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DEGRADATION OF CYTOCHROME P-450 HEME IN ASCORBIC ACID-DEFICIENT GUINEA PIGS*†

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Abstract—Degradation of hepatic cytochrome P-450 heme in ascorbic acid-deficient and ascorbic acid-dosed (p.o., 25 mg/100 g/body wt/day) guinea pigs was investigated by determining the turnover of radioactive cytochrome P-450 and the formation of *in vivo* expired carbon monoxide (^{14}CO). ^{14}CO is a specific degradation product of the labeled heme. Hepatic levels of cytochrome P-450 and cytochrome b_5 were decreased significantly (61 per cent, $P < 0.001$, and 29 per cent, $P < 0.01$, respectively) in scorbutic animals compared to ascorbic acid-adequate animals. After the administration of δ-aminolevulinic acid [3,5-^{3}H], the disappearance of radioactivity from the fast-phase and slow-phase components of cytochrome P-450 heme (CO-binding pigments) exhibited half-lives of 4.2 and 30.8 hr, respectively, in the ascorbic acid-depleted guinea pigs. The turnover of cytochrome P-450 heme was similar for both phases in animals fed the ascorbic acid-deficient diet and dosed with ascorbic acid or animals fed a normal stock diet. In guinea pigs deficient in ascorbic acid and injected with [5-^{14}C]δ-aminolevulinic acid, the cumulative expired ^{14}CO was similar to guinea pigs adequate in ascorbic acid. These studies demonstrate that the decrease in hepatic cytochrome P-450 content in ascorbic acid deficiency is not due to a defect in cytochrome heme catabolism. The role of ascorbic acid in stabilizing cytochrome P-450 remains to be determined.

Ascorbic acid deficiency in guinea pigs results in a decrease in hepatic mixed-function oxygenase system [1,2], related in some way to its effects on microsomal cytochrome P-450 content [3–9]. The biochemical basis for the effect of ascorbic acid depletion on drug metabolism is unknown. The possibility that the decreased quantity of cytochrome P-450 in ascorbic acid-deficient guinea pigs may result from an impairment in heme synthesis was studied, in the past and recently, with inconsistent results. Luft *et al.* [10] found that cytochrome P-450 levels returned to normal when scorbutic guinea pigs were injected with δ-aminolevulinic acid (ALA), a key substrate for heme biosynthesis. However, others have demonstrated that there is no significant difference in the key enzymes involved in heme synthesis, such as ALA synthetase, ALA dehydratase, or ferrochelatase, and ascorbic acid deficiency did not affect the availability of heme for cytochrome P-450 synthesis [11–13].

Recently in our laboratory, we found that ascorbic acid deficiency in guinea pigs does not result in any appreciable induction of microsomal heme oxygenase (MHO) [14]. MHO is the rate-limiting enzyme which catalyzes the oxidative degradation of heme to form biliverdin [15], and biliverdin is subsequently reduced to bilirubin by biliverdin reductase in the soluble cell fraction [16,17]. In rat liver and kidney, MHO undergoes adaptive regulation or induction in response to a substrate load [18,19].

In an attempt to confirm our previous findings [14], we investigated the effect of ascorbic acid deficiency on *in vivo* expired carbon monoxide, an obligatory by-product of heme catabolism via MHO [20,21]. In addition, we were interested in determining whether or not the lack of ascorbic acid intake might result in cytochrome P-450 heme breakdown by mechanisms other than by the induction of MHO. Therefore, we investigated the influence which ascorbic acid status in guinea pigs might have on the turnover of hepatic cytochrome P-450 heme labeled with radioactive ALA.

MATERIALS AND METHODS

Animals and diet. Young male, Hartley guinea pigs (Charles River, Wilmington, MA), with an initial weight of 180–230 g, were housed individually in stainless steel wire-bottomed cages equipped with an automatic watering system. The animal room was maintained at 25° with a 12-hr light–dark cycle (7:00 a.m. to 7:00 p.m.). A purified pelleted diet, deficient in ascorbic acid, was made up according to specifications developed by Reid and Briggs [22]. Both food and tap water were made available at all times to the animals. The ascorbic acid content of the purified diet was less than 0.03 mg/g of diet [23]. Guinea pigs were weighed daily in order to calculate the daily dose for ascorbic acid supplements and to compare the daily body weight changes of the two treatment groups. Fifty-four guinea pigs were divided randomly into two treatment groups and fed

* The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

† In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Revision of the Guide for Laboratory Animal Resources, National Research Council.

the ascorbic acid-deficient diet for 21 days. Other animals (twenty-four total) were fed a commercial stock diet for a similar period of time. Half the animals fed the ascorbic acid-deficient diet, hereafter referred to as supplemented guinea pigs, were dosed (p.o.) daily, once a day, with an aqueous solution of 25 mg ascorbic acid/100 g body weight. This aqueous solution of ascorbic acid was made fresh daily and was administered via the mouth by micropipet. The dosage volume was 100 μl/100 g body wt.

Chemicals. Subtilisin, L-ascorbic acid, hyamine hydroxide (methyl benzethonium hydroxide), glutathione and nicotinamide were obtained from the Sigma Chemical Co., St. Louis, MO.; δ-amino[5-^{14}C]levulinic acid ([^{14}C]ALA, 30 mCi/mmole) and [3,5-^{3}H(N)]δ-aminolevulinic acid ([^{3}H]ALA, 5.0 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. Aqueous Counting Scintillant (ACS) was obtained from Amersham, Arlington Heights, IL. All other chemicals used were of the highest purity obtainable from commercial sources.

Tissue preparation. Animals were weighed and then decapitated. A small portion of liver, about 0.5 g, was removed, minced and homogenised in 10% trichloroacetic acid for ascorbic acid analysis. The remaining liver was perfused with 1.15% KCl, excised and minced while cold, and then homogenized in 2 vol. of cold 0.1 M Na^+/K^+ phosphate buffer (pH 7.4) made up in 1.55% KCl containing 10 mM nicotinamide and 2 mM glutathione. Homogenization was done in a glass and Teflon homogenizer by three strokes of the pestle. The homogenate was centrifuged for 15 min at 10,000 *g* in a Sorvall RC2-B centrifuge with an SS-34 rotor to remove nuclear, mitochondrial and cellular debris. The resulting supernatant fraction was centrifuged for 60 min at 100,000 *g* in a Beckman–Spinco ultra centrifuge with a type 50 Ti rotor. The microsomal pellet was resuspended in buffer and recentrifuged for 30 min at 100,000 *g*. The final pellet was then suspended in buffer and kept at 4° until used.

Labeled cytochrome P-450 from CO-binding particles. Twenty-five days after starting the dietary regimen, ascorbic acid-deficient and supplemented guinea pigs were injected intracardially with [^{3}H]ALA, which has been diluted with 0.9% saline. Intracardiac injections were performed, without the aid of anesthesia, by restraining the animal on its back and inserting the needle (25 gauge) in the xiphoid area, aiming for the left ventricle. All animals received 200 μCi and 28 μg ALA/kg body wt. The dosage vol was 50 μl/kg body wt.

After tissue preparation and centrifugation, the washed microsomes were incubated with subtilisin, 10 μg/mg of protein, in buffer and glycerol, 20% by volume.* Incubation was done with constant shaking at 4° for 15 hr. The mixture was then centrifuged for 60 min at 100,000 *g* and the resulting pellet, hereafter referred to as CO-binding particles, was resuspended in buffer and recentrifuged for 30 min at 100,000 *g*. The final CO-binding particles were suspended in buffer and portions were assayed for radioactivity, cytochrome P-450, cytochrome *b*$_5$ and protein. The portion that was analyzed for radioactivity was first solubilized in hyamine hydroxide, neutralized with acetic acid, and counted in ACS using a liquid scintillation spectrometer.

Cytochrome P-450 and cytochrome b_5. By using a Beckman DK2A split-beam recording spectrophotometer, microsomal cytochrome content was estimated on a resuspended microsomal pellet or resuspended CO-binding particles (2–3 mg protein/ml). Cytochrome P-450 was analyzed by measuring the dithionite-reduced carbon monoxide difference spectrum [24]. Cytochrome *b*$_5$ was analyzed by measuring the dithionite-reduced difference spectrum [25].

Ascorbic acid and proteins. Ascorbic acid was determined on trichloroacetic acid preserved liver samples by a procedure similar to that developed by Roe [26] and modified for tissues [23]. Proteins were determined by the method for small samples of Miller [27].

^{14}CO in expired air. Ascorbic acid-deficient and supplemented guinea pigs were dosed by intracardiac injections with [^{14}C]ALA (50 μCi/kg body wt.) at zero time. They were then put into a CO-collection device (Fig. 1) similar to the one described by Landaw and Winchell [20], and ^{14}CO was trapped and measured.

Statistics. Data were analyzed statistically by Student's *t*-test. Differences between values of group means were considered significant only where $P < 0.05$.

RESULTS

Ascorbic acid deficiency. At the end of 25 days, gross clinical symptoms of ascorbic acid deficiency were becoming apparent in the group of guinea pigs receiving no ascorbic acid. Signs included diarrhea, marked hind limb weakness, ataxia, and cessation of normal weight gain, which started approximately at day 17. On each day they were killed, post-[^{3}H]-ALA injection, the deficient animals weighed 7.7 to 35.7 per cent less than the ascorbic acid-supplemented guinea pigs. Table 1 shows the combined group means of body weights, taken at the end of the experiment, which demonstrated that the ascorbic acid-deficient guinea pigs weighed 27.3 per cent ($P < 0.001$) less than the animals dosed with 25 mg ascorbic acid/100 g body wt. Combined mean liver weights were not significantly different between the two treatment groups. Table 1 also demonstrates that liver ascorbic acid was decreased 95.2 per cent ($P < 0.001$) in guinea pigs fed a diet lacking ascorbic acid compared to supplemented animals, thus indicating the severity of the deficiency.

Microsomal hemoprotein. Ascorbic acid depletion markedly decreased the levels of cytochrome P-450 and cytochrome *b*$_5$, the two hemoproteins which occur in liver microsomes (Table 2). In general, on each of the days post-[^{3}H]ALA injections, the cytochrome P-450 and cytochrome *b*$_5$ levels were 49.8 to 68.5 and 7.6 to 39.8 per cent less, respectively, in the deficient animals. Mean values for cytochrome P-450 and cytochrome *b*$_5$ demonstrated 61.0 per cent

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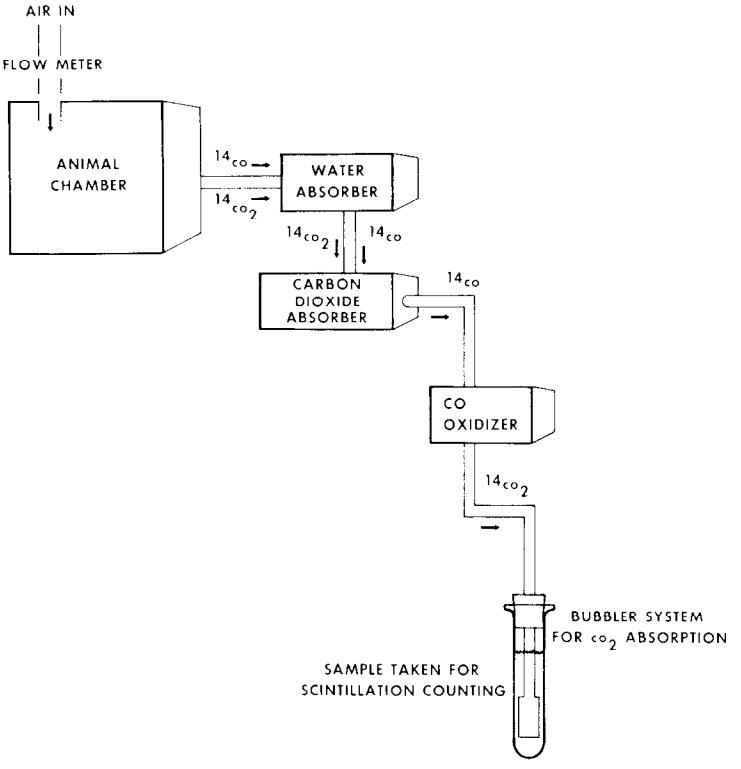

Fig. 1. *In vivo* breath collection system. The pathway of air (200 ml/min) is shown by arrows. The water absorber was composed of anhydrous $CaSO_4$. The carbon dioxide absorber was composed of sodalime and Ascarite (A. H. Thomas Co., Philadelphia, PA) (250 g and 100 g respectively). The carbon monoxide oxidizer is composed of 17 g of Hopcalite (No. 41566 MSA "CO" absorber, Mine Safety Appliances Co., Pittsburgh, PA) (a mixture of magnesium and copper oxides with other catalytic agents) and serves to oxidize CO to CO_2 at low ambient temperature.

($P < 0.001$) and 28.9 per cent ($P < 0.01$) respective decreases in deficient animals compared to supplemented guinea pigs.

In a preliminary experiment using guinea pigs fed a normal stock diet, we found that cardiac injections of [^{3}H]ALA produced labeling of hepatic cytochrome P-450 with reproducible variation. Two hr post-injection and after isolation, subcellular fractionation, and solubilization of cytochrome b_5, the mean values of radioactivity found in the CO-binding particles had coefficients of variation of about 6–20 per cent. Calculations based on a previous report [28] indicate a range of coefficients of variation of 2.6 to 8.7 per cent. In that investigation [28], the tail vein was used. Lack of readily accessible veins prevented us from using the intravenous route in the guinea pig; however, we found that cardiac injections could be performed rapidly. If we used some animal restraint, we did not need to anesthesize them.

Figure 2 shows the rate of disappearance of radioactive heme from the hepatic CO-binding (microsomes devoid of cytochrome b_5) of guinea pigs fed

Table 1. Effects of ascorbic acid on body weight, liver weight, and liver ascorbic acid after injection with [^{3}H]-ALA*

Treatment	Body weight (g)	Liver weight (g)	Liver ascorbic acid (μg/g)
−C	221 ± 8 (16)	9.3 ± 0.4 (16)	13.8 ± 2.8 (16)
	304 ± 10†	10.5 ± 0.6‡	286.0 ± 122†
+C	(18)	(18)	(18)

* Each value represents the mean ± S. E. M. Key: ascorbic acid-deficient animals (−C), and ascorbic acid-dosed animals (+C). Numbers in parentheses represent total N per treatment group.

† $P < 0.001$.

‡ $P > 0.05$.

Table 2. Effects of ascorbic acid on hepatic cytochrome P-450 and cytochrome b_5 after injection with [^{3}H]-ALA*

Treatment	Cytochrome P-450 (nmoles/mg protein)	Cytochrome b_5 (nmoles/mg protein)
−C	0.48 ± 0.04 (15)	0.23 ± 0.02 (15)
+C	1.22 ± 0.04† (18)	0.33 ± 0.02‡ (16)

* Each value represents the mean ± S. E. M. Key: ascorbic acid-deficient animals (−C), and ascorbic acid-dosed animals (+C). Numbers in parentheses represent total N per treatment group.
† $P < 0.001$.
‡ $P < 0.01$.

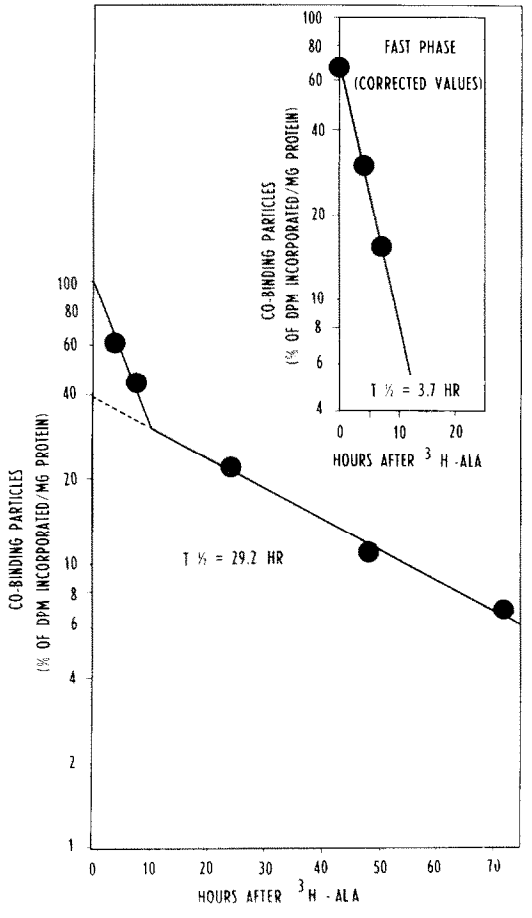

Fig. 2. Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from guinea pigs fed stock diet. Groups of animals (N = 3–4) were killed 4, 7, 24, 48 and 72 hr post-[^{3}H]ALA injection. Points represent mean values. The zero-time intercept of the uncorrected fast-phase was set equal to 100 per cent. Values used for the determination of the corrected half-life of the fast-phase (insert) were obtained by first extrapolating the slow-phase line to zero time and then subtracting the values of the extrapolated slow-phase from values of the uncorrected fast-phase.

a normal stock diet. The disappearance of cytochrome CO-binding particles was biphasic, indicating the existence of at least two forms or states of the hemoprotein [28, 29]. The half-life of the slow-phase component was estimated to be 29.2 hr, whereas the half-life of the fast-phase component, corrected for contribution of the slow phase, was 3.7 hr.

Figure 3 illustrates the rates of disappearance of radioactive heme from cytochrome P-450 of guinea pigs fed an ascorbic acid-deficient or a supplemented diet. The half-life of the corrected fast-phase component from the ascorbic acid-dosed groups was 4.6 hr. In the ascorbic acid-depleted group, the half-life of the corrected fast-phase fractions was 4.2 hr, similar to the half-life found for the ascorbic acid-dosed group. The half-life of the slow-phase component was 30.5 hr in the ascorbic acid-dosed group, and 30.8 hr in the ascorbic acid-depleted animals. Essentially, there appeared to be no difference in either the corrected fast-phase or slow-phase half-lives among animals fed the stock diet, the ascorbic acid-deficient diet alone, or fed the deficient diet and dosed with ascorbic acid.

Formation of CO *from* [^{14}C]-ALA. Figure 4 illustrates the expiration of ^{14}CO after the injection of the heme precursor [^{14}C]ALA. The expiration of ^{14}CO has been correlated well with the excretion of [^{14}C]bilirubin [21]. Both products represent the oxidation of the C-labeled δ-methylene bridge of the heme moiety by the enzyme heme oxygenase. There appears to be no effect of ascorbic acid deficiency on ^{14}CO production.

DISCUSSION

As found in our previous study and confirming many earlier studies of others [3–9], we found an influence of ascorbic acid on cytochrome P-450 content in guinea pigs. We also found a significant decrease in hepatic cytochrome b_5 which confirms the findings in an earlier study [7]. Others [4–6] have failed to find this decrease. Inasmuch as these animals were scorbutic, the drop in cytochrome b_5 may reflect this devastating condition. Ascorbic acid deficiency could influence the level of cytochrome P-450 by either heme synthesis impairment or enhancement of heme catabolism or both [30,31].

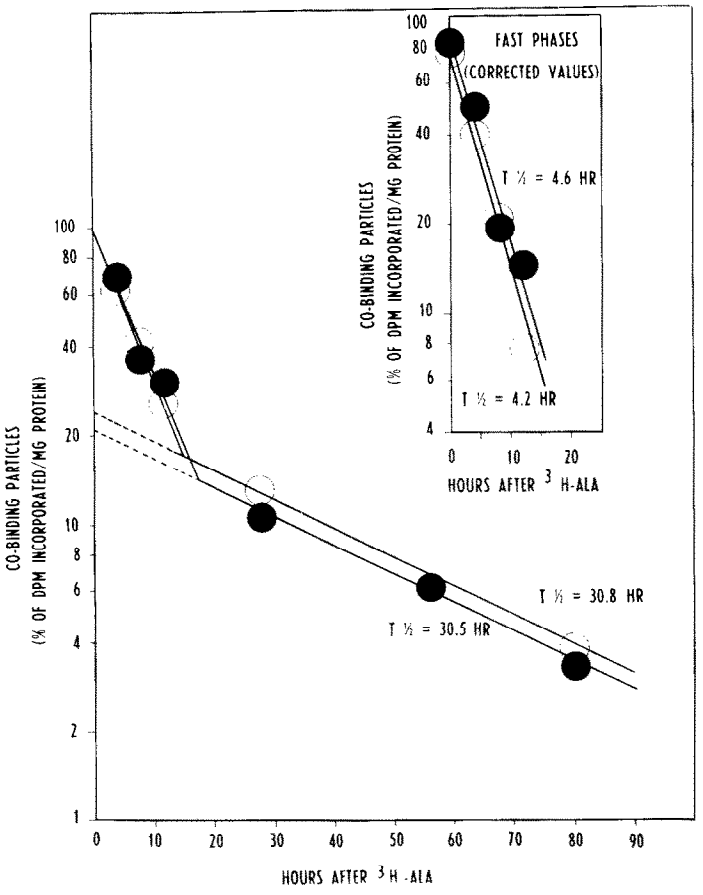

Fig. 3. Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from guinea pigs fed ascorbic acid-deficient (—○—) or ascorbic acid-supplemented (—●—) diets. Groups of animals (N = 2–4) were killed 4, 8, 12, 56 and 80 hr post-[^{3}H]ALA injection. The half-lives of the various fractions were calculated as described in the legend of Fig. 2.

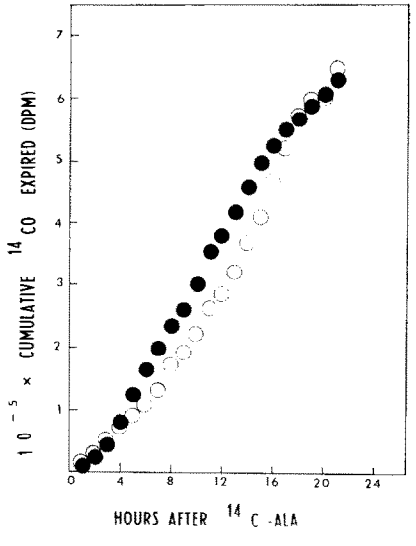

Fig. 4. ^{14}CO expired by ascorbic acid-deficient (—○—) and ascorbic acid-supplemented (—●—) guinea pigs. Aliquots of CO trapping solution were taken at hour intervals for 21 hr and measured for radioactivity. Values in the figure represent one pair of animals which are similar to values found in two other pairs of animals.

Several have postulated that hepatic heme synthesis is defective in ascorbic acid deficiency, but more recent investigations failed to demonstrate such a defect [10–13]. Lipid peroxidation and microsomal heme oxygenase (MHO) represent the two most well documented degradative pathways for cytochrome P-450 [32–34]. Also, the influence of inadequate ascorbic acid-promoting lipid peroxidation seems unlikely since others failed to find changes in oxygen consumption, NADPH disappearance or formation of the by-product of lipid peroxidation, malonaldehyde, in ascorbic acid-depleted microsomes [11–13]. Also, phosphatidylcholine in ascorbic acid-deficient liver microsomes was only decreased 18 per cent compared to a drop of 29 per cent in starved controls when both were compared to normal controls [12]. There was also no difference between the chromatographic migration of phosphatidylcholine or phosphatidylethanolamine in normal vs ascorbic acid-deficient animals [12]. Finally, no evidence was found for increased cytochrome P-420 levels in ascorbic acid-deficient microsomes, the cytochrome species often found when lipid peroxidation occurs [8].

The synthetic and degradative pathways for hepatic microsomal cytochrome P-450 heme are

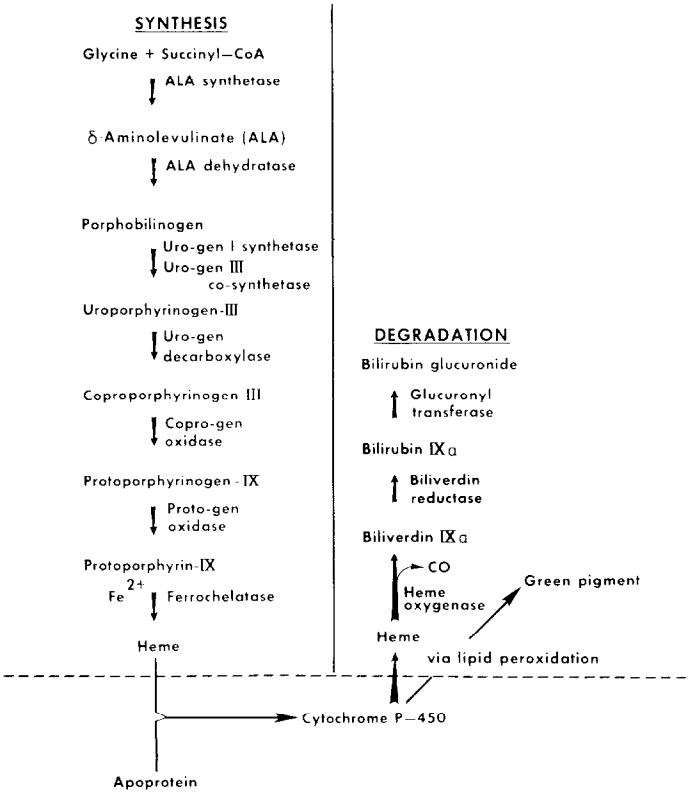

Fig. 5. Pathways for hepatic cytochrome P-450 synthesis and degradation.

shown in Fig. 5. Previously, we found no evidence that ascorbic acid deficiency results in increased heme catabolism via MHO [14], but that MHO was related directly to the liver levels of ascorbic acid. Carbon monoxide along with the excretion of bilirubin are products of MHO action on hepatic heme. In this present study, we were not able to find any difference in expired CO from ascorbic acid-deficient or supplemented guinea pigs, confirming our previous findings on hepatic MHO.

Since unknown mechanisms of heme degradation may exist, we sought a general method to define such degradation. At the present time, the most used technique for the measurement of hepatic cytochrome P-450 turnover is by the loss of radioactivity from isolated CO-binding particles obtained from animals whose hemoprotein was prelabeled [30,31]. Selective solubilization of the microsomal cytochrome b_5 ensures that only cytochrome P-450 will be detected [35]. Investigations utilizing such techniques have provided evidence of the existence of more than one CO-binding hemoprotein, as suggested by the biphasic nature of the decrease in radioactive hemoproteins. These assumptions have been supported by spectral studies of microsomal cytochrome P-450 hemoprotein [36,37]. The most commonly used animal model for this technique is the rat, where the biological half-life of the rapidly disappearing hemoprotein fraction was on the order of 7–8 hr and that of the slowly disappearing fraction was about 30–50 hr. In the guinea pig, we found that the slowly disappearing fraction was about the same as that found for rat; however, the rapidly disappearing hemoprotein, in contrast, was less than that found for the rat (3.7–4.6 hr) (Fig. 3). It is interesting that this fast-phase degradation found in guinea pigs is more on the order of that seen with the induction of heme degradation by cadmium or other exogenous agents [38]. Whether or not this difference reflects just species difference should be explored further.

Essentially, the ascorbic acid status did not appear to influence the degradation of microsomal CO-binding particles. These findings, along with the past investigations on heme synthesis, suggest that ascorbic acid has no influence on heme catabolism or heme synthesis. The possibilities still remain that ascorbic acid may influence: (1) apo-cytochrome P-450 synthesis, (2) iron and perhaps copper metabolism (by way of its reducing properties) [39,40] which could influence, in turn, the incorporation of ferrous iron into the heme moiety of cytochrome P-450, and (3) the binding of heme and apo-cytochrome P-450 to form active cytochrome P-450. Changes in the disc gel electrophoretic patterns of partially purified cytochrome P-450 from ascorbic acid-deficient animals have led others to suggest that apo-cytochrome P-450 synthesis may be impaired [13]. Recently, indirect evidence has provided some support for the second hypothesis. It was found that ascorbic acid-depleted animals had significantly elevated ceruloplasmin levels when compared to

ascorbic acid-adequate animals [41]. Ceruloplasmin is a copper-containing protein which may maintain the oxidation state of iron in the Fe^{+3} state [42]. The subsequent oxidation state of iron may, in turn, make iron unavailable for incorporation into protoporphyrin.

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