, it is not clear why, with ALA, the heme peaks in the subcellular liver fractions occur earlier than the corresponding peaks in whole liver. Newly synthesized heme

in transit from mitochondria to microsomes (7, 8) may perhaps account for this difference. The pulse of ALA-[^{14}C] might represent a transient excess of substrate, leading to a transient excess of highly labeled heme. Disposition of this heme might be delayed during transit through the cytosol, accounting for its prolonged appearance in whole liver but not in the microsomal and mitochondrial fractions. Previous studies have shown that exogenous ALA does increase net heme synthesis (15) and catabolism (16) in the liver, although the amounts of ALA administered were larger than those used in the present experiments.

If most heme is synthesized in the mitochondria, a larger and more rapid peak of mitochondrial heme synthesis might have been anticipated. Possibly, this was missed because measurements were not made at sufficiently early times. Alternatively, heme newly made in the mitochondria and destined for other sites such as the endoplasmic reticulum may be transferred there immediately. A sudden excess of labeled heme might not be so readily accommodated, leading to its temporary accumulation in the cytosol.

Although ALA does not provide clear discrimination of the two early peaks of hepatic heme labeling, this bimodal pattern is undoubtedly real. Independent studies of bile pigment production in both intact rats (11) and isolated rat liver (17), with either ALA-[^{14}C] or glycine-[^{14}C] as precursor, have demonstrated substantial loss of labeled heme at a time corresponding to the trough between the two peaks. In rats up to 20% of ALA-[^{14}C] is excreted as early-labeled bilirubin 1–2 hr after isotope administration (11), exceeding the fall in heme labeling in whole liver during the same interval of time (Fig. 2). It is unlikely that this discrepancy is due to degradation of early-labeled heme in other tissues such as the kidney (18). Thus, the trough between the two heme peaks in the liver probably represents a composite of heme catabolism and continuing heme synthesis, the latter eventuating in the second peak of early heme formation.

The nature of these two early peaks of hepatic heme labeling is not yet clear. We have speculated (1, 2) that the first peak may represent a fraction of free or unassigned heme (12, 16, 18) with a rapid rate of turnover and with important regulatory functions; the second peak may be related to the formation of specific hemoproteins. Recent pilot experiments, however, have suggested that the first heme peak is also associated with specific apoproteins. Biochemical characterization of these peaks should provide new insights into the mechanisms of hepatic heme synthesis.

Summary. The early phases of heme synthesis were measured with ALA-[^{14}C] as precursor. Two early peaks of heme labeling were observed in the liver, as found in earlier studies with glycine-2-[^{14}C]. However, hepatic heme labeling with ALA differed from that observed with glycine in several respects. There was much poorer discrimination of the two early heme peaks, with more rapid formation of the second component. Formation of both peaks occurred more rapidly in microsomes and mitochondria as compared to whole liver, and there was a larger contribution of the microsomal fraction to total hepatic heme labeling. Also in contrast to the findings with glycine-[^{14}C], there was no distinct early heme peak in the peripheral blood. These differences may be due to the fact that ALA enters the pathway of heme biosynthesis beyond the primary rate-limiting step and could have selective access to certain sites of heme formation. ALA is often employed as a selective label for liver hemes. However, glycine may be required for more definitive assessment of the kinetics and magnitude of labeled heme synthesis.

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