To the Graduate Council:

I am submitting herewith a dissertation written by Michelle Laborde Vineyard entitled "An Evaluation of ^{14}C-delta Aminolevulinic Acid as a Non-invasive Measure of the Mixed Function Oxidase System of Mice." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Home Economics.

John T. Smith

John T. Smith, Major Professor

We have read this dissertation
and recommend its acceptance:

Robert H. Kirk

Ada Marie Campbell

Mary Nell Taylor

Accepted for the Council:

L. Edwin Pell

Vice Chancellor
Graduate Studies and Research

AN EVALUATION OF 5-^{14}C-DELTA-AMINOLEVULINIC ACID AS A NON-INVASIVE MEASURE OF THE MIXED FUNCTION OXIDASE SYSTEM OF MICE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Michelle Laborde Vineyard

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ABSTRACT

The mixed function oxidase system has been postulated as a factor in the development of obesity due to alterations of energy metabolism. Previous measurements of the mixed function oxidase system used an in vitro procedure. A non-invasive measurement of the mixed function oxidase system which would be convenient to use, cause minimal stress to the test subject, and produce consistent results would be desirable.

5-^{14}-C-delta-aminolevulinic acid was evaluated as a non-invasive measurement of the mixed function oxidase system in mice. ALA (20 mg/ml) was administered by intraperitoneal injections to mice and the ^{14}CO excreted via the lungs was collected. A closed circulation apparatus was devised to collect the ^{14}CO excreted. The ^{14}CO was trapped in a scintillation liquid for radioactive counting by bubbling the gas through a solution of palladium chloride (0.8 g/liter) which converted the ^{14}CO to $^{14}CO_2$.

Two age groups of mice, consisting of six pairs each of obese and lean littermates, were studied. The collection of ^{14}CO from the mice was over a three-hour time period 13 to 16 hours after injection of ALA. Immediately following the ^{14}CO collection, the hepatic cytochrome P-450 level of each mouse was determined by isolation of liver microsomes and spectrophotometric examination. The results of the two procedures were compared.

Five normal mice were selected to evaluate the pattern of ^{14}CO excretion by collecting ^{14}CO at hourly time intervals from 1 through 15

hours after the injection of ALA. The incorporation of injected ALA into the blood, heart, lungs, kidneys, spleen, and liver of randomly selected groups at eight time periods after injection was also measured. To determine the consequences of an oral dose of ALA, ten rats were administered 4-^{14}C-ALA on a weight basis. Urine and feces were collected for each rat for 24 hours and then the animal was sacrificed. The incorporation of ALA by the liver, heart, lungs, spleen, and kidneys was measured.

To verify that the ^{14}CO collection was a measurement of the mixed function oxidase system, known inducers of the enzyme system were administered to lean mice. Two groups of five mice each received sodium pentobarbital (2mg/ml) for different induction time periods. One group of five mice received injections of benzo-α-pyrene in corn oil (12 mg/ 5 ml). The ^{14}CO excreted from each mouse after ALA injection was collected.

Results of this investigation indicated that the administration of ALA and subsequent ^{14}CO collection was an effective measure of ^{14}CO excretion. The pattern of ^{14}CO excretion over a prolonged time period was a sharp initial peak within three hours followed by a steady decline to a stable amount of ^{14}CO excretion by the sixth hour. A similar pattern of ALA incorporation into some of the organs of the mice was noted.

There was a significantly greater excretion of ^{14}CO by lean mice than by obese mice at both ages studied. No difference was noted between the age groups for the lean or the obese mice. It was not possible to correlate the ^{14}CO values with the hepatic cytochrome P-450 values.

Pentobarbital induction for a sufficient time period resulted in a significantly greater ^{14}CO excretion. Benzo-α-pyrene did not appear to cause an increase in the ^{14}CO excretion. Administration of an oral dose of ALA to rats resulted in a large portion of the ALA being excreted in the urine. However, ALA incorporation into various organs was determined. Differences in incorporation between male and female rat organs were measured.

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CHAPTER I

INTRODUCTION

Many influences have been reported as contributing factors in excessive weight gain and the development of obesity. Primary attention is usually given to aspects of overeating or insufficient activity levels which lead to a positive energy balance and adipose tissue formation. However metabolic efficiency of energy utilization in some individuals has received strong support as another causative agent in obesity development. Considerable evidence suggests that certain animal species can adapt to increased efficiency of food conversion to lipid and slower catabolism of stored tissue (1-3). It has been postulated that the hepatic microsomal mixed function oxidase system may be a factor in this increased efficiency of metabolism. The presence of an increased metabolic rate was detected in rats given known inducers of hepatic microsomal enzymes as measured by increases in oxygen consumption and changes in body weight (4).

The mixed function oxidases involve endogenous as well as exogenous substrates. Cytochrome P-450, which is recognized to function as a terminal oxidase in drug metabolism, accounts for a major portion of this oxidase system (5). These oxidation enzymes are inefficient because they utilize oxygen directly with oxidation of both the substrate and the cofactor, NADPH, but without coupling to the electron transport system. The energy produced is dissipated as heat instead of being used for generation of ATP (6). Up to 6 percent of an individual's oxygen

utilization has been estimated to occur via the hepatic mixed function oxidase system (7). Changes in the activity of this system could have a significant effect on net energy balance of an individual.

The laboratory rodent has served as a useful model for studying the physiological basis for genetic differences in obesity. Investigations in this laboratory[1] have used the C57BL/6J-ob mouse strain[2] to determine differences in levels of hepatic cytochrome P-450 in normal and obese (ob/ob) mice. The results of these studies indicate a significantly lower level of cytochrome P-450 in obese mice when compared to corresponding lean controls at ages of 6 weeks to 7 months. The genetic and physiologic characteristics of the obese (ob/ob) mouse have been extensively reviewed.[1] The determination of hepatic cytochrome P-450 was done according to the in vitro technique of Omura and Sato (8,9) by isolation and purification of liver microsomes from liver homogenate.

To further test the hypothesis of increased energy efficiency in obesity, it would be desirable to utilize a non-invasive technique of measuring cytochrome P-450. This method should be convenient and consistently reproducible. One approach for doing this involves a measurement of carbon monoxide (CO) excreted via the lungs as a result of the catabolism of heme.

This investigation was conducted to evaluate the use of 5-^{14}C-delta-aminolevulinic acid (ALA) as a non-invasive measurement of the degradation

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[1]Dorsey, J. L. (1976) An investigation of the hepatic cytochrome P-450 levels of normal and obese (ob/ob) mice. Unpublished Doctoral Dissertation, The University of Tennessee, Knoxville.

[2]Jackson Memorial Laboratory, Bar Harbor, Maine 04609.

of hepatic cytochrome P-450 in normal (?/+) and obese (ob/ob) mice. ALA is an intermediate compound in the heme biosynthetic pathway. Administered doses of ALA are used primarily for the synthesis of non-hemoglobin hemeproteins. Catabolism of these hemeproteins, including cytochrome P-450, results in excretion of CO. It was postulated that administration of 5-^{14}C-ALA will result in excretion of ^{14}CO in quantities which will serve as comparative measurements of the mixed function oxidase system, especially cytochrome P-450.

CHAPTER II

REVIEW OF LITERATURE

I. MICROSOMAL MIXED FUNCTION OXIDASE SYSTEM

Enzymes which incorporate a single oxygen atom into carbon chains of intermediate metabolites without equilibration with water are designated monoxygenases (10). These monoxygenases are present in the microsomal fraction of the liver, kidneys, lungs, intestinal mucosa, adrenal gland, and pancreas, and the mitochondrial portion of organs which synthesize steroid hormones (11). Because molecular oxygen, the source of the hydroxyl group in the enzymatic reaction, is reduced while the substrate is oxidized, these monoxygenations are collectively referred to as mixed function oxidations (12) or microsomal hydroxylations (13). The following reaction is catalyzed by microsomal mixed function oxidases:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \qquad (11,14).$$

The liver microsomal mixed function oxidases are membrane-bound and function as a multicomponent electron transport system responsible for the metabolism of a variety of endogenous substrates (steroids, fatty acids, bile acids) and exogenous substances (drugs, carcinogens, insecticides, and others). Some studies also indicate that a small amount of ethanol may be metabolized by a microsomal fraction of hepatocytes (15). The rate of metabolism of various compounds depends on the species,

strain, age, tissue nutritional status, and pretreatment of the animal (13,14,16). The drug metabolizing mixed function oxidation system has been extensively reviewed including the reviews of Kuntzman (17) and Conney (18).

During the course of studies on fatty acid ω-hydroxylation by liver microsomes, the mixed function oxidase system was solubilized and resolved into three components: cytochrome P-450, NADPH cytochrome P-450 reductase, and a heat stable factor now identified as phosphatidylcholine (13,16). Cytochrome P-450 is the most important of the three components because of its vital role in oxygen activation and substrate binding.

Cytochrome P-450

Cytochrome P-450 is best characterized by the presence of an absorbance band at about 450 nm for the carbon monoxide adduct of the reduced pigment. The hemeprotein nature of cytochrome P-450 was established by Omura and Sato (8,9) who also described a millimolar extinction coefficient for the substance.

Much of the present knowledge on the function of cytochrome P-450 has been derived from spectrophotometric observations obtained by examining special perturbations occurring on addition of various substrates, oxygen, or reduced pyridine nucleotides to microsomal fractions (19). Cytochrome P-450 is the dominant hemeprotein of microsomes (20). Although many tissues also contain some cytochrome b_5 (19), Levin et al. reported that cytochrome b_5 does not appear to be an obligatory component of the mixed function oxidase system (21).

Estabrook et al. (20) summarized what is currently known about the oxidation reaction catalyzed by cytochrome P-450 (Figure 1): (1) There is an interaction of the ferric form of cytochrome P-450 with a substrate to form a complex; (2) this complex is reduced by an electron donated by the flavoprotein, NADPH-cytochrome P-450 reductase; (3) oxygen interacts with the reduced hemeprotein to form an oxycytochrome P-450 complex still tightly bound by a molecule of substrate; (4) there is a further reduction of the oxygen-substrate-P-450 complex by an electron donated from either a reduced flavoprotein or possibly reduced cytochrome b_5; (5) one atom of oxygen is reduced to water while the other atom of molecular oxygen is introduced as a hydroxyl group into the substrate molecule; (6) finally there is dissociation of the product with regeneration of the ferric hemeprotein.

Multiple Forms of P-450

The broad substrate specificity of cytochrome P-450 is due to occurrence of multiple forms of the cytochromes which differ on the basis of decreasing mobility and increasing subunit molecular weight (14). Evidence of at least six separate forms of cytochrome P-450 in rat liver has been demonstrated (22) although not all strains are responsive to each of the different inducers. Multiple forms of the mixed function oxidase system have been identified on the basis of which types of drugs will induce each of the fractions of the enzyme system. Basically there are two types of enzyme inducers. The polycyclic hydrocarbons such as 3-methylcholanthrene, benzo-α-pyrene, and 1,2,5,6-dibenzanthracene induce one form of the enzyme system referred to as P-448, P-446, or P_1-450.

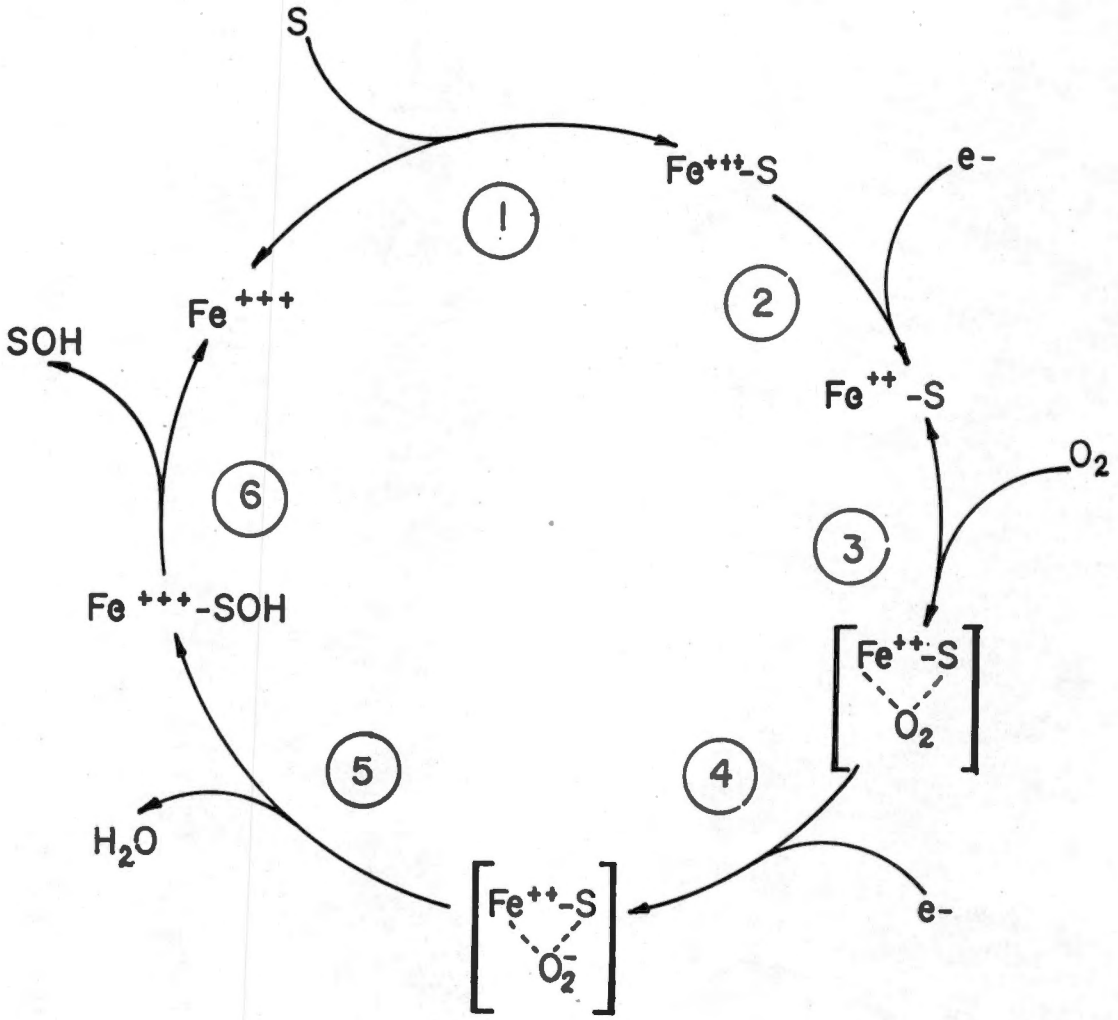

Fig. 1. Scheme of the cyclic function of cytochrome P-450 (20).

Barbiturates will induce another spectrally distinct form—P-450 (17,18, 23-29). When either of these forms is reconstituted with lipid and NADPH cytochrome C reductase it is catalytically active although the different forms exhibit different substrate specificities (30).

The drug metabolizing enzymes are concentrated in the smooth endoplasmic reticulum. Phenobarbital causes a proliferation of the smooth endoplasmic reticulum and thus an increase in cytochrome P-450 as observed both in vivo (18,28,31-34) and in vitro. No influence of phenobarbital on cytochrome b_5 levels has been observed (35). Nebert reported that one form of cytochrome P-448 is induced by polycyclic aromatic compounds in the liver of the C57BL/6N (B6) mouse strain (22). Wood et al. (36) found that highly purified P-448 metabolized benzo-α-pyrene to highly mutagenic metabolites while purified P-450 was relatively poor in metabolizing the benzo-α-pyrene.

II. DELTA-AMINOLEVULINIC ACID

Proteins such as cytochrome P-450 which contain the characteristic ring of heme are generally classified as hemeproteins (37). The heme molecule is distinct from other tetrapyrroles in that the four linked pyrrole rings hold iron in complex formation. The sequence of reactions leading to heme synthesis starts with the formation of delta-aminolevulinic acid (ALA) (38).

The Role of ALA in Heme Biosynthesis

The amino acid glycine has been demonstrated to be a common substrate for the biosynthesis of heme (39,40). Early studies by Neuberger and

Scott (41) determined in rats that two molecules of glycine condensed with one molecule of succinic acid to form δ-ALA, a five carbon ketone. Gibson, Laver, and Neuberger (42) later showed that preparations of freeze-dried particles of chicken erythrocytes synthesized δ-ALA from succinyl Co A and glycine. Numerous researchers have confirmed these results in a large number of cells and in many avian and mammalian tissues (5). This condensation reaction is the first rate-limiting step in heme biosynthesis. It is controlled by the enzyme ALA synthetase which requires pyridoxal phosphate as a cofactor (5,37,38,43).

The next reaction is the formation of the precursor pyrrole ring, porphobilinogen (PBG). This reaction is catalyzed by the enzyme ALA dehydrase (5,37). The synthesis of ALA and PBG is diagrammed in Figure 2. Isotopically labelled carbons from either 2-^{14}C-glycine or 5-^{14}C-ALA precursors are indicated.

Finally the characteristic protoporphyrin of heme is formed, comprised of four of the PBG rings linked by methene bridges. Further studies have shown that the glycine molecule is the source of the four nitrogen atoms and eight of the carbon atoms of the protoporphyrin molecule (5,44,45). The carbons are from the α-carbon of glycine, four of which are attached to the nitrogen on the pyrrole ring while the other four form the methene bridges which link the pyrrole rings. The carboxyl carbon of glycine is not used in the synthesis of the pyrrole ring (45). Figure 3 depicts the pathway of heme biosynthesis.

While ALA has been demonstrated to be utilized in pathways other than heme biosynthesis, the principal pathway for utilization of ALA is

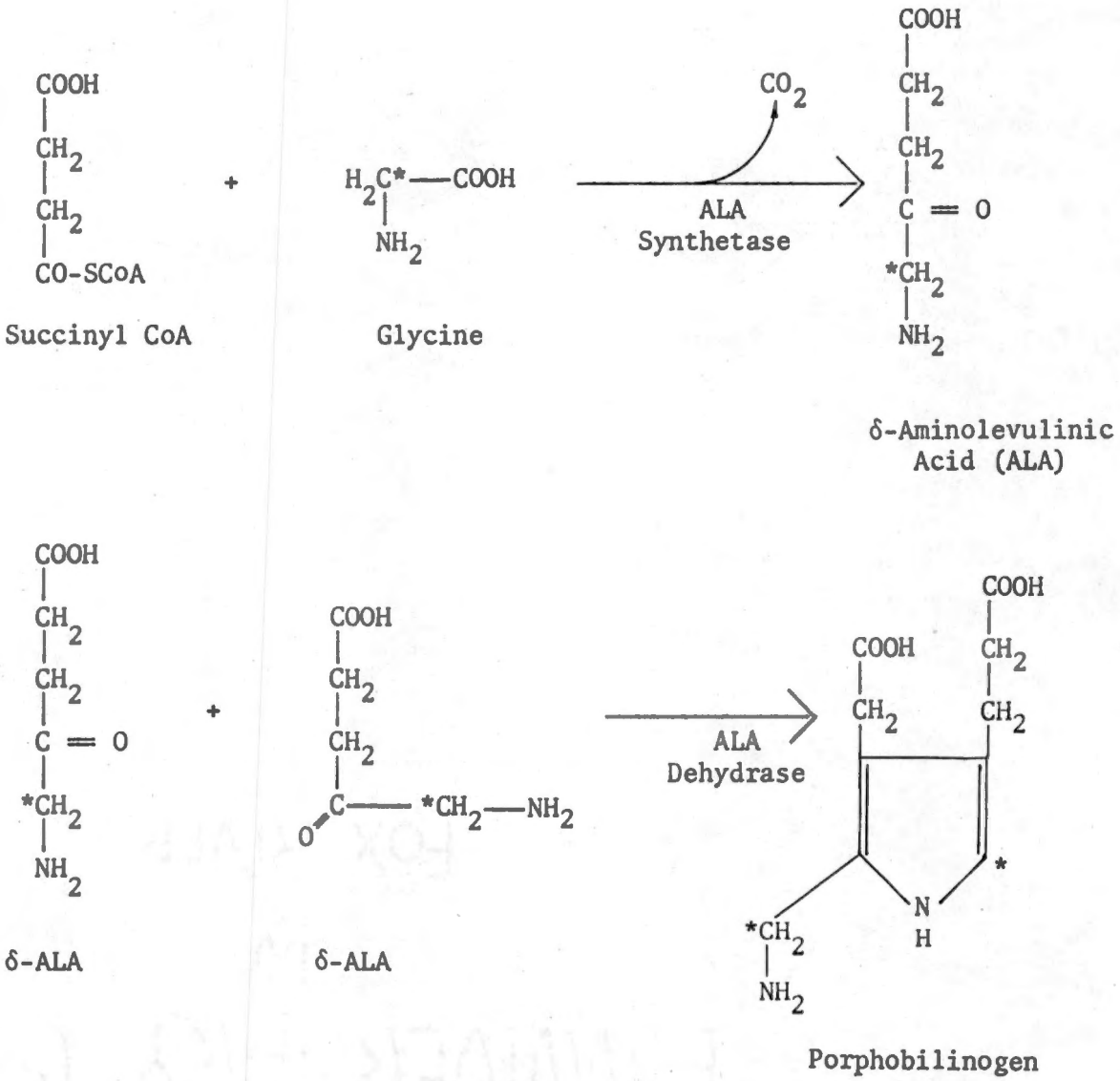

Fig. 2. Synthesis of δ-aminolevulinic acid and porphobilinogen. (*) Represents radioactive carbons (5.37).

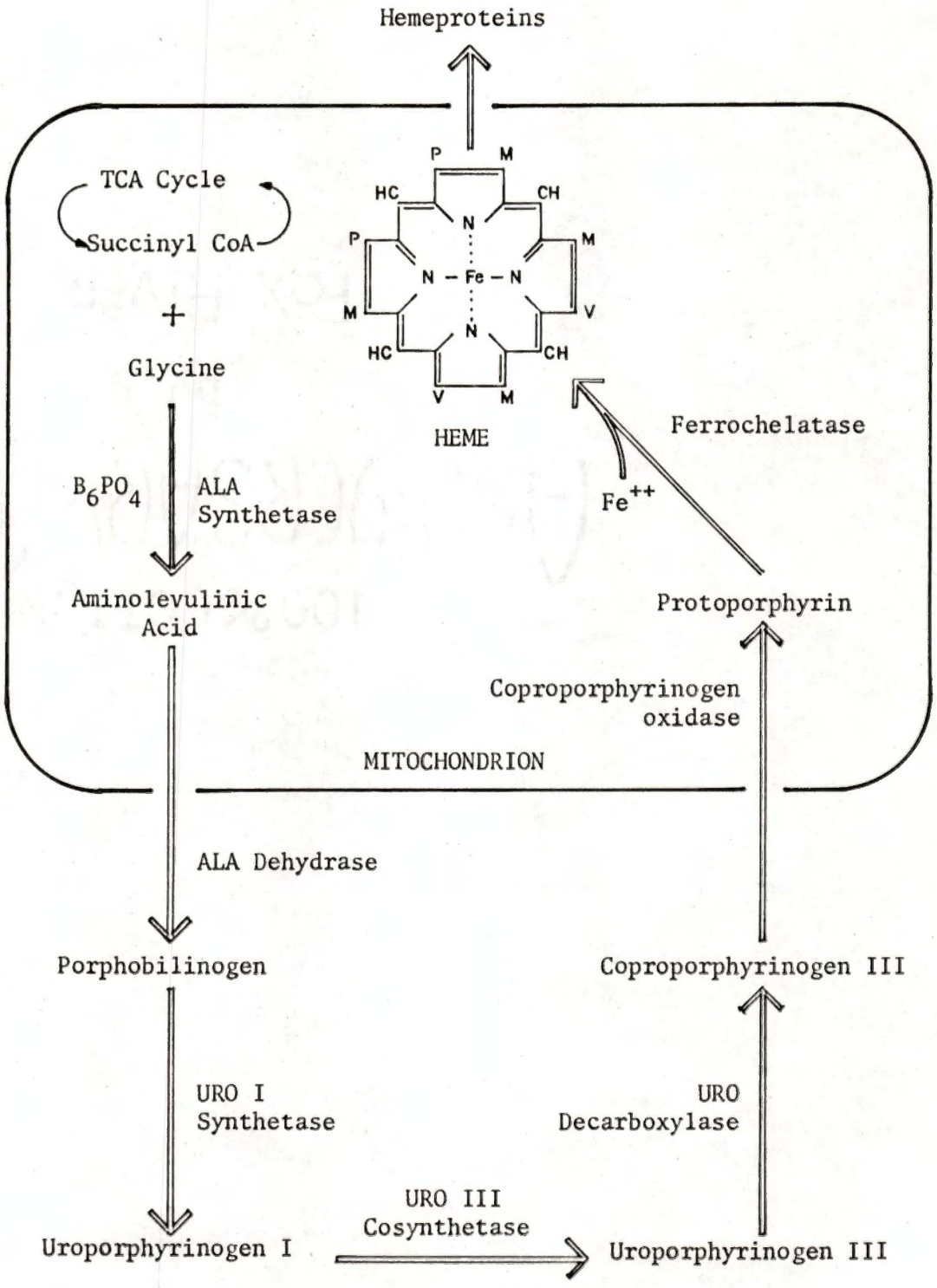

Fig. 3. Pathway of heme biosynthesis (38).

the formation of PBG (46). In intact eukaryotic cells, ALA synthetase is found only within mitochondria (47). This dependence on intact mitochondria may account for the fact that ALA formation does not take place in mature mammalian erythrocytes (48).

The ferrous protoporphyrin formed has side chains of methyl, vinyl, and propionic acid. While there is a possibility of 15 isomers of the heme molecule, only the protoporphyrin IX isomer seems to occur naturally (37).

Metabolism of Heme

When a heme protein is catabolized the apoprotein is degraded and returned to the amino acid pool (49). The remaining cyclic heme is converted to bilirubin by a complex enzymatic mechanism. Figure 4 illustrates the metabolism of heme which proceeds by the following reaction:

$$\text{Heme}(Fe^{+2}) + 6\text{NADPH} + 3O_2 \rightarrow \text{Biliverdin} + CO + Fe^{+3} + 3H_2O + 3NADP^{+} \quad (50).$$

The initial reaction is the cleavage of the ferroprotoporphyrin ring at its α-methene bridge by microsomal heme oxygenase requiring NADPH and O_2 as cofactors (50). This reaction occurs exclusively at the α-position, probably due to the specificity of the catalyzing enzyme (51). One mole each of biliverdin, CO, and iron will result. Biliverdin is then catabolized to bilirubin by the soluble enzyme bilirubin reductase, also requiring NADPH (52). It was originally thought cytochrome P-450 was involved as the terminal oxidase in the initial reaction of this system (52-54). However, while evidence strongly suggests that heme oxygenase

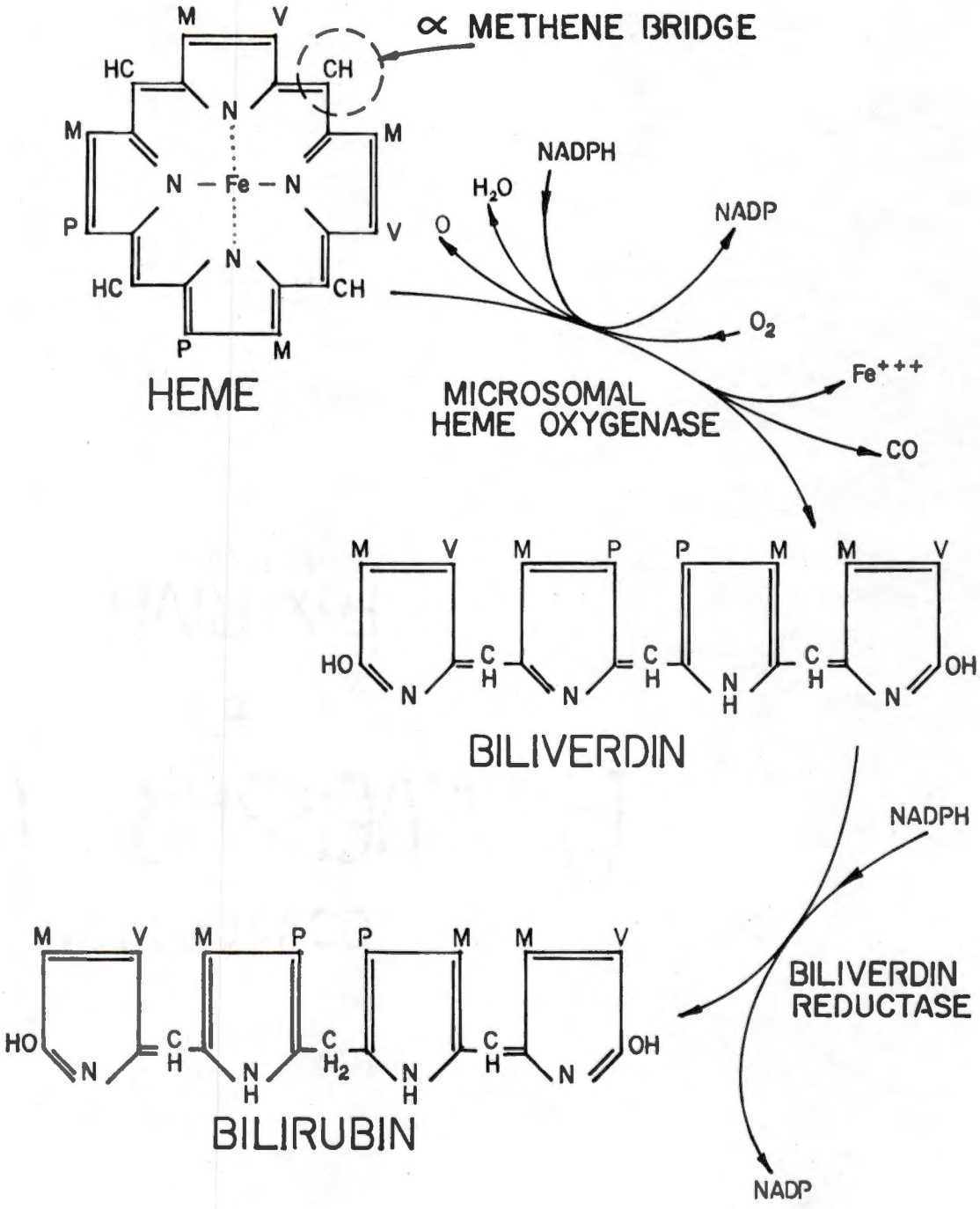

Fig. 4. Degradation of heme to bilirubin showing both enzymatic steps involved (53).

is a mixed function oxidase (55,56), it appears to be distinct from cytochrome P-450 (57-60).

While more than 80 percent of bilirubin[3] is from hemoglobin catabolism (61,62), a small portion results from non-hemoglobin sources (52,63). Probably the first definite evidence for bilirubin formation from non-erythroid sources was the observation that a patient with aplastic anemia and absent erythropoietic activity continued to produce bile pigment in a substantial quantity (64). Degradation of non-hemoglobin containing hemeproteins also results in bilirubin synthesis and the release of CO (62).

Isotopic Labelling of Heme Metabolism

Determination of the production rate of the end products of heme metabolism can be utilized as a measure of heme in vivo. Because of the molar production of bilirubin and CO during heme degradation, either of these excreted substances may serve as an accurate measure of heme catabolism (31,54,65,66). The accurate estimation of bilirubin production is of considerable importance to a variety of physiologic investigations because it reflects the turnover of biologically important hemeproteins. Since the bulk of bilirubin is derived from hemoglobin catabolism, the measurement of bilirubin production is also of potential clinical value as a rapid, if indirect, means of estimating erythrocyte turnover (67). Analysis of endogenous production of CO is also applicable to the study

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[3]Robinson, S. H., Tsong, M., Brown, B. W. & Schmid, R. (1965) Sites and kinetics of bile pigment formation in the rat. J. Lab. Clin. Med. 66, 1015-1016 (abstract No. 114).

of various aspects of heme catabolism in rats[4] and in man (63,65), under both normal and pathological states.

Isotopically labelled 2-^{14}C-glycine or 5-^{14}C-ALA has been used frequently as a precursor of heme to measure the resulting labelled bilirubin and/or CO. Glycine and ALA are on opposite sides of the rate-limiting step of heme biosynthesis (ALA synthetase), thus each will measure different aspects of heme metabolism. Administered glycine seems to conform better to the physiological steps of total heme synthesis because of its freer access to multiple sites of heme synthesis (68,69). Labelled ALA appears to be a better measure of non-erythroid sources of heme especially the hepatic hemes (70,71).

ALA As A Preferential Precursor of Cytochrome P-450

Labelled ALA enters hepatocytes and labels hepatic heme rather than hemoglobin heme (62,70). Comparisons of incorporation of glycine and ALA into the heme of hemoglobin reveal glycine is more rapidly incorporated into the erythroblasts than ALA by a factor of five to seven times (41,72-75). Sites of heme synthesis in the liver readily permit the entry of ALA. Having gained access to these extra-erythroid sites, ALA is unimpeded by normal mechanisms of rate limitation and is preferentially incorporated into non-hemoglobin hemeproteins (76). Schwartz et al.[5] in the dog and Robinson et al. (68) in the rat noted

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[4]Landaw, S. A. & Winchell, H. S. (1966) C-14 labelled carbon monoxide and heme catabolism. Clin. Res. 16, 319 (abstract).

[5]Schwartz, S., Ibrahim, G. & Watson, C. J. (1964) The contribution of nonhemoglobin hemes to the early labelling of bile bilirubin. J. Lab. Clin. Med. 64, 1003 (abstract No. 119).

more than 99 percent of bile bilirubin appearing after the injection of ALA was non-erythropoietic in origin. While not all ALA labelling is in the non-erythropoietic hemeproteins in humans (77) only an extremely small percentage of an oral dose of ALA in human subjects is incorporated into hemoglobin heme (78). And this hemoglobin incorporation of ALA in humans is still significantly smaller than incorporation of glycine (79).

As ALA goes primarily into hepatocytes, it can be used for labelling heme compounds in the liver (80). Although the administration of labelled ALA results in the labelling of non-hemoglobin hemeproteins in tissues other than the liver, there is no convincing evidence that turnover of these extra-hepatic hemeproteins gives rise to appreciable bilirubin formation under physiologic conditions in vivo (81). Cytochrome P-450 accounts for the major part of total hepatic heme synthesized. It has been reported that more than 50 percent of available hepatic heme is utilized in cytochrome P-450 synthesis (5). Thus, it should be possible to measure the degradation of cytochrome P-450 in terms of the rate of appearance of labelled degradation products of heme—bilirubin and CO—when 5-^{14}C-ALA is administered (82). Several investigations using labelled ALA have indicated that the radioactivity measured in hepatic CO-binding particles is cytochrome P-450 heme (83,84).

There are several advantages to the use of ALA as an isotopic precursor to cytochrome P-450. ALA is not incorporated to a significant extent into any other cellular components except heme (71). Incorporation into the heme of hemoglobin is low possibly due to: (1) the rapid destruction or transformation of ALA to compounds not effective as

porphyrin precursors, (2) failure to penetrate hemopoietic sites due to relative impermeability (5,54,76), and/or (3) rapid excretion (72). This rapid excretion of ALA from the body eliminates any problem due to a prolonged tissue half-life (74). Since the rate-limiting step in heme biosynthesis is the formation of ALA by ALA synthetase no significant body pool of the precursor ALA exists (85). The possible reutilization of heme has been considered and indications are that heme reutilization is probably not a major difficulty in studying the synthesis and turnover of hepatic cytochrome P-450 (86,87).

Effects of Administration of ALA

Researchers have fed or injected doses of ALA to determine the metabolic consequences. Injection or oral administration of labelled ALA to humans or animals results in excretion of a large portion of the unaltered compound in the urine (62) usually approximately 30 percent of the administered dose. Most of this excreted ALA appears within 24 hours of administration, primarily in the first six hours (72-74). Only 20 percent of the total initial dose is still in the body four days after administration. Only a small fecal fraction appears. It is believed that this fraction arises entirely from metabolized ALA rather than unabsorbed ALA (74).

It appears that ALA is poorly reabsorbed, if at all, by the renal tubules (73). Humans who have been exposed to lead have been studied to determine the metabolic pathways of ALA. Little renal reabsorption has been reported (88,89). Conflicting results in animals indicate that

some species such as the rabbit (89,90) have a better fractional reabsorption of ALA than the reabsorption reported in humans.

III. ENDOGENOUS CARBON MONOXIDE

Background

The existence of small amounts of a combustible gas in the blood was first demonstrated in dogs in the late nineteenth century and later demonstrated in human blood (91). It was speculated that this gas was CO. In the 1940's Sjostrand confirmed that this combustible gas in human blood was CO (92). He used a rebreathing technique to measure the CO concentration of alveolar air in humans in order to determine the effects of increased levels of CO in environmental air. After studying large numbers of patients with various diseases, Sjostrand noted a relationship between the CO concentration and intravital hemolysis. He also showed the CO was constantly produced in small quantities. Under certain pathological conditions of erythropoietic destruction this formation of CO was considerably increased. Further investigations determined that the CO resulted from the catabolism of hemoglobin with one molecule of CO produced for every molecule of heme degraded (91). This led to the eventual development of calculations which allow determination of total body hemoglobin by measuring CO production (93,94). Later studies have consistently supported the data of Sjostrand. Several reviews on endogenous CO production have appeared in the literature (54,63,65).

Ludwig, Blakemore, and Drabkin[6,7] demonstrated the in vitro production of CO by oxidation of hemoglobin and hemin. The α-methene bridge of heme was identified as the source of the CO on the basis of the specific activity of the recovered radioactive carbon. Experimental results in dogs[8] also confirmed that CO is a catabolic end-product of hemoglobin metabolism. Intravenous injections of damaged erythrocytes increased ^{14}CO production in dogs (95). Similar results were observed in rats (31) and in humans (96). The hepatic heme formed was converted predominantly to bilirubin and CO in equimolar amounts.

Source of the Endogenous CO

Carbon monoxide is produced in organs that catabolize heme, especially the liver and the spleen (97). Endogenously produced CO diffuses in the blood, reacts rapidly with hemoglobin, and mixes in the body stores of CO. Excretion occurs almost entirely via the lungs at rates that are influenced by blood alveolar ventilation, the CO diffusing capacity of the lungs, pulmonary capillary oxygen tension, and other variables (65,97). Only three carbon containing compounds have been identified in expired air: CO_2, CO, and ketone bodies (98). One

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[6]Ludwig, G. S., Blakemore, W. S. & Drabkin, D. L. (1957) Production of carbon monoxide by hemin oxidation. J. Clin. Inves. 36, 912 (abstract).

[7]Ludwig, G. S., Blakemore, W. S. & Drabkin, D. L. (1957) Production of carbon monoxide and bile pigment by haemin oxidation. Biochem. J. 66, 38 P (abstract).

[8]Coburn, R. F. Williams, W. J., Kahn, S. B., Ludwig, G. D. & Forster, R. E. (1964) Endogenous CO production in dogs. Fed. Proc. 23, 469 (abstract No. 2208).

estimation of the average normal rate of CO production in man, measured with a rebreathing system, is 0.42 ml/hour (99).

Some CO in the body is metabolized to CO_2 (100) but the amounts are considered insignificant (63,97,101). Luomanmaki (102) demonstrated that the rate of metabolism of CO in vivo is extremely slow. Labelled CO_2 in expired breath of humans administered ^{14}CO comprised only a small amount (about 3 percent) of the carbon production in the breath (103). As a mechanism of elimination from the body, metabolism is probably insignificant compared to excretion via the lungs. The metabolism of CO to CO_2 might become more important only at higher than normal blood carboxy-hemoglobin levels (104). Evidence was also presented by Coburn (105) that transdermal CO exchange in man and in dog is not a significant determinant of body CO stores under normal conditions.

Some investigators have found that CO may be produced from endogenous sources other than heme (106-109), from exogenous sources (110), and by enteric routes (110,111). Despite these potential sources, the ratio of CO production to bilirubin turnover observed in humans suggests that the extent of production of CO from non-heme sources by such processes is small or negligible (112). The greatest portion of excreted CO in man results from the breakdown of hemoglobin in senescent red blood cells (61). Isotopic data on the molar production rate of CO are consistent with this concept (86).

CO Production from Non-Hemoglobin Hemes

Under physiologic conditions it has been estimated that anywhere from 15 to 23 percent of the bile pigment and CO produced endogenously is

attributed to hemeproteins other than hemoglobin, of which the most important are the cytochromes P-450 and b_5 (52,63,99,113,114). Measures of CO production in rats and in guinea pigs indicated more CO was produced than could be accounted for by hemoglobin turnover alone (115). Another indication that CO is also produced by non-hemoglobin sources is the demonstration of White (116) that rat liver homogenates incubated in vitro with glycine or ALA will produce CO and bilirubin. Goldsmith and Landaw (117) and Ibrahim et al. (118) noted that non-erythropoietic components were a source of bilirubin and CO production.

Correlation of Bilirubin and CO as a Measure of Heme Catabolism

The availability of labelled precursors of heme such as glycine and ALA have enabled investigators to study the fate of heme compounds by measuring carbon monoxide or bilirubin production. Several studies confirm that the measurement of CO production in gas phase or blood phase analysis correlates highly with the measurement of bilirubin turnover (63,67,98,112). Gydell (119) reported an increase in serum bilirubin and carboxy-hemoglobin levels after injection of nicotinic acid or non-viable red cells. CO production closely paralleled bilirubin production rates in rats (31,66), and measures of CO in mice were used to determine the survival time of circulating red blood cells (66). Engel[9] also found both bilirubin and CO production provided equivalent information about

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[9]Engel, R., Berk, P. D., Rodkey, F. L., Howe, R. B. & Berlin, N. I. (1969) Estimation of heme turnover (HT) and erythrocyte survival (RBCLS) in man from clearance of bilirubin (BR) -^{14}C and from carbon monoxide production (COP). Clin. Res. 17, 325 (abstract).

heme turnover and the half-life of red blood cells. Landaw formulated equations for calculation of total heme activity as a function of time after measurement of CO and bilirubin production from injected labelled isotope (120).

Alterations in heme metabolism result in a parallel increase of serum bilirubin levels and CO production such as after the injection of damaged erythrocytes (96). Coburn et al. (121) and White et al. (79) each investigated endogenous production of CO in relationship to red blood cell and hemoglobin catabolism in patients with anemia or thalassemia. Each showed that CO was a measure of the changes in hemoglobin metabolism during abnormal states. The production of CO in human subjects was found to depend on the total heme mass at the time of measurement (122) and fluctuations in the rate of CO production in healthy humans has been correlated with changes in serum iron levels and serum unconjugated bilirubin levels (123).

In one study however, Berk et al. (124) found that a simultaneous measurement of bilirubin turnover and CO production in humans revealed CO production exceeded bilirubin turnover by 14 percent. Perhaps this excess is related to the evidence of CO production from non-heme sources as previously discussed. Or this may be due to a discrepancy in the measure of bilirubin since 13 to 21 percent of bilirubin produced (125) is excreted directly into the bile without passage into the plasma (65).

Determination of total hemeprotein catabolism appears to be better accomplished by measurement of CO production than of bilirubin turnover. The measurement of CO includes red blood cell destruction, erythropoiesis,

and hepatic heme turnover, while bilirubin turnover measurements tend to exclude hepatic heme turnover (65). The fact that CO is stable and not metabolized to other substances allows more time for its measurement. In contrast bilirubin is readily degraded to non-identifiable substances limiting the reliable measurement time (116).

IV. EARLY-LABELLED PEAK OF HEME CATABOLISM

Administration of a precursor to heme synthesis results in a characteristic rate of production of the metabolic end products—CO and bilirubin. With 2-^{14}C-glycine as the heme precursor, the excretion of these products occurs in two peaks (66,76,126). The early-labelled peak (ELP), which is 10 to 20 percent of the total bile pigment or CO produced (76,116), begins within minutes of administration of the precursor. It reaches a maximum in a few hours[10] and subsequently declines asymptomatically over the next few days (54,65). The later peak appears at a time corresponding to the average degradation time of red blood cells (66,76). In rats this late peak has been reported at 65 days[11] and 40 to 70 days[10]; in mice it was reported at 55 days[11] and between 42 and 82 days (68).

Source of the Early-Labelled Peak

The source of the early-labelled peak of heme catabolism has been the subject of several investigations. Initially it was thought the ELP

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[10]Landaw, S. A. & Winchell, H. S. (1966) Studies of heme metabolism using exhaled C-14 carbon monoxide. Clin. Res. 16, 141 (abstract).

[11]Landaw, S. A. & Winchell, H. S. (1967) In vivo studies of RBC lifespan distribution using endogenous ^{14}CO production. Clin. Res. 15, 106 (abstract).

was related to heme synthesis during erythroid cell development in bone marrow (79,127). The marrow is a source of the ELP in patients with ineffective erythropoiesis (116). Later evidence indicated the ELP probably resulted from non-erythropoietic hemeprotein sources (5,61), principally from the degradation of the hepatic cytochromes and unassigned heme which may serve as a precursor in formation of hepatic hemeproteins (63,128).

Israels et al. (70) identified two components of this early labelling in man. The first is independent of marrow erythropoietic heme synthesis. It appears within the first hour and is maximum at 1 to 24 hours after administration. The second component is dependent on heme synthesis and is maximum three to four days after administration of the heme precursor. Levitt et al. (129) and Yamamoto et al. (130) also reported the existence of two components of the ELP. The first appears rapidly and is independent of red blood cell synthesis and the second corresponds to the time red blood cells emerge from the marrow.

Experiments with rat liver homogenates have demonstrated that the liver is the source of the early bilirubin and CO production (126,131). This same conclusion, that the ELP is most closely associated with hepatic hemeprotein turnover, was reached in studies on intact animals (120). It appears from published results that the primary sources of the hepatic component of the ELP are cytochromes P-450 and b_5 (80).

The rapid appearance of the first portion of the ELP (often within 30 minutes) suggests that there may also be a pool of free or unassigned heme (70). This free heme may be converted rapidly to bilirubin after

administration of a heme precursor (80). This rapid initial peak has been identified in tissues which synthesize heme[12] indicating a presence of a heme pool in each of these tissues (132).

Principal evidence that the ELP is primarily hepatic heme comes from the research of Robinson and coworkers (61,76). Larger amounts of administered ALA are incorporated into early bilirubin production than into hemoglobin. Only minimal amounts of glycine appear in the early-labelled bilirubin. A patient with congenital non-hemolytic hyperbilirubinemia was administered 3-5-^{3}H-ALA (127). The rapid initial peak represented ALA incorporation into bilirubin. No labelling of hemoglobin heme by ALA was measured; therefore the conclusion was that non-hemoglobin heme in the liver and other tissues was responsible for the ELP.

Effect of ALA and Glycine on the Peaks of Production

The rate of synthesis of the ELP of bilirubin or CO can be estimated by measurement of the incorporation of isotopically labelled 5-^{14}C-ALA. Differences in the effect of administration of 2-^{14}C-glycine and 5-^{14}C-ALA have been reported. With ^{14}C-ALA the ELP bilirubin formed during the first few days after the isotopic pulse makes up 62 percent of all labelled bilirubin in animals. This ELP is only 13 to 15 percent when ^{14}C-glycine is given (54).

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[12]Schmid, R. (1972) The formation of bilirubin. Scan. J. Clin. Lab. Invest. 29, (Suppl. 126), 11 (abstract).

With ALA as a precursor in humans (130) and in animals (68,118), the early phase of the ELP was strikingly increased whereas the later phase was much reduced. As previously discussed ALA labelling of hemoglobin heme is minimal. The peak of production of bilirubin after ALA administration occurs within the first 24 hours (118). This early peak of ALA bilirubin or CO production is limited to the first few days after administration.[13] The late peak associated with glycine as a precursor is virtually absent when ALA is used as the labelled substance (76).

With ^{14}C-ALA the initial effect is the appearance of a single very tall peak, maximal at about 1 to 2 hours which then falls rapidly to baseline values (69,76). This peak appears to be from a heme fraction with a very rapid turnover (82), perhaps from the injected ALA (49) or from the heme pool previously described (129). After this initial peak the turnover of more slowly degraded tissue hemeproteins appears. This is believed to be almost entirely cytochrome P-450 from liver tissue. Renal hemeproteins are only a minor portion of the ELP (129).

Data obtained by Bissell and Hammaker (82) showed an appearance of labelled CO in 30 minutes. This peaked rapidly at 2.5 hours and then fell during the subsequent 7 to 9 hours. At about 12 hours it assumed a log-linear decline. This CO excretion beyond the initial peak closely approximated the rate of cytochrome P-450 degradation. The slope of the curve suggested a half-life of the heme source similar to that reported

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[13]Schwartz, S., Ibrahim, G. & Watson, C. J. (1964) The contribution of nonhemoglobin hemes to the early labelling of bile bilirubin. J. Lab. Clin. Med. 64, 1003 (Abstract No. 119).

for cytochrome P-450. Twenty-two hours after injection, 60 to 70 percent of the total hepatic ^{14}C-heme in the liver was in cytochrome P-450. The data implied that 18 to 30 hours after ALA administration hemeproteins other than cytochrome P-450 contributed little to the total ^{14}CO production by the liver. Thus ALA administration appears to be an excellent measurement of hepatic hemeprotein degradation after the initial peak of the ELP.

V. MEASUREMENT OF ENDOGENOUS CO

Several methods have been used in animals and in humans to determine the CO produced when heme is catabolized. Methods employed consist of: The determination of blood CO content (as carboxy-hemoglobin), with or without correction for exogenous CO sources; measurement of CO in expired air; and determination of the linear increase in CO content of blood or gas in a closed rebreathing system (65).

Early Measurements of CO

The initial demonstration by Sjostrand (91,92) of endogenous CO production was done by collection of a sample of expired air in a rubber bag and comparison of the air to the CO content of the surrounding atmosphere. Later, the carboxy-hemoglobin levels were correlated with the amount of excreted CO (91).

In vitro measurements of CO production from hemoglobin oxidation by Ludwig et al.[14,15] included the following methods for determination of

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[14]See footnote 6, page 19. [15]See footnote 7, page 19.

CO: an infrared gas analyzer; spectrophotometric recording of CO complexes of hemoglobin; chemical reactions with iodine pentoxide or palladium; and production of radioactive $BaCO_3$ following oxidation of CO.

Rebreathing Method

A rebreathing system was described by Coburn et al. (97,99) in 1963 and has been used frequently since that time. A closed air trap was designed so as to stop CO excretion as well as uptake of exogenous CO via the lung. Under these conditions of rebreathing, the CO production is equal to the rate of increase in body CO stores, computed from measures of the rate of increase in blood carboxy-hemoglobin and a factor that relates changes in blood carboxy-hemoglobin to changes in total body CO (133). This dilution factor is obtained by adding a known amount of CO to the body stores and measuring the increase in blood CO levels.

Rodkey and Berk (112) modified the rebreathing system to provide an accurately controlled oxygen input in the closed system. Another modification was by Logue et al. (134) who measured the rate of CO production from changes in the concentration of CO in expired gas rather than changes in blood CO levels. Gas phase analysis and blood measurements[16] yield equivalent results of CO production (112). White and coworkers (79) measured CO in the blood—by a spectrophotometric technique using palladium chloride—and CO in the breath—by a washout procedure which increased the partial pressure of oxygen in a rebreathing system.

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[16]See footnote 9, page 21.

Isotope Studies

Isotopes of glycine and/or ALA can be used to study the kinetics of endogenous CO (65). The resulting ^{14}CO excreted from administration of a tracer pulse dose has been detected. Landaw and Winchell injected rats with a pulse dose of labelled glycine to measure simultaneous CO and bilirubin production (31,66). A closed circulation system was used to detect the ^{14}CO expired. The air was drawn from the animal chamber through a system consisting of a water absorber, then an ionization chamber, followed by a CO_2 absorber of sodalime and Ascarite. Next the remaining ^{14}CO was converted to $^{14}CO_2$ by passing through a Hopcalite cannister at room temperature. Hopcalite is a trade name for a mixture of magnesium and copper oxides with other catalytic agents which works only if the air is dry. Finally the $^{14}CO_2$ was collected in a scintillation liquid as described by Jeffay and Alvarez (135) for radioactive counting.

More recently Bissell and Hammaker have determined the rate of degradation of hepatic cytochrome P-450 in rats by administering labelled 5-^{14}C-ALA. Their method was also an air train essentially the same as that used by Landaw and Winchell with the modification that the Hopcalite cannister is heated (82). In this method also, the final $^{14}CO_2$ collected was trapped in a scintillation liquid for radioactive counting.

Palladium Chloride

When CO is liberated from a substance it can be oxidized to CO_2 by several suitable reagents including palladium chloride (136). Palladium is one of the platinum metals in group VIII of the periodic system of

elements (137). It is the lightest of the platinum metals and complexes of palladium are used for numerous chemical catalytic reactions (138,139). The ability of palladium to oxidize CO under a variety of conditions has been reported (140,141) although frequently the oxidation reaction is studied at high temperatures (142).

Palladium chloride is a red hygroscopic solid (138) that has been demonstrated to effectively catalyze reactions of CO with both organic and inorganic compounds. In the absence of other reducing substances the following reaction is an old test for the presence of CO either colorimetrically or by titration:

$$PdCl_2 + CO + H_2O \rightarrow Pd + CO_2 + 2HCl \qquad (143,144).$$

Allen and Root (143) found a fairly high and consistent recovery of CO with oxidation by $PdCl_2$. The quantity of CO in the blood was determined by Fabre et al. (144) and Le Moan (145) by the spectrophotometric analysis of changes in $PdCl_2$. White et al. (79) measured CO in excreted air by drawing the air through a train to trap CO_2 (in Ascarite) and converting the CO to CO_2 by passing through a column of 0.1M $PdCl_2$ in 0.06N HCl. The final CO_2 was quantitated by titration.

Measurement of CO by Induction of Hemeproteins

Repeated administration of a drug often results in the induction of enzymes that metabolize that drug (18). One method to determine whether a system is measuring the mixed function oxidase system or any portion of it would be to administer doses of different enzyme inducing drugs such as phenobarbital and benzo-α-pyrene and observe the effects.

Phenobarbital enhances the synthesis of cytochrome P-450 and not b_5 (62). It has been shown to increase the non-erythropoietic component of early-labelled bilirubin and CO excretion in rats (31). Coburn gave phenobarbital to humans[17] and noted an increased CO excretion while reticulocytes and blood hemoglobin counts remained normal (146). The increase observed in the early-labelled components of heme degradation was strong evidence that a certain portion of the bile pigment originates from non-hemoglobin sources in the liver (32,146,147).

The two types of inducers of the mixed function oxidases differ in their course and intensity of action (18). Phenobarbital induction of cytochrome P-450 as observed requires at least three to five days to reach a maximal increase of three to ten fold. The polycyclic hydrocarbons will reach maximal induction of five to ten fold of cytochrome P-448 within one to two days of administration (11,18,25). Previous investigators have administered benzo-α-pyrene in concentrations of 20mg/kg dissolved in either corn oil or sesame oil (28,34,148,149). Phenobarbital has been administered in doses of 75 to 80 mg/kg in isotonic saline solution (28,34,148,149). Studies in this laboratory indicated the animals were not able to tolerate a dose of sodium pentobarbital higher than 25 mg/kg.

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[17]Coburn, R. F. (1967) Effect of phenobarbital on endogenous carbon monoxide production in normal man. J. Clin. Inves. 46, 1046 (abstract).

CHAPTER III

EXPERIMENTAL PROCEDURE

In order to measure the degradation of hepatic cytochrome P-450 in normal and obese mice, a closed circulation apparatus for the collection of labelled ^{14}CO excreted via the lungs was devised. This apparatus allowed the evaluation of injected 5-^{14}C-delta-aminolevulinic acid as an isotopic tool for detection of changes in the activity of hepatic cytochrome P-450. The amount of ^{14}CO excreted via the lungs was measured at specific time intervals after the injection of the labelled ALA to establish an optimal time for collection of the ^{14}CO. This in vivo measure of hepatic cytochrome P-450 in obese and normal mice was compared to results obtained by using the in vitro technique of Omura and Sato (8) for determination of cytochrome P-450 in isolated liver microsomes. The consequences of injected doses of ALA on specific organs of mice and of oral doses of ALA on specific organs of rats were also investigated. Finally, known inducers of the microsomal mixed function oxidase system, sodium pentobarbital and benzo-α-pyrene, were administered to normal mice and the effects on the excretion rate of ^{14}CO were measured.

I. EXPERIMENTAL ANIMALS

Mice used in this study were the progeny of mice heterozygous for the obese gene (ob/+) bred in the laboratory of The University of Tennessee. The original colony was bred from the C57BL/6J-ob mouse

strain obtained from the Jackson Laboratory.[18] The techniques for care and breeding of the mice were identical to previously described methods.[19] The mice were identified as obese (ob/ob) or non-obese (?/+) on the basis of gross appearance at 8 weeks of age. Obese and non-obese littermates of the same sex were housed together with not more than four mice to a cage. Plastic breeding cages with bedding of wood shavings[20] and wire rack lids were used to house the animals. Distilled water and the laboratory stock diet[21] were supplied ad libitum.

Male and female rats from the Long-Evans, Sprague-Dawley strain bred in this laboratory were used to determine the effects of an oral dose of ALA. The five male rats used weighed between 330 and 410 g and the five female rats weighed between 235 and 270 g. During the 24-hour investigation the rats were housed individually in metal metabolism cages[22] to allow urine and fecal collections. Distilled water and the laboratory stock diet were supplied ad libitum.

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[18]See footnote 2, page 2.

[19]See footnote 1, page 2.

[20]Ab-sorb-dri, Garfield, New Jersey.

[21]Ralston Purina Company, St. Louis, Missouri 63188.

[22]Hoeltage, Inc., Cincinnati, Ohio.

II. NON-INVASIVE MEASUREMENT OF CYTOCHROME P-450

Administration of ALA

For the measurement of excreted ^{14}CO, the mice received intraperitoneal injections of 5-^{14}C-ALA hydrochloride[23] in isotonic saline (20 mg/ml) with an approximate specific activity of 4.0×10^6 cpm/ml. Intraperitoneal injections of 4-^{14}C-ALA[24] in isotonic saline (10 mg/ml) were used to measure the levels of ALA incorporated into specific organs of the mice. The specific activity of the 4-^{14}C-ALA solution was 1.7×10^6 cpm/ml. The amount of ALA administered to each animal was on a weight basis of 167 mg/kg body weight. Each animal was weighed on a top-loading balance just prior to receiving the dose of ALA.

Collection of ^{14}CO

After administration of the ALA the mouse was placed in a small chamber in a closed circulation system for collection of the labelled CO excreted from the lungs. Air movement through the system was controlled by a forced air pump.[25] The experiment was conducted at room temperature, 20-25°.

In this system, CO_2 was first removed from the air entering the animal chamber. The air exiting the animal chamber was bubbled through

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[23]NEC-601 Aminolevulinic Acid Hydrochloride δ-5-^{14}C, New England Nuclear, Boston, Massachusetts.

[24]Amersham, Arlington Heights, Illinois.

[25]Whisper 400, Willinger Bros., Inc., Ft. Lee, New Jersey 07024.

NaOH to remove excreted CO_2. Next the gas passed through a solution of palladium chloride ($PdCl_2$, 0.8 g/liter) which oxidized the ^{14}CO to $^{14}CO_2$. This final quantity of $^{14}CO_2$ was collected in a scintillation liquid for radioactive counting. All radioactive quantities for these investigations were measured in a liquid scintillation counter,[26] and the data collected were recorded as counts per minute (cpm). For comparative purposes the data were expressed as counts per minute as a percentage of the specific activity of the dose given (cpm as % of dose).

Closed Circulation Apparatus

The apparatus used to collect the labelled CO is diagrammed in Figure 5. The rate of air flow through the forced air pump was regulated by a variable electrical transformer.[27] The initial container which removed CO_2 from the entering air held approximately 50 ml of 2N NaOH. The animal chamber was a dark glass container of approximately 180 ml volume, a size that was sufficient for the experimental mouse but did not allow excessive air space. After exiting the animal chamber the air bubbled through a series of three tubes made from 24/40 Pyrex ground glass joints. Each tube contained approximately 30 ml of 2N NaOH. In the next series of three tubes, each was made of a 14/20 Pyrex ground glass joint (Corning No. 6640) welded to a 30 ml Pyrex test tube so that each was one long tube 30 cm in length with a 1 1/4 cm diameter. Each tube held approximately 50 ml of $PdCl_2$ solution. The elongation of these

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[26]Beckman LS 100 C, Fullerton, California.

[27]Powerstat, Superior Electric Co., Bristol, Connecticut.

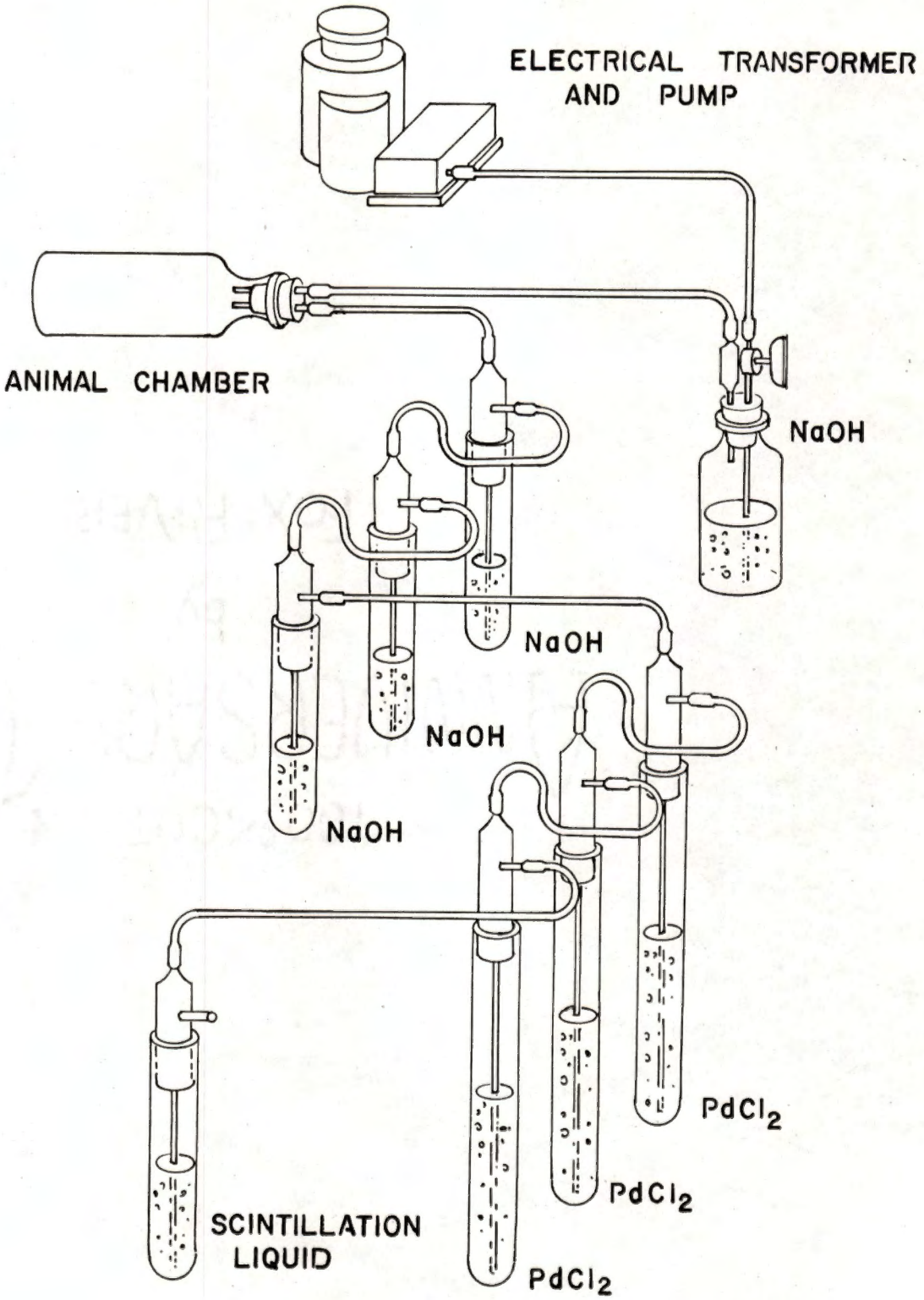

Fig. 5. Closed circulation apparatus for ^{14}CO collection.

tubes allowed increased transit time of the gas through the $PdCl_2$ and thus more efficient conversion of CO to CO_2.

The final tube was also made from a 24/40 Pyrex ground glass joint and contained the scintillation solution. This solution consisted of 2 ml Soluene-100[28] in 20 ml of PPO[29] in toluene[30] (12 g/liter). At the end of the collection time the scintillation liquid was transferred to a screw-top vial for radioactive counting.

Palladium Chloride Solution. The $PdCl_2$ was prepared according to the method of Fabre et al. (144). Eight-tenths of a gram of $PdCl_2$[31] was dissolved in 10 ml of 1N HCl brought to boiling. After cooling, this solution was diluted to 100 ml with distilled water. The $PdCl_2$ was stored in a dark glass container in an area free of direct light to prevent spontaneous reduction or ionization. Just prior to use, the solution was diluted 10-fold with distilled water.

Measurement of ^{14}CO Excretion from Normal and Obese Mice

On the basis of pilot studies and reports in the literature (82), the collection of excreted ^{14}CO from obese and normal mice was for a three-hour time period, 13 to 16 hours after the injection of ALA. Both

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[28]0.5N Quaternary Ammonium Tissue Solubilizer, Packard Instruments, Downers Grove, Illinois 60515.

[29]2,5-Diphenyloxazole, Fisher Scientific Co., Fair Lawn, New Jersey.

[30]J. T. Baker Chemical Co., Phillipsburg, New Jersey.

[31]Fisher Scientific Co., Fair Lawn, New Jersey.

male and female mice at two age levels were studied. One experimental group consisted of six pairs of obese and normal littermates at 3 1/2- to 4-months of age. The second experimental group consisted of six pairs of obese and normal littermates 7- to 8-months old. These age groups were chosen because previous studies in this laboratory[32] indicated that hepatic cytochrome P-450 activity peaked at 3- to 4-months of age in mice and then declined steadily. In addition, a significant difference between hepatic cytochrome P-450 levels of obese and normal littermates was detected. The levels of ^{14}CO excreted were expected to reflect these same differences between the experimental groups.

One mouse per day was studied and littermates were tested on consecutive days whenever possible. At 6:00 P.M. on the evening prior to the test, each animal was injected with 5-^{14}C-ALA on a weight basis as described. On the following day the animal was placed in the collection apparatus at 7:00 A.M. for three hours. The injection time was designated as 0 hour and the collection time period was during hours 13 to 16. At the end of the collection period the animal was removed from the apparatus and sacrificed for the in vitro determination of hepatic microsomal cytochrome P-450. The quantity of ^{14}CO collected in the scintillation liquid was measured in the radioactive counter and recorded as counts per minute.

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[32]See footnote 1, page 2.

III. IN VITRO MEASUREMENT OF CYTOCHROME P-450

Isolation of Liver Microsomes

This procedure is a modification of the procedure of Omura and Sato (8) as has been used in earlier studies in this laboratory. Immediately following the ^{14}CO collection, the mouse was stunned by a blow to the head and then quickly decapitated. The liver was excised and removed to a cold solution of isotonic (1.15 percent) KCl. The liver and subsequent fractions were kept cold throughout the procedure. A top-loading balance was used to determine the net weight of the liver which was recorded to two decimal places. The liver was homogenized in the isotonic KCl—4 volumes for obese mouse livers and 9 volumes for lean mouse livers—by using a motor driven homogenizer[33] with a Teflon pestle. The homogenate was centrifuged at 12,000 × g for 25 minutes in a refrigerated centrifuge[34] to sediment the nuclei and mitochondria. The supernatant fluid was centrifuged at 96,592 × g for 40 minutes in a preparative ultracentrifuge[35] to sediment microsomes. The supernatant fluid was decanted and discarded. Any lipid present on the sides of the centrifuge tube was removed by a cotton swab. The microsomal pellet was resuspended in one volume of isotonic KCl to give a microsomal suspension with a protein concentration of approximately 10 mg/ml.

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[33]Thomas Size B Homogenizer, Thomas Co., Philadelphia, Pennsylvania.

[34]Lourdes Betafuge Model A, Lourdes Instrument Corp., Brooklyn, New York.

[35]Beckman L5-50 Ultracentrifuge and Type 50 Rotor, Spinco Division, Beckman Instruments, Inc., Palo Alto, California 94304.

Determination of Cytochrome P-450

This procedure is also the described modification of that of Omura and Sato (8). To the microsomal suspension were added five volumes of a 0.1 M phosphate buffer to give a protein concentration of about 2 mg/ml. The phosphate buffer was prepared by adding 500 ml of 0.1 M NaH_2PO_4 to 790 ml of 0.1 M Na_2HPO_4 and adjusting to pH 7.0 with the appropriate solution. The microsomal suspension was very turbid necessitating the addition of sufficient buffer beyond the initial five volumes so that the initial absorbance reading of the sample was between 0.3 and 0.5. Sometimes three to four times the amount of initial buffer was required to achieve this reading. Duplicate 3 ml aliquots were accurately pipetted into spectrophotometric tubes. The cuvettes were wiped off and initial absorbance readings taken at 450 nm[36] for each sample. This first reading was used only as a check and not in the calculations of cytochrome P-450 content.

CO was then bubbled through the sample for 20 seconds and absorbance readings were recorded again. Solid sodium dithionite ($Na_2S_2O_4$) was quickly mixed into the sample in quantities of 15 mg $Na_2S_2O_4$ per 1 ml of sample in the cuvette. The final absorbance reading of the reduced cytochrome P-450-CO complex was recorded within one minute of addition of the dithionite. All absorbance readings were taken at room temperature, 20-25°.

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[36]Beckman Quartz Spectrophotometer, Model DU, Beckman Instruments, Fullerton, California.

The cytochrome P-450 content of the microsomal preparation was calculated by determining the average absorbance difference (absorbance of reduced P-450-CO complex minus absorbance of microsomal suspension saturated with CO). This was then converted to nanomoles of cytochrome P-450 by using the extinction coefficient of 91 $cm^{-1}mM^{-1}$ (8).

The protein content of the microsomal suspension which did not have added sodium dithionite was determined. The final results were expressed as nanomoles of cytochrome P-450 per mg of microsomal protein. The Folin Ciocalteu method for protein determination as described by Lowry et al. (150) was used.

Folin-Ciocalteu Method of Protein Determination

One ml samples were prepared so that they contained 30-200 µg of protein in 1 ml of 0.5N NaOH. This was done by combining 1 ml of the microsomal suspension with 1 ml of 1.0N NaOH and 2 ml of 0.5N NaOH. Duplicate 1 ml aliquots were pipetted into test tubes. The protein standard was prepared by weighing 0.02 g of bovine serum albumin and diluting to a volume of 100 ml with 0.5N NaOH. This gave a concentration of 200 µg of protein per ml. One ml of the standard was treated in the same manner as the samples.

Reagent A was prepared by adding 1 ml of 2.7 percent sodium potassium tartrate•4 H_2O and 1 ml of 1 percent $CuSO_4$•5 H_2O to 100 ml of 2 percent Na_2CO_3. Reagent B was commercial Folin-Ciocalteu reagent diluted with enough distilled water so that it contained 1N acid. Five ml of reagent A were mixed with each sample and allowed to stand at room temperature for at least 10 minutes. Five-tenths ml of reagent B was quickly added and

mixed within a few seconds. After at least 30 minutes, the absorbance at 750 nm was determined. The protein concentration of the sample (mg protein per ml sample) was calculated by comparing the absorbance of the sample to that of the protein standard as shown below:

$$\frac{\text{abs}_{\text{sample}}}{\text{abs}_{\text{standard}}} \times \text{Conc.}_{\text{std.}} \times 4 = \text{mg protein/ml}$$

IV. INVESTIGATIONS OF ALA ADMINISTRATION AND ^{14}CO EXCRETION

Timed Interval Collections of ^{14}CO

A group of five normal mice (four female and one male) weighing from 27 to 32 g was selected to determine the quantity of ^{14}CO excreted at various time intervals after injection of 5-^{14}C-ALA. One animal per day was studied. The ALA was administered by intraperitoneal injections on a weight basis as described. Most of the injections were given between 7:30 and 9:00 A.M. on the day of the investigation. The exceptions to this were two animals injected at 11:00 P.M. on the night preceding the ^{14}CO collection.

For the first part of the study, each animal was placed in the collection apparatus for eight hours immediately after the ALA injections. The animal did not have access to food or water during this time period. The ^{14}CO was collected in the scintillation liquid at hourly intervals so that a total of eight samples for each one hour period was obtained. The second part of the study was conducted a few days later. Each animal was again injected with ALA but not placed in the ^{14}CO collection

apparatus until eight hours after the injection. Then the mouse was put in the circulation system from the ninth through fifteenth hour after the injection time. Hourly samples of the scintillation liquid were collected so that an additional seven samples were obtained for each animal.

The radioactivity of each sample was determined in the liquid scintillation counter. The data were expressed as counts per minute as a percentage of the isotope given. The mean value at each time interval was plotted graphically to illustrate the pattern of ^{14}CO excretion.

Measure of the Effect of Injected ALA on Mouse Organs

Both obese and normal, male and female mice were used to determine the incorporation of 4-^{14}C-ALA into blood and several different organs at various time intervals after intraperitoneal injections. A total of eight groups was studied, each group consisting of three to five mice at least 6-months of age. The time periods tested were: 1.5, 2.5, 3.5, 4.5, 5.5, 8, 12, and 16 hours after the injection. The ALA was administered on a weight basis as described previously.

For the first five groups (1.5 to 5.5 hours), not more than two mice per day were tested. Each animal from these five groups was placed in the ^{14}CO collection apparatus immediately following the injection and removed at the specified time to be sacrificed. The animal was stunned by a blow to the head and then decapitated. Blood was collected immediately in a 15 ml test tube containing isotonic saline. The liver, kidneys, heart, spleen, and lungs were excised and stored separately in screw-top vial containers at -20°.

For the 8, 12, and 16 hour time intervals, groups of five normal mice were injected on the same day. The animals were sacrificed at the same time by stunning and decapitation. The liver, kidneys, heart, lungs, and spleen were removed and stored separately at -20° until ready for radioactivity determination.

The radioactivity incorporated into each organ was measured by homogenizing the organ in 2 to 4 ml of Soluene-100. This homogenate was poured into a scintillation vial and about 15 ml of PPO in toluene (12g/liter) was added. The radioactivity was counted in the liquid scintillation counter. The data were recorded as counts per minute.

For two groups of the animals (8 and 16 hours), the liver was separated into fractions and the radioactivity of each fraction was determined. This was done by first homogenizing each liver in 2 volumes of isotonic KCl. A portion of the homogenate, 0.5 ml, was added to 20 ml of the scintillation liquid and the radioactivity determined.

The remaining portion of the homogenate was centrifuged at 900 × g for 10 minutes to sediment the nuclei. The supernatant was decanted and centrifuged again at 12,000 × g for 25 minutes to isolate the mitochondria. This supernatant was again decanted and centrifuged in the preparative ultracentrifuge at 96,000 × g for 40 minutes to obtain the microsomal pellet. Each fraction, the nuclei, mitochondria, and microsomes, was solubilized in 2 to 4 ml of Soluene-100 and poured into a scintillation vial. About 15 ml of the scintillation liquid was added and the radioactivity of each fraction was measured. The radioactive count of 0.5 ml of the final supernatant remaining after isolation of the microsomes was also measured.

To measure the incorporation of the ALA into the blood, each sample of blood in isotonic saline was immediately centrifuged[37] at 965 × g for five minutes. The supernatant was decanted and the cells washed with isotonic saline and centrifuged a second time. After the second supernatant was poured off, enough isotonic saline was added to the red blood cells in a volumetric flask to equal 1 ml. Five-tenths ml was pipetted into a scintillation vial containing 20 ml of the scintillation liquid and the radioactivity of the sample was determined. The quantity of red blood cells was determined by use of a hemocytometer[38] with a Levy-Hauser counting chamber.

Administration of an Oral Dose of ALA to Rats

Five male and five female rats were administered a single oral dose of 4-^{14}C-ALA on a weight basis of 167 mg/kg. The purpose of this study was to determine the fate of ALA when ingested. The effects were measured on the basis of the amount of ALA incorporated into certain organs of the rats 24 hours after the administration of the ALA. A 24-hour collection of urine and feces was done for each animal. At the end of the 24 hours the rats were sacrificed by decapitation. The liver, heart, lungs, kidneys, and spleen were excised and stored at -20°. The urine sample was filtered and diluted to 25 ml. One gram of the dried feces was added to 20 ml of distilled water. This was shaken and allowed to

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[37]International Equipment Co., Model V, Size 2, Boston, Massachusetts.

[38]American Standard Haemocytometer, Arthur H. Thomas Co., Philadelphia, Pennsylvania.

settle. A 0.5 ml aliquot of each sample of the urine and feces solutions was added to the scintillation liquid for radioactive counting.

The radioactive incorporation of the isotope into the heart, lungs, kidneys, and spleen was determined by homogenizing each organ separately in 5 to 10 ml of Soluene-100. The homogenate was added to the scintillation liquid and the radioactivity counted. The livers were lyophilized[39] and then powdered with a mortar and pestle. The total freeze-dried liver tissue was weighed on a top-loading balance. A sample of 0.25 g of the powdered liver was accurately weighed and dissolved in 2 ml Soluene-100 and added to the scintillation liquid for radioactivity determinations. All data were expressed as counts per minute as a percentage of the dose of ALA administered. The mean radioactive incorporation values for each organ were compared between the two groups.

Induction of the Mixed Function Oxidase System

Known inducers of the mixed function oxidase system were administered to normal mice prior to ALA administration and ^{14}CO collection. This was done to establish further that the ^{14}CO collected from the closed circulation apparatus was a measurement of the mixed function oxidase system, especially cytochrome P-450. Sodium pentobarbital[40] in isotonic saline (2mg/ml), which induces cytochrome P-450, and benzo-α-pyrene[41]

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[39]VirTis Freeze Dryer, VirTis, Gardiner, New York.

[40]Abbott, No. 3114, North Chicago, Illinois 60064.

[41]Sigma Chemical Co., P. O. Box 14508, St. Louis, Missouri.

in corn oil (12mg/5ml), which induces cytochrome P-448, were used for the investigation.

Two groups of five normal mice each were selected for the pentobarbital induction effect. One group was 6 to 8 months of age weighing 25 to 30 g, and the other group was 3 1/2-months old weighing 23 to 27 g. Each mouse was administered the sodium pentobarbital by a single daily intraperitoneal injection on a weight basis of 25 mg/kg. The injections were given in the morning for at least three days prior to the ^{14}CO collection for the 3 1/2-month-old group. The other group only received the injections for two days prior to the ^{14}CO collection.

On the evening prior to the test of CO excretion, each mouse received labelled ALA (167 mg/kg) at 6:00 P.M. The following day the ^{14}CO excreted was collected for a three-hour time period as described earlier. Data from normal mice used in the comparison of obese and normal ^{14}CO excretion were used as the control data for comparison of the effect of the pentobarbital.

The effects of the injection of benzo-α-pyrene on ^{14}CO excretion after ALA administration were tested on six normal male mice. The mice tested were 2 1/2- to 3-months-old. A control group of four littermates of these mice was injected with corn oil alone to test the influence of corn oil on the ^{14}CO excretion rate.

Each mouse weighed approximately 23 g and received single daily injections of the inducer (20 mg/kg) or the sterile corn oil. The doses were administered three to four days prior to the injection of ALA. A three-hour ^{14}CO-excretion sample was collected for each mouse 13 to 16 hours after the isotope administration as described previously.

V. STATISTICS

The data were analyzed by using the Student's t-test either for paired comparisons or for unpaired observations where appropriate as described by Steele and Torrie (151). All computations were done using the Olivetti-Underwood Programma 101.

CHAPTER IV

RESULTS

I. TIMED INTERVAL MEASUREMENT OF ^{14}CO EXCRETION

One aspect of this study was designed to investigate the pattern of ^{14}CO excreted from the lungs of mice after injection of 5-^{14}C-ALA. This was measured on a group of normal mice for a 15-hour time period. Figure 6 depicts the pattern of ^{14}CO excretion as determined by the mean ^{14}CO collected at each hour. The results indicated a sharp increase in the excretion of ^{14}CO between the first and second hours. After the second hour, the ^{14}CO excretion gradually declined to a fairly constant plateau by the fifth hour. Approximately the same amount of ^{14}CO excretion was measured from the sixth through the fifteenth hour after ALA administration.

II. COMPARISON OF ^{14}CO EXCRETION IN NORMAL AND OBESE MICE

Excretion of ^{14}CO as a measurement of differences in the level of the mixed function oxidase system activity in normal and obese mice was investigated. Two age groups of mice consisting of normal and obese littermate pairs were injected with 5-^{14}C-ALA and the ^{14}CO excreted via the lungs was collected. The amounts of radioactivity detected in a three-hour sample from 3- and 4-month-old mice are reported in Table 1. The cytochrome P-450 values determined for each mouse by isolation of liver microsomes are also listed in this table. The data revealed that

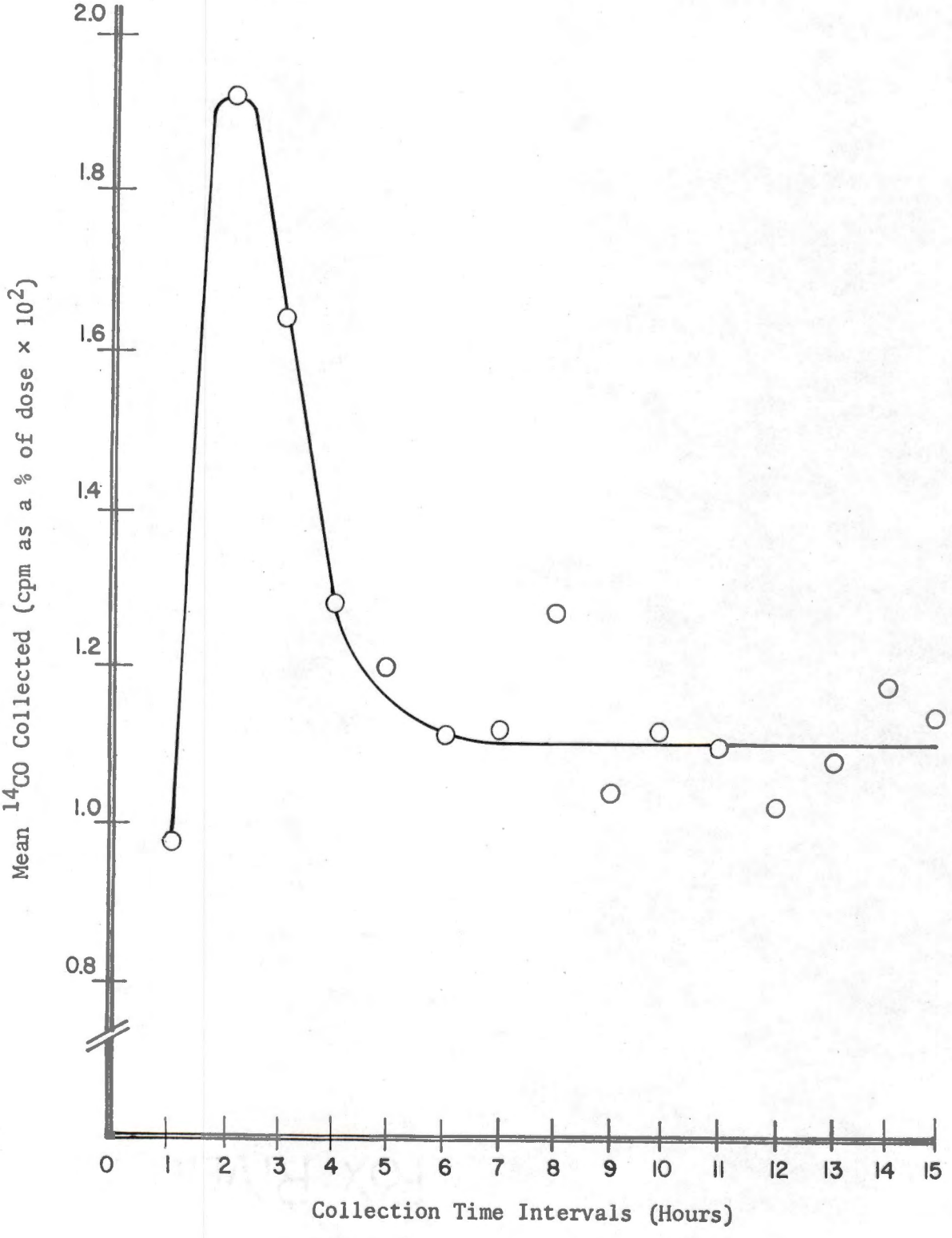

Fig. 6. The mean ^{14}CO excreted at specific time intervals after injection of ALA into 5 normal mice.

TABLE 1

^{14}CO EXCRETION VIA THE LUNGS AND HEPATIC CYTOCHROME P-450 LEVELS OF 3- AND 4-MONTH-OLD NORMAL AND OBESE MICE

	^{14}CO Excretion[a] (cpm as % dose × 10^3)		Cytochrome P-450 (nanomoles/mg protein)	
	Normal	Obese	Normal	Obese
Females	3.92 (991)[b]	2.36 (990)	3.66 (991)	3.60 (990)
	4.12 (994)	1.98 (992)	4.08 (994)	3.88 (992)
	3.39 (995)	4.44 (993)	4.00 (995)	3.50 (993)
Males	3.35 (988)	2.12 (989)	3.60 (988)	4.00 (989)
	3.63 (987)		4.08 (987)	
	2.24 (1006)	1.87 (1005)	ND (1006)[c]	ND (1005)[c]
Mean ± S.E.	3.44 ± 0.27	2.48 ± 0.40	3.88 ± 0.10	3.74 ± 0.12
	$P < 0.1$		N. S.[d]	

[a] ^{14}CO was collected for a three-hour time period.

[b] Numbers in parentheses represent serial numbers assigned to mice.

[c] Hepatic cytochrome P-450 level not determined.

[d] Mean values are not significantly different.

there was a significant difference between the mean values of ^{14}CO excreted by the normal and the obese mice. The obese mice excreted 26 percent less ^{14}CO than the normal mice at this age group. There was no difference noted for the cytochrome P-450 levels between the normal and the obese mice. Analysis of the data included an attempt to correlate the ^{14}CO and the cytochrome P-450 values. No correlation between the two values was measured.

The ^{14}CO and cytochrome P-450 values determined for 7- and 8-month-old normal and obese mice are given in Table 2. The mean ^{14}CO excreted from obese mice is 47 percent less than that excreted from the normal mice. This difference is statistically significant. There was a trend detected between the cytochrome P-450 levels of the normal and obese mice in this group. While the statistical difference was not significant, the mean cytochrome P-450 was slightly greater for the normal mice than the obese mice. No correlation between the ^{14}CO and cytochrome P-450 values was observed when these data were analyzed.

Comparison of the data for the ^{14}CO values for normal mice at the two different age levels revealed no significant difference between the means. There was also no significant difference between the mean ^{14}CO values of the obese mice at the two age levels.

III. ^{14}CO EXCRETION AFTER INDUCTION OF THE MIXED FUNCTION OXIDASE SYSTEM

Two known inducers of the mixed function oxidase system were administered to groups of normal mice. The inducers chosen were sodium

TABLE 2

^{14}CO EXCRETION VIA THE LUNGS AND HEPATIC CYTOCHROME P-450 LEVELS OF 7- AND 8-MONTH-OLD NORMAL AND OBESE MICE

	^{14}CO Excretion[a] (cpm as % of dose × 10^3)		Cytochrome P-450 (nanomoles/mg protein)	
	Normal	Obese	Normal	Obese
Females	5.35 (969)[b]	1.82 (968)	3.33 (969)	3.14 (968)
	3.23 (970)	1.43 (967)	4.02 (970)	2.79 (971)
	5.25 (1027)	4.62 (1025)	2.94 (1027)	2.67 (1025)
Males	3.71 (959)	1.63 (962)	2.66 (959)	2.32 (962)
	3.22 (957)	1.28 (960)	4.10 (957)	4.40 (960)
	2.61 (958)	1.72 (961)	3.66 (958)	3.00 (961)
Mean ± S.E.	3.89 ± 0.46	2.08 ± 0.51	3.45 ± 0.24	3.05 ± 0.29
	$P < 0.01$		$P < 0.2$	

[a] ^{14}CO was collected for a three-hour time period.

[b] Numbers in parentheses represent serial numbers assigned to mice.

pentobarbital and benzo-α-pyrene. The effect on ^{14}CO excretion by each inducer was then measured.

The ^{14}CO detected in normal mice after sodium pentobarbital treatment is reported in Tables 3 and 4. Four of the 7-month-old mice (Table 3) were administered the inducer for only two days prior to the ^{14}CO collection. When compared to a control group which received no sodium pentobarbital, no difference in ^{14}CO excretion was detected in the experimental group. When the number of sodium pentobarbital injections was increased, as in the 3-month-old group (Table 4), there was a significant increase in the ^{14}CO excreted by the experimental group as compared to the control group. The mean ^{14}CO excretion value of the induced group was almost double that of the control group. This was also true of the difference between the two experimental groups. The mean value of the ^{14}CO excreted by the 7-month-old group was significantly lower than that of the 3-month-old group.

Table 5 presents the data from the study of the effects of benzo-α-pyrene on ^{14}CO excretion. It is difficult to draw positive conclusions from these data because of lack of an adequate control sample. The benzo-α-pyrene was administered in corn oil, so it was necessary to test the effect of corn oil alone. Only two of the four control mice survived the corn oil administration, while all six mice tested with the benzo-α-pyrene survived the procedure. The mean ^{14}CO values obtained were not significantly different between the two groups. This indicated that while the ^{14}CO value for the benzo-α-pyrene group was higher than detected previously in normal mice, the difference may

TABLE 3

EXCRETION OF ^{14}CO FROM 7-MONTH-OLD NORMAL MICE INJECTED WITH SODIUM PENTOBARBITAL VS. A CONTROL GROUP OF MICE

Pentobarbital Mice		Control Mice[a]
Number of Injections	^{14}CO (cpm as % of dose × 10^3)	(cpm as % of dose × 10^3)
2	5.45 (1)[b]	5.35 (969)
2	4.09 (11)	3.23 (970)
2	3.03 (10)	5.25 (1027)
2	3.43 (26)	3.71 (959)
4	3.83 (966)	3.22 (957)
		2.61 (958)
Mean ± S.E.	3.97 ± 0.41	3.89 ± 0.46
N.S.[c]		

[a]Control data obtained from the group of normal animals used to test the closed circulation apparatus.

[b]Numbers in parentheses represent serial numbers assigned to mice.

[c]Difference between means is not significant.

TABLE 4

EXCRETION OF ^{14}CO FROM 3-MONTH-OLD NORMAL MICE INJECTED WITH SODIUM PENTOBARBITAL VS. A CONTROL GROUP OF MICE

Pentobarbital Mice		Control Mice[a]	
Number of Injections	^{14}CO (cpm as % of dose × 10^3)		^{14}CO (cpm as % of dose × 10^3)
3	5.82	(998)[b]	3.92 (991)
3	10.93	(1001)	4.12 (994)
4	6.36	(8)	3.39 (995)
5	5.06	(9)	3.35 (988)
6	4.77	(12)	3.63 (987)
			2.24 (1006)
Mean ± S.E.	6.59 ± 1.12		3.44 ± 0.27

$P < 0.02$

[a]Control data obtained from the group of normal animals used to test the closed circulation apparatus.

[b]Numbers in parentheses represent serial numbers assigned to mice.

TABLE 5

^{14}CO EXCRETION FROM BENZO-α-PYRENE-TREATED AND CORN OIL-TREATED MICE

Benzo-α-pyrene[a]	Corn Oil[b]
(cpm as % of dose × 10^3)	
4.52(1)[c]	5.24(4)
5.56(2)	
3.97(3)	
3.82(5)	4.74(6)
4.82(7)	
4.82(8)	———
Mean ± S.E. 4.59 ± 0.26	4.99 ± 0.25
N.S.[d]	

[a]Benzo-α-pyrene administered i.p. in sterile corn oil.

[b]Insufficient control data obtained because control animals did not survive the procedure.

[c]Numbers in parentheses represent serial numbers assigned to animals.

[d]Difference between means is not significant.

have been due to the effect of corn oil rather than the benzo-α-pyrene. It should be noted that the mean ^{14}CO value for the benzo-α-pyrene group was significantly less ($P < 0.1$) than the mean ^{14}CO value for the pentobarbital group.

IV. INCORPORATION OF ALA BY SPECIFIC ORGANS AND BLOOD OF MICE

An investigation of the amount of ALA incorporated into certain organs and the blood of mice was conducted. Measures of the amount of radioactivity in the kidneys, lungs, spleen, heart, and liver were determined at specific time intervals after the injection of 4-^{14}C-ALA.

Table 6 shows the mean level of radioactivity present in the blood of five groups of mice sacrificed at 1.5 to 5.5 hours after injection. Approximately the same amount of radioactivity was detected in the blood at 1.5, 2.5, and 3.5 hours after injection. At 4.5 and 5.5 hours after the injection, there was a slight increase in the blood radioactivity. This incorporation pattern of ALA in the blood did not follow the ^{14}CO excretion pattern from the lungs, which was a sharp increase at two hours after injection followed by a gradual decline.

The mean values of radioactivity in the heart at eight different time periods after injection of ALA, as listed in Table 7, revealed a greater incorporation of ALA by the heart tissue than by the blood. The radioactivity in the heart was greater at 3.5, 8, and 16 hours after the injection than at other time periods.

The mean values for incorporation of ALA in the spleen were similar to the values measured in the heart. While the mean radioactivity

TABLE 6

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO THE BLOOD OF MICE[a]

Hour After Injection	Number of Mice	Radioactivity (cpm as % of dose × 10^3)
1.5	4	0.19 ± 0.08[b]
2.5	4	0.18 ± 0.04
3.5	4	0.18 ± 0.06
4.5	5	0.57 ± 0.21
5.5	3	0.54 ± 0.21

[a]Each group consisted of a random selection of both male and female, normal and obese mice.

[b]Mean ± S.E.

TABLE 7

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO THE HEART OF MICE[a]

Hour After Injection	Number of Mice	Radioactivity (cpm as % of dose × 10^3)
1.5	4	1.2 ± 0.3[b]
2.5	4	3.7 ± 1.5
3.5	5	6.2 ± 1.8
4.5	5	3.9 ± 2.1
5.5	4	3.6 ± 0.5
8	5	13.5 ± 0.9
12	5	2.4 ± 0.3
16	5	7.1 ± 1.6

[a]Each group consisted of a random selection of both male and female, normal and obese mice.

[b]Mean ± S.E.

reported in Table 8 indicated a peak of radioactivity for the second group sacrificed 2.5 hours after the injection, increases in radioactivity were also recorded at 8 and 16 hours after the injection.

Table 9 lists the mean values for ALA incorporation into the lungs at eight time intervals after the injection of the isotope. Once again there appeared to be a peak at 2.5 hours after injection with a decline afterwards. The trend seemed to compare to the pattern of ^{14}CO excretion via the lungs—a sharp initial peak followed by a fairly steady level of radioactivity. The mean incorporation values obtained for the lungs were comparable to the values obtained for the heart and the spleen.

The mean incorporation values of ALA in the kidneys were much higher than the amount of radioactivity incorporated into the heart, spleen, or lungs. These values for the kidneys are reported in Table 10. A sharp increase in radioactivity at 2.5 hours after injection was noted. This was followed by a steady decline at each of the later time periods measured.

There was a high degree of variability in the mean values of radioactivity measured in the livers, reported in Table 11. This was true within each group, as evidenced by the large standard errors of the means, and between the groups at the different time periods. For some of the time periods (3.5, 4.5, 8, and 16 hours after injection), the ALA incorporated in the liver was higher than the ALA activity measured in the other organs. At the other time periods the values were similar for the liver and the other organs. No defined pattern of ALA incorporation by the liver was identified.

TABLE 8

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO THE SPLEEN OF MICE[a]

Hour After Injection	Number of Mice	Radioactivity (cpm as % of dose × 10^3)
1.5	4	2.2 ± 0.7[b]
2.5	4	10.4 ± 2.9
3.5	5	4.8 ± 1.2
4.5	5	3.0 ± 0.4
5.5	4	3.1 ± 0.8
8	5	16.2 ± 2.6
12	5	5.9 ± 2.3
16	4	16.1 ± 2.1

[a]Each group consisted of a random selection of both male and female, normal and obese mice.

[b]Mean ± S.E.

TABLE 9

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO THE LUNGS OF MICE[a]

Hour After Injection	Number of Mice	Radioactivity (cpm as % of dose × 10^3)
1.5	3	2.0 ± 0.7[b]
2.5	3	6.5 ± 3.8
3.5	5	3.6 ± 1.6
4.5	5	4.6 ± 2.0
5.5	4	4.2 ± 1.1
8	5	4.9 ± 0.4
12	4	5.3 ± 2.4
16	5	3.7 ± 0.6

[a]Each group consisted of a random selection of both male and female, normal and obese mice.

[b]Mean ± S.E.

TABLE 10

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO THE KIDNEYS OF MICE[a]

Hour After Injection	Number of Mice	Radioactivity (cpm as % of dose × 10^3)
1.5	4	15.6 ± 3.3[b]
2.5	4	57.7 ± 8.8
3.5	5	25.2 ± 1.6
4.5	4	21.6 ± 5.0
5.5	4	11.8 ± 3.1
8	5	11.3 ± 0.5
12	5	5.5 ± 0.9
16	5	9.8 ± 1.1

[a]Each group consisted of a random selection of both male and female, normal and obese mice.

[b]Mean ± S.E.

TABLE 11

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO THE LIVER OF MICE[a]

Hour After Injection	Number of Mice	Radioactivity (cpm as % of dose × 10^3)
1.5	4	13.0 ± 5.5[b]
2.5	4	9.9 ± 8.4
3.5	5	28.1 ± 22.5
4.5	4	30.6 ± 21.9
5.5	3	4.2 ± 2.1
8	5	31.2 ± 3.1
16	5	44.3 ± 11.4

[a]Each group consisted of a random selection of both male and female, normal and obese mice.

[b]Mean ± S.E.

Fractions of the liver were measured for radioactivity at two time periods, 8 and 16 hours. Mean values for the radioactivity detected in the nuclei, mitochondria, microsomes, and the final supernatant are listed in Table 12. The data for the muclei, mitochondria, and microsomes were similar within each time period. However, a greater amount of the radioactivity at 16 hours appeared in the nuclei than in the mitochondria. At 8 hours the opposite was true. The radioactivity detected in the mitochondria was greater than in the nuclei. In the microsomes, the radioactive level was about the same at both time periods. Only a small amount of the radioactivity was found in the final supernatant after isolation of the microsomal pellet.

V. INCORPORATION OF AN ORAL DOSE OF ALA INTO RAT ORGANS

The consequences of oral administration of ALA were measured on rats for a 24-hour period. During this time period, urine and fecal collections were obtained. The mean values for these collections for males and females are given in Table 13. The amount of radioactivity excreted in the urine was a large portion of the total dose administered.

While less ALA was excreted in the urine of the male rats than the female rats (52.4 percent vs. 43.6 percent), there was no significant difference. Only a small percentage of the ALA administered was detected in the feces for either group (3.6 percent and 2.5 percent) and the means for each were not significantly different.

Data for measures of the incorporation of ALA into various organs after 24 hours are presented in Table 14. The radioactivity detected in

TABLE 12

MEAN INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO FRACTIONS OF THE LIVERS OF TWO GROUPS OF LEAN MICE

Liver Fraction	Radioactivity[a] (cpm as % of dose × 10^3)	
	8 Hours[b]	16 Hours[b]
Nuclei	8.51 ± 0.46[c] (4)[d]	13.09 ± 3.13 (5)
Mitochondria	10.70 ± 1.04 (4)	8.03 ± 2.47 (4)
Microsomes	9.44 ± 1.14 (4)	8.95 ± 1.35 (5)
Final Supernatant	1.70 ± 0.30 (4)	1.06 ± 0.17 (5)

[a]Radioactive values represent the total of each liver fraction.

[b]Time animal was sacrificed after ALA injection.

[c]Mean ± S.E.

[d]Numbers in parentheses represent number of samples studied.

TABLE 13

MEAN RADIOACTIVITY DETECTED IN A 24 HOUR COLLECTION OF URINE AND FECES OF RATS ADMINISTERED AN ORAL DOSE OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID

Sample	Radioactivity (cpm as % of dose)		
	Females (N = 5)	Males (N = 5)	
Urine	52.4 ± 8.2[a]	43.6 ± 6.6	N.S.[b]
Feces	3.6 ± 0.7	2.5 ± 0.4	N.S.

[a]Mean ± S.E.

[b]Difference between two means is not significant.

TABLE 14

MEAN RADIOACTIVE INCORPORATION OF 4-^{14}C-DELTA-AMINOLEVULINIC ACID INTO FIVE ORGANS OF RATS 24 HOURS AFTER ORAL ADMINISTRATION

Organ	Radioactivity (cpm as % dose × 10^3)		
	Females (N = 5)	Males (N = 5)	
Liver	900 ± 300[a]	2,510 ± 300[a]	$P < 0.02$
Heart	22.1 ± 4.5	10.1 ± 2.5	$P < 0.1$
Lung	19.8 ± 5.9	27.4 ± 4.5	N.S.[b]
Kidney	18.3 ± 6.9	22.1 ± 4.8	N.S.[b]
Spleen	14.1 ± 3.0	7.2 ± 1.0	$P < 0.1$

[a]Mean ± S.E.

[b]Difference between two means is not significant.

the liver of the male rats was at least 100 times greater than the radioactivity detected in the other organs measured. The mean liver values for the female group was about 45 times greater than the mean value for the next highest organ, the heart. Differences were detected in the amount of radioactive incorporation of the males and females. In the liver of the males, a significantly higher mean value of radioactivity was measured, almost three times greater than in the females. There was also significantly less radioactivity determined in the heart and the spleen of the male rats than in the female rats. The data indicate 50 percent less radioactivity in each of these groups. No significant difference was noted in measures of the radioactive incorporation into the lungs or the kidneys. However for male rats the second and third highest values obtained were for the lungs (27.4×10^{-3} percent) and kidneys (22.1×10^{-3} percent), respectively. The radioactivity in the heart (10.1×10^{-3} percent) was the second lowest value. In females the order in quantity detected differed. The heart was the second highest mean value (22.1×10^{-3} percent) and the lungs (19.8×10^{-3} percent) and kidneys (18.3×10^{-3} percent) were next, although all three values were similar. For both groups the spleen had the lowest amount of radioactive incorporation.

CHAPTER V

DISCUSSION

Metabolic efficiency as a contributing cause to development and/or maintenance of obesity has drawn support from numerous researchers. The hypothesis that cytochrome P-450 and the mixed function oxidase system may be related to alterations in energy metabolism has been evaluated in this laboratory.

Dorsey[42] documented that obese mice have significantly lower levels of hepatic cytochrome P-450 than lean controls. The measurement of cytochrome P-450 was done by isolating microsomes of liver homogenate. Such a procedure is not effective to apply to other species because of distress to the subject. Ideally, a non-invasive measure of cytochrome P-450 would be valuable to test the hypothesis of metabolic efficiency on other subjects including humans. This research project was undertaken to evaluate the use of labelled delta-aminolevulinic acid as an in vivo measure of the mixed function oxidase system especially cytochrome P-450.

ALA, a precursor in the heme biosynthetic pathway, was selected as an isotopic pulse because the labelled carbon is incorporated into the heme molecule at the methene bridges of the porphyrin ring. Upon the degradation of the hemeprotein to bilirubin, one of the labelled carbons is released as CO and excreted in the breath. It was hypothesized that if the quantity of CO excreted after the administration of ALA could be

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[42]See footnote 1, page 2.

determined, the CO would serve as a comparative measurement of the mixed function oxidase system. Administered ALA is preferentially utilized in the synthesis of non-hemoglobin hemeproteins of which cytochrome P-450 is in the largest quantity (62). One difficulty in measurement of CO excreted in the breath was the need to convert the CO to CO_2 for collection in the scintillation liquid for radioactive counting.

The results of this study indicated that ^{14}CO excretion is a possible evaluative technique of the degradation of the mixed function oxidases. The apparatus devised to collect the ^{14}CO excreted from the animal appeared consistent in the results obtained. One advantage of this apparatus over those used by other researchers (98,82) was that the $PdCl_2$ solution used allowed the ^{14}CO conversion to $^{14}CO_2$ at room temperature. Other systems have required high temperatures for adequate conversion. The system devised was also convenient to use.

Only a small percentage of the initial dose of the isotope was recovered in the three-hour collection of ^{14}CO. Such a small value would be expected, however. Of the initial dose, a large portion (50 percent in the rats in this investigation, 30 to 70 percent reported in the literature) was probably excreted intact in the urine. The remaining dose was utilized for heme synthesis. Each heme molecule contained eight of the labelled carbons of which only one would be released as ^{14}CO. In the first few hours, a greater amount of this ^{14}CO would be excreted as shown by the early peak shown with the time interval investigation. The remaining ^{14}CO excreted is from cytochrome P-450 or the other non-hemoglobin hemeproteins, but this ^{14}CO was excreted over a

prolonged time period. Some radioactivity has been reported as much as 80 days after the initial injection (69). Therefore it was not unusual that a three-hour collection of ^{14}CO would be only a small percentage of the total dose administered.

Several procedures were used to evaluate the effectiveness of the circulation system. One such procedure was the determination of the pattern of excretion of ^{14}CO at specific time intervals over a prolonged period. The results obtained, as illustrated in Figure 6, page 50, revealed a pattern very similar to previous literature reports (69,76,118). The sharp initial peak (at two hours after ALA administration) with a gradual decline has been reported for both CO and bilirubin excretion. This peak is thought to be either from the administered ALA or from a pool of free heme in the body. The plateau period, which began at five hours after the injection of ALA in this study, is thought to represent the degradation of hepatic non-hemoglobin hemeproteins such as cytochrome P-450 (82). Comparison of this pattern of ^{14}CO excretion with that reported by other researchers indicates that the system was measuring ^{14}CO from hemeprotein degradation. The time period for collection of ^{14}CO excretion in other aspects of this study (13 to 16 hours after isotope injection) was determined on the basis of this pattern of ^{14}CO excretion. Once the relative amount of ^{14}CO excreted is stabilized, any time period should result in consistent comparative data.

The data obtained from the measure of ^{14}CO excreted by lean and obese littermates reinforces the hypothesis that there is a difference between mixed function oxidase levels of obese and normal mice. There

was a significantly lower level of ^{14}CO excreted by obese mice than by the lean controls. This was true of both groups tested, the 3- to 4-month-old mice and the 7- to 8-month-old mice.

Even more relevant was the close agreement of the results of this investigation with those of Dorsey.[43] There was 26 percent less ^{14}CO excreted by the obese mice at 3- to 4-months of age than by the lean mice at this age. Dorsey measured 24 percent less hepatic cytochrome P-450 in both 3- and 4-month-old obese littermates of lean mice. A greater difference between the ^{14}CO excretion of the 7- to 8-month-old mice was determined. The obese mice excreted 47 percent less ^{14}CO than the controls. The hepatic cytochrome P-450 levels of 7-month-old obese mice were 36 percent less than those of the 7-month-old lean mice as reported by Dorsey. The similarity of results from these two investigations strongly supports the assumption that the ^{14}CO collection apparatus measured relative values of the degradation of the mixed function oxidase system.

No difference between the ^{14}CO levels of the two age groups was noted. The measure of hepatic cytochrome P-450 by Dorsey revealed a peak of cytochrome P-450 at 3- and 4-months of age with a steady decline in levels of the enzyme to 7-months of age for both the normal and obese mice. The ^{14}CO level may not have shown a difference with age because it was a measure of the total mixed function oxidase system. Perhaps with age, while the level of cytochrome P-450 decreases, the level of other

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[43]See footnote 1, page 2.

cytochromes increases so that the net effect is no change in the total mixed function oxidase enzymes.

It was expected that the values of hepatic cytochrome P-450 and ^{14}CO excretion for each mouse in this investigation would be correlated if the measurement of ^{14}CO was a measurement of the mixed function oxidase system. No correlation between the measurement of hepatic cytochrome P-450 and the ^{14}CO values was seen. It is hypothesized that the lack of correlation is explained by experimental difficulties with the measurement of the hepatic cytochrome P-450. Estabrook and Werringloer (19) discussed difficulties encountered with the spectrophotometric method including dilution effects, insufficient light transmission due to turbidity, and unexplained shifts in the baseline measure. While numerous attempts were made by the investigator to pinpoint the cause of the problem, it was not determined. One difficulty could have been the concentration of the sample. According to the experimental procedure the microsomal solution was diluted with phosphate buffer to a concentration of approximately 2 mg per ml protein. In order to obtain the specified initial reading with the spectrophotometer it was necessary to further dilute the microsomal solution. As mentioned in the experimental procedure, this often required a three- to five-fold increase in the buffer quantity added to the microsomal suspension due to the turbidity of the solution. Such an increased dilution probably affected the accuracy of the determinations. Contamination of the microsomal pellet with hemoglobin could also have interfered with accurate readings.

Despite the difficulties with the cytochrome P-450 technique, the ^{14}CO collection still appeared to be a valid measure of the cytochrome

P-450 and/or mixed function oxidase system. This was especially true in view of the close comparison to results of cytochrome P-450 measures previously documented in this laboratory as discussed above. Evidence obtained from the measure of the excretion rate of ^{14}CO and the effect of inducers of the mixed function oxidase system supports the hypothesis.

It was expected that if the circulation apparatus measured the mixed function oxidase system, administration of an inducer of the enzyme system would result in increased ^{14}CO excretion. The 3-month-old mice given sodium pentobarbital for at least three days did show a significantly greater ^{14}CO excretion than the normal control mice. In fact the reported values of the pentobarbital mice were double the values of the uninduced mice. To add further support to the measurement of the mixed function oxidases, no increase in ^{14}CO excretion was seen in the second group of induced mice which received pentobarbital for only two days. It has been reported that at least three days of phenobarbital administration are required to induce cytochrome P-450 (18). Induction of the enzyme had not occurred with the 7-month-old mice. Thus these mice showed no difference in ^{14}CO values from their uninduced control group.

The administration of benzo-α-pyrene induces cytochrome P-448. Induction of cytochrome P-448 occurs about 24 hours after benzo-α-pyrene administration (18). If the ^{14}CO collected was representative of the mixed function oxidase system, there should have been an increase in ^{14}CO excretion after benzo-α-pyrene induction. Benzo-α-pyrene is not water-soluble so it was necessary to use an oil as the injection medium.

Corn oil was chosen for this procedure. In order to determine what effect corn oil administration might have on the excretion of ^{14}CO, a control group received daily injections of corn oil alone in amounts similar to the amounts of benzo-α-pyrene received by the experimental group. It was difficult to draw definite conclusions from the control group, because only two of the four control animals survived the corn oil injections. All of the benzo-α-pyrene animals survived the procedure.

The two control animals that did survive the procedure did not appear to have ^{14}CO values any different from the benzo-α-pyrene group. The ^{14}CO excretion of the benzo-α-pyrene animals was higher than normal 3-month-old mice but significantly lower than the ^{14}CO excretion of the sodium pentobarbital-treated animals. If cytochrome P-448 was induced, then it was to a degree much less than pentobarbital induction of cytochrome P-450. It appeared that there was actually no induction of the mixed function oxidase system by benzo-α-pyrene. Rather, it seemed that corn oil administration induced the system and caused the increased ^{14}CO. There was more than sufficient time for cytochrome P-448 induction, so this would not be the cause of a lack of increased ^{14}CO. One possible conclusion from these results is that the use of ALA with ^{14}CO collection was more selective for the measure of cytochrome P-450 than for other hemeproteins in the mixed function oxidase system.

The measure of ALA incorporation into various organs of the mice was also undertaken to evaluate the ALA administration. It has been observed that mature red blood cells are relatively impermeable to ALA

(54,76), thus administered ALA would be preferentially incorporated into sites of synthesis of non-hemoglobin hemeproteins. The results obtained reflected this. The level of ALA measured in the blood was much lower than measured in any of the organs. The amount of radioactivity detected in the liver was higher than most of the organs at some but not all time periods. A higher level in the liver would be expected because cytochrome P-450 is found in greater quantities in the liver than in other organs.

One of the organs that had consistently higher radioactive levels was the kidney. This could be associated with the poor reabsorption of ALA by the renal filtration system which results in a large portion of the dose being excreted in the urine (62). Also the activity of the mixed function oxidase system is relatively high in the kidneys.

There was little noticeable difference in the quantity of ALA detected in the heart, lungs, and spleen. Several of the organs exhibited a pattern of incorporation of ALA similar to the pattern of ^{14}CO excretion, although not as clearly defined. The heart, spleen, lungs, and kidneys showed a peak radioactivity at 2.5 to 3.5 hours after the ALA injections and then a decline afterwards. These increases in the initial hours may have been associated with unmetabolized ALA from the administered dose or may have coincided with rapid incorporation into the free heme pool (70).

Differences were noted in the radioactive incorporation of the liver fractions of the 8 and 16 hour samples. These differences could have resulted from different stages of the heme biosynthetic pathway occurring at the two time periods. Or the results may not be indicative of any

differences between the time periods but the consequence of inadequate sedimentation of the nuclei and mitochondria especially for the 16-hour samples.

Oral doses of ALA administered to rats revealed the fate of ALA 24 hours after dosage. Variations noted between male and female rats most probably reflect differences in levels of the mixed function oxidase system. It has been reported that male animals have a greater quantity of hepatic cytochrome P-450 than female animals (13). The incorporation of ALA into the liver was significantly greater in the male rats than in the female rats. The differences in radioactivity of various organs may also have resulted from different rates of metabolism or degrees of utilization of the ALA. These may explain the greater levels of radioactivity in the heart and spleen of the female rats than of the male rats.

Urine and fecal collections for a 24-hour period contained about half of the administered isotope. Previous observations have indicated that anywhere from 30 to 70 percent of injected oral doses was excreted in the urine (72-74), and the major portion of the urinary excretion occurred in the first few hours consisting almost entirely of intact ALA. The very low percentage of radioactivity detected in the feces (3 percent) concurred with previous reports (72,73). Nearly all of a dose of ALA, either injected or fed, is absorbed from the intestinal tract.

The implications of this investigation to future research are numerous. The study of obesity is an on-going process in human health research. Treatments currently utilized for obesity have very limited

success. No single cause for obesity development has been identified, although most health professionals attribute obesity to energy intake in excess of energy expenditure. A conflict with this arises when an individual appears to have an energy intake much less than the average person and a typical energy expenditure, yet is still extremely overweight. In such a situation some type of metabolic alteration in energy is suggested.

The mixed function oxidase system is an energy wasteful system in the body and data indicate the activity of this system is decreased in obese mice. Therefore the obese mice are energy conserving and would be expected to store excess energy as adipose tissue. If the hypothesis that the mixed function oxidases are involved in alterations of energy metabolism is valid, then it must be tested on other species including humans.

It is difficult to measure mixed function oxidase activity by available procedures because liver tissue is required for these procedures. While it is possible to obtain human liver tissue samples by a liver biopsy technique, this procedure is stressful to the individual. A system of measurement which does not cause undue stress to the subject would be more desirable.

It is possible to conceive that the mixed function oxidase system could be measured in humans by a non-invasive technique just as this investigation measured it in mice. The total body heme level has been successfully measured in humans by determination of carboxy-hemoglobin levels and/or CO excretion (94). Carbon monoxide measures have been

utilized to study disorders of hemoglobin (65). If ALA is preferential to non-hemoglobin heme synthesis in humans as it is in animals, then the administration of ALA to humans could be a tool for the measurement of the mixed function oxidase enzymes in humans. The CO excreted would be selectively representative of the non-hemoglobin hemeproteins of the mixed function oxidase system particularly cytochrome P-450.

CHAPTER VI

SUMMARY

The mixed function oxidase system is an energy wasteful enzyme system in the body which may be associated with the development of obesity. A decreased level of the mixed function oxidase system may contribute to an energy-saving mechanism which can lead to increased fat deposition. This hypothesis was documented in an earlier investigation in this laboratory. The hepatic cytochrome P-450 levels of obese and non-obese mice were shown to differ significantly, with the non-obese mice having the greater hepatic cytochrome P-450 values. The previous measurement utilized for determination of hepatic cytochrome P-450 was an in vitro spectrophotometric technique. This method required a liver tissue homogenate. A procedure which could measure the mixed function oxidase system without requiring sacrifice of the animal would be desirable to further evaluate the hypothesis. If available, such a procedure could eventually be applied to other species including humans.

In this investigation, an in vivo method for measuring the hepatic mixed function oxidase system was evaluated. Delta-aminolevulinic acid is a preferential precursor to hepatic hemeprotein synthesis. The degradation of a hemeprotein results in the excretion of CO in the breath as well as the formation of bilirubin. The source of the carbon in the CO is the fifth carbon of aminolevulinic acid. Therefore it was postulated that when 5-^{14}C-delta-ALA is administered, the degradation of the hepatic hemeproteins, specifically the mixed function oxidases, can

be measured by the collection of ^{14}CO excreted in the breath. In order to collect labelled CO it must be converted to CO_2 so that it can be trapped in a scintillation liquid for counting of the radioactivity.

A tracer pulse dose of 5-^{14}C-delta-aminolevulinic acid was administered to the animals tested. Subsequently ^{14}CO excreted via the lungs of the mice was collected in scintillation liquid for radioactive counting. The apparatus used to collect the ^{14}CO was a closed circulation system controlled by a forced air pump. A solution of palladium chloride was used to convert the ^{14}CO to $^{14}CO_2$ so that it could be trapped for the determination.

Analysis of the data obtained revealed that the system evaluated was an effective measure of the hepatic mixed function oxidase system. The closed circulation apparatus devised for the procedure was convenient to use. It provided consistent results with no observable stress to the animal. The palladium chloride solution adequately converted the ^{14}CO excreted in the breath to $^{14}CO_2$ so that the radioactivity could be determined.

The ^{14}CO values obtained from groups of obese and normal mice were in agreement with hepatic cytochrome P-450 values obtained in the previous investigation. A significant difference was found between the ^{14}CO excreted by the obese and the non-obese mice in both age groups studied. The lean mice excreted a greater amount of ^{14}CO than the obese mice. It was not possible to correlate hepatic cytochrome P-450 values obtained from this study with the ^{14}CO values, most probably due to experimental technique difficulties rather than an error in the hypothesis.

Other aspects of the investigation supported the hypothesis that the ^{14}CO measurement of ALA was a comparative measurement of the mixed function oxidase system. Pentobarbital treatments also caused an increase in ^{14}CO excretion when the pentobarbital was administered for a sufficient time period to cause induction of cytochrome P-450. Benzo-α-pyrene injections did not appear to increase ^{14}CO excretion which may have suggested that the system was more selective in the measurement of cytochrome P-450 rather than cytochrome P-448.

Data from the oral administration of delta-aminolevulinic acid to rats indicated that while a large portion of the isotope is excreted in the urine a quantifiable amount is incorporated in several major organs. The greatest quantity of the dose incorporated in an organ 24 hours after administration was found in the liver. A significantly larger amount of the radioactivity in the liver was measured in the male rats than in the female rats. It appeared that the aminolevulinic acid was metabolized differently in the males than the female rats. This could have resulted from differences between the two sexes in cytochrome P-450 levels.

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VITA

Michelle Marie Laborde was born in Columbus, Georgia on May 31, 1952. She attended parochial schools in New Orleans and Lafayette, Louisiana. Following graduation from Cathedral-Carmel High School in 1970, she entered The University of Southwestern Louisiana and was awarded the Bachelor of Science degree from USL in May 1975. She interned in dietetics at Methodist Hospital of Indiana, Inc., in Indianapolis. Upon completion of the dietetic internship in August 1975, the author entered The University of Tennessee to begin graduate work. She was awarded the Master of Science degree in August 1976. In December 1977 she married James Benjamin Vineyard. From September 1976 to June 1979, Ms. Vineyard served as Instructor of Nutrition and Diet Therapy at Fort Sanders Presbyterian Hospital School of Nursing in Knoxville. In June 1979, the author accepted employment with the State of Tennessee Department of Public Health as Director of Nutrition Services for the East Tennessee Region. She received the Doctor of Philosophy degree in August 1979.