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

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Abstract—Studies were carried out in the guinea pig to compare the effects of ascorbic acid (AA) status on hepatic and blood hemoproteins and to evaluate the role of Δ-aminolevulinic acid (ALA) injections in the modulation of the adverse actions produced by AA depletion. Hepatic microsomal cytochrome P-450 concentration, drug metabolism and blood heme parameters are significantly ($P < 0.05$) lower in AA-deficient guinea pigs than in AA-adequate animals. Intraperitoneal injections of ALA were not effective in reversing hepatic cytochrome P-450 or blood heme parameters in AA- deficient guinea pigs. Urinary excretion of porphyrins and porphyrin precursors was lower or not changed in AA-depleted animals compared to AA-adequate guinea pigs. These studies support the hypothesis that AA deficiency does not block heme synthesis. However, the incorporation of [^{3}H]ALA into hepatic cytochrome P-450 heme was less in AA-depleted animals than in AA-supplemented animals. This finding is consistent with a possible error in assembly of holoprotein from heme and apoprotein. Two additional findings were noted: (1) AA status had no significant effect on blood catalase activity, and (2) the anemia found in scorbutic guinea pigs was not associated with macrocytic changes.

Levels of hepatic and extrahepatic heme proteins may be determined by factors which affect synthesis or degradation of the heme or protein components or which influence the assembly of these two components to form the holoprotein. Synthesis of the protein component occurs in the rough endoplasmic reticulum, while heme synthesis is a mitochondrial function [1]. Degradation of the protein component is probably by proteolytic, perhaps lysosomal, enzymes, while degradation of the heme components may be any of at least five pathways [2].

In spite of several efforts, the role of ascorbic acid (AA) in hepatic metabolism of drugs has not been established. AA influences hepatic cytochrome P-450 levels [3–7] and other hemoproteins [8, 9]. Luft *et. al.* [10] suggested that the decreased levels of cytochrome P-450 in AA-deficient guinea pigs is due to an impairment in heme synthesis. However, investigation into enzymes responsible for heme synthesis failed to provide supporting evidence that AA deficiency results in impaired synthesis [11–13]. In addition, recent studies in our laboratory, and others, failed to demonstrate any influence of AA deficiency on the general turnover of hepatic cytochrome P-450 [14], as well as any specific induction of cytochrome heme degradation via lipid peroxidation [4] or microsomal heme oxygenase induction [7].

In this study, we focused our attention on heme metabolism in AA-deficient guinea pigs, using *in vivo* [15] as well as *in vitro* measurements. Also, we investigated what influence AA deficiency might have on other hemoproteins.

MATERIALS AND METHODS

Animals and diet. Eighty-two male guinea pigs (Hartley strain, Charles River, Wilmington, MA), age 14–18 days and weighing 200–250 g, were housed individually in stainless steel wire-bottom cages. The cage racks were equipped with an automatic watering system and the animal room was maintained at 25° with a 12-hr light–dark cycle (7:00 a.m. to 7:00 p.m.). Both food and tap water were made available at all times to the animals. On arrival from the supplier, the animals were fed on a standard stock diet for 5 days. For the next 3 days the stock diet was gradually deleted and replaced with a purified pelleted diet. The purified pelleted diet, deficient in AA, was made up according to the specifications developed by Reid and Briggs [16]. AA content of the purified diet was less than 0.02 mg/g of diet. Guinea pigs were weighed daily in order to compare daily body weight change between treatment groups and to calculate the daily dose for AA supplements. The animals were divided randomly into two treatment groups and fed the AA-deficient diet for 21–25 days. One-half of the animals fed the AA-deficient diet, hereafter referred to as supplement guinea pigs, were dosed (p.o) daily, once a day, with an aqueous solution of 25 mg AA/100 g body wt. This aqueous solution of AA was made fresh daily and was administered perorally by micropipet. The dosage volume was 100 μl/100 g body wt.

Materials. Porphobilinogen, coproporphyrin, uroporphyrin, δ-aminolevulinic acid (ALA), subtilisin, L-ascorbic acid, methyl benzethionium hydroxide

* 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 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.

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(hyamine hydroxide), NADP, NADPH, glutathione, glucose-6-phosphate, and nictoinamide were obtained from the Sigma Chemical Co., St. Louis, MO. Aniline hydrochloride, hydrogen peroxide, trichloroacetic acid (TCA), and 2,5-dinitrophenylhydrazine (DNPH) were obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. *p*-Aminophenol was obtained from the Eastman Kodak Co., Rochester, NY, and aminopyrine was obtained from the Aldrich Chemical Co. Inc., Milwaukee, WI. [3,5-^{3}H(N)]^{1}δ-Aminolevulinic acid ([^{3}H]-ALA, 5.0 mCi/mole) was obtained from the New England Nuclear Corp., Boston, MA. Aqueous counting scintillant (ACS) was obtained from Amersham/Searle, Arlington Heights, IL. All other chemicals used were of the highest purity obtainable from commercial sources.

Treatment with δ-aminolevulinic acid. Two experiments were done in order to ascertain any influence the heme precursor, ALA, might have on hemoproteins in AA-deficient guinea pigs. The procedure used in experiment A was similar to the procedure followed by Lift *et al.* [10]. The animals were injected (i.p.) once every 12 hr for 48 hr with 5 mg ALA/kg prior to killing. In experiment B we were concerned that injections of ALA 48 hr prior to killing the animals would not allow sufficient time for incorporation of precursor into the cytochrome. Consequently, the animals were injected (i.p.) once every 24 hr for 10 days before killing. δ-Aminolevulinic acid was dissolved in 0.9% saline and injected in a dosage volume of 5 ml/kg body wt.

Preparation of subcellular fractions. Animals were weighed and then decapitated. Blood was collected into tubes containing heparin and kept at 4° until catalase was determined. The livers were weighed, and a small portion weighing about 0.5 g was removed, weighed, minced and homogenized in 5% trichloroacetic acid (TCA) for ascorbic acid analysis. The remaining liver was excised and minced cold, and then homogenized in 3 vol. of cold 0.1 M Na^+/K^+ phosphate buffer, pH 7.4, made up in 1.15% 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. Portions of the 10,000 *g* post-supernatant fraction were centrifuged at 100,000 *g* for 60 min in a Beckman–Spinco ultracentrifuge with a type 50 Ti rotor. The resultant supernatant (cytosol) fraction was removed and discarded. To remove residual hemoglobin concentrations from microsomes isolated from livers, resuspended (washed) microsomes were recentrifuged at 100,000 *g* for 30 min. The 100,000 *g* post-supernatant fracture was discarded, and the microsomal pellet was resuspended again in a small volume of buffer for cytochrome P-450 determination.

Assay of enzyme activities and microsomal cytochrome levels. The Nash procedure for formaldehyde [17] was used to determine aminopyrine *N*-demethylase activity in the 10,000 *g* post-supernatant fraction [18]. The hydroxylation of aniline in the 10,000 *g* post-supernatant fraction was determined by measuring the formation of *p*-aminophenol [19]. Microsomal cytochrome content was estimated on 4–5 mg protein/ml resuspended microsomal pellet or resuspended microsomal CO-binding particles (see below) by using a Beckman DK2A split-beam recording spectrophotometer. Cytochrome P-450 was analyzed by measuring the dithonite-reduced carbon monoxide (CO) difference spectrum [20]. Cytochrome b_5 was analyzed by measuring the dithionite-reduced difference spectrum [21]. Catalase was determined in dilute whole blood by following the rate of H_2O_2 decomposition [22].

Measurement of protein and ascorbic acid. Protein was determined in homogenates and subcellular fractions by the method of Miller [23]. Ascorbic acid was determined on acid stabilized liver homogenates by a procedure similar to that developed by Roe [24], modified for tissues [25].

Determination of porphyrin precursors in urine. Urinary ALA and porphobilinogen were determined by the method of Davis and Andelman [26]. Coproporphyrin and uroporphyrin were determined fluorometrically as described by Schwartz *et al.* [27].

Isolation of labeled cytochrome P-450. Twenty-one days after starting the dietary regimen, ascorbic acid-deficient and supplemented guinea pigs were injected intracardially with [^{3}H]-ALA, which had 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 29 μg of ALA per kg body wt. The dosage volume was 50 μl/kg body wt. Groups of animals were killed 15, 30, 45 and 60 min post [^{3}H]ALA injection.

After the preparation of subcellular fractions, the washed microsomes were incubated with subtilisin, 10 μg/mg 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.

Statistics. Data were analyzed statistically either by Student's *t*-test or by analysis of variance.

RESULTS

In experiment A, no gross clinical symptoms of AA deficiency, other than a non-significant loss in body weight, were observed in the group of guinea pigs receiving no AA, or the group of guinea pigs receiving no AA and injected with ALA, compared to AA-supplemented guinea pigs. Animals in experiment A were observed to day 21, upon which they were killed for biochemical evaluation. At the end of 25 days in experiment B, gross clinical symptoms of AA deficiency were becoming apparent in both groups of guinea pigs receiving no AA. Signs included diarrhea, marked hind limb weakness and

Table 1. Effect of AA on urinary excretion of porphyrins and porphyrin precursors*

Treatment	Excretion			
	δ-Aminolevulinate (μg/24 hr)	Porphobilinogen (μg/24 hr)	Uroporphyrin (μg/24 hr)	Coproporphyrin (μg/24 hr)
AA-deficient	28.1 ± 2.4†	11.9 ± 2.5	0.93 ± 0.16	1.14 ± 0.31‡
AA-supplemented	40.1 ±2.2	7.1 ± 1.9	1.13 ± 0.10	3.67 ± 0.82

* Values are expressed as means ± S.E.M.; N = 6.
† $P < 0.001$ vs AA-supplemented group.
‡ $P < 0.02$ vs AA-supplemented group.

Table 2. Effect of AA and ALA on the levels of cytochrome P-450 and cytochrome b_5 in liver microsomes*

Expt.	Treatment	Cytochrome P-450 (nmoles/mg protein)	N	b_5 (nmoles/mg protein)
A	−AA	0.747 ± 0.001	4	
	+AA	1.680 ±0.076†	7	
	−AA, +ALA (48 hr)	0.749 ± 0.226‡	4	
B	−AA	0.870 ± 0.090	18	0.367 ± 0.063
	+AA	1.700 ± 0.110†	25	0.530 ±0.040
	−AA, +ALA (10 days)	0.665 ± 0.141‡	8	0.432 ± 0.063

* Values are expressed as means ± S.E.M. In experiment A, ALA-treated animals were injected with 5 mg/kg once every 12 hr before killing. In experiment B, they were injected once every 24 hr for 10 days before killing.
† $P < 0.05$ vs −AA group.
‡ $P < 0.05$ vs +AA group.

Table 3. Effect of AA and ALA on drug metabolism*

Expt.	Treatment	Aminopyrine demethylase (nmoles/mg protein)	N	Aniline hydroxylase (nmoles/mg protein)
A	−AA	1.83 ± 0.095	4	0.150 ± 0.042
	+AA	2.34 ± 0.093†	7	0.525 ± 0.044†
	−AA, +ALA (48 hr)	1.93 ± 0.097‡	4	0.176 ± 0.039‡
B	−AA	3.15 ± 0.092	18	0.228 ± 0.030
	+AA	4.60 ± 0.480†	25	0.710 ± 0.093†
	−AA, +ALA (10 days)	2.90 ± 0.470‡	8	0.265 ±0.041‡

* Values are expressed as means ± S.E.M. In experiment A, ALA-treated animals were injected with 5 mg/kg once every 12 hr before killing. In experiment B, they were injected once every 24 hr for 10 days before killing.
† $P < 0.05$ vs −AA group.
‡ $P < 0.05$ vs +AA group.

ataxia. Body weights ± S.E.M. were 381 ± 11.0, 306.5 ± 16.7 and 248.4 ± 13.8 g in guinea pigs receiving daily doses of AA, no AA, or no AA and injections of ALA, respectively. Upon analysis of liver AA ± S.E.M., it was found that animals receiving no AA and animals receiving no AA and injections of ALA were 8.3 ± 1.5 and 7.6 ± 1.5 per cent, respectively, in experiment A, and 15.9 ± 3.5 and 8.9 ± 1.4 per cent, respectively, in experiment B, of liver AA found in AA-supplemented guinea pigs [7].

Urinary porphyrins and porphyrins precursors are often increased by enzyme defects in the heme-synthetic pathway. The urinary excretion of these substances is shown in Table 1. The 24-hr δ-aminolevulinate excretion by AA-supplemented guinea pigs was significantly increased by 42.7 per cent compared to AA-deficient animals. No significant effect of AA status on excretion of porphobilinogen or uroporphyrin was found. AA-supplemented animals excreted more coproporphyrins than did AA-deficient guinea pigs. Although there was a tendency for AA-deficient guinea pigs to excrete more porphobilinogen than AA-supplemented animals, owing to large variations, the results were not significant.

The data presented in Table 2 confirm previous observations of decreases in hepatic microsomal cytochrome P-450 in ascorbic acid deficiency [3–7],

but show that no statistically significant change in levels of cytochrome b_5 was seen. In additon, we failed to confirm that the parenteral administration of ALA every 12 hr for 48 hr would increase the level of cytochrome P-450 of AA-deficient animals. Since it is conceivable that the influence of parenteral administered ALA may be a function of the turnover of the cytochrome heme, we also injected animals once every 24 hr for 10 days to ensure adequate time for synthesis. We found that ALA treatment of AA-deficient animals resulted in a further 23.6 per cent in cytochrome P-450 compared to just AA- deficient animals.

Results tabulated in Table 3 confirm previous observations that hepatic aminopyrine demethylase and aniline hydroxylase activities are a function of AA status. Like cytochrome P-450, the injection of ALA did not significantly increase drug metabolism in AA-deficient animals. Also, there was a significant decrease in aminopyrine demethylase activity after injecting AA-deficient animals once a day for 10 days, compared to AA-deficient guinea pigs.

Conflicting reports can be found on whether AA deficiency results in anemia. It has also been questioned whether, when anemia is found, it is an indirect result of the failure of the scorbutogenic diet to provide other nutrients such as vitamin B_{12} or folic acid. Table 4 documents the results of AA deficiency and of ALA treatment on blood heme parameters. Anemia was clearly found in both treatments. In experiment B, the addition of 10-day daily treatments of the AA-deficient animals with ALA enhanced their anemia. Of particular interest is that in neither experiment were the MCV values changed. Changes in MCV values would be indicative of microcytic or macrocytic anemia, the latter characteristic of megloblastic forms found in B_{12} and/or folic deficiency [28, 29]. This clearly indicates that the normocytic anemia is not the result of the failure of the scorbutogenic diet to provide vitamin B_{12} or folic acid.

The incorporation of [^{3}H]ALA into the CO-binding particles is shown in Fig. 1. Animals were killed at various times, and the radioactivity incorporated into the CO-binding particles was measured. Studies have been showing that radioactivity appears in the particles within 1 min after injection of [^{3}H]ALA and reaches a maximum within 30–60 min [30]. It appears that for the first 30 min there is less incorporation of the label into CO-binding particles isolated from AA-deficient guinea pigs. The difference is statistically significant ($P < 0.05$) at 20 min post-injection.

As an attempt to determine whether or not other hemoproteins would be affected by AA status, catalase activity was determined in whole blood. Table 5 reveals that no statistical statements can be made.

Table 4. Effect of AA and ALA on blood heme*

Expt.	Treatment	RBC (10^6 cell/mm^3)	Hgb (g/dl)	Hct (%)	MVC (μ^3)	MCHC (%)	MCH (pg)	N
A	−AA	3.57 ± 0.45	9.40 ± 1.28	28 ± 3.2	77.8 ± 0.77	33.8 ± 0.12	26.3 ± 0.73	3
	+AA	4.81 ± 0.11†	12.74 ± 0.23†	38 ± 0.8†	77.6 ± 1.63	33.2 ± 0.02	26.7 ± 0.61	5
	−AA, +ALA (48 hr)	4.15 ± 0.12†	10.67 ± 0.55‡	32 ± 1.5‡	75.8 ± 1.33	33.9 ± 0.02	25.7 ± 0.60	3
B	−AA	4.15 ± 0.26	11.2 ± 0.76	32 ± 2.1	77.2 ± 0.61	35.0 ± 0.34	27.0 ± 0.8	18
	+AA	4.78 ± 0.22†	13.7 ± 0.33†	39 ± 0.9†	77.9 ± 0.34	35.0 ± 0.24	27.2 ± 0.18†	25
	−AA, +ALA (10 days)	3.11 ± 0.33†,‡	8.1 ± 0.97†,‡	24 ± 2.5†,‡	76.3 ± 0.93	33.1 ± 0.95†,‡	25.3 ± 0.81†	8

* Values are expressed as means ± S.E.M. In experiment A, ALA-treated animals were injected with 5 mg/kg once every 12 hr for 48 hr before killing. In experiment B, they were injected once every 24 hr for 10 days before killing.
† $P < 0.05$ vs −AA group.
‡ $P < 0.05$ vs +AA group.

DISCUSSION

Ascorbic acid has been known to play a significant role in the metabolism of xenobiotics. Initial observations demonstrated that pentobarbital sleeping times were prolonged and that plasma half-lives of a number of drugs were increased in scorbutic guinea

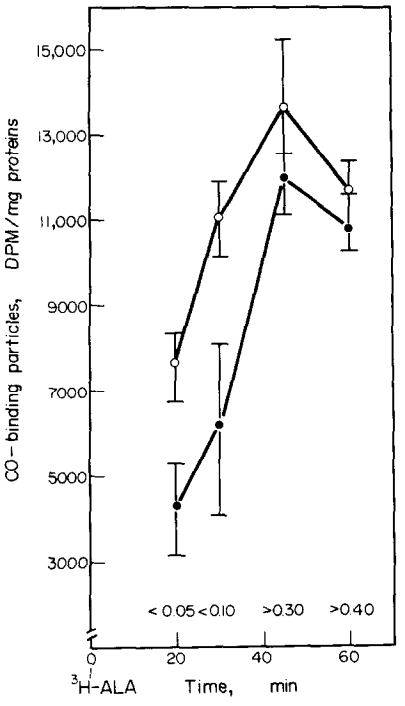

Fig. 1. Incorporation of [^{3}H]ALA into the CO-binding particles isolated from ascorbic acid-deficient (—●—) and ascorbic acid-supplemented (—○—) guinea pig liver microsomes. Mean ±S.E.M.; N = four to seven animals per point.

pigs [31, 32]. This extended drug effect in animals depleted of AA was correlated subsequently with a decreased rate of hepatic microsomal drug-toxicant metabolizing enzymes [33]. Several reports [5, 34, 35] suggest that AA deficiency may decrease overall hepatic mixed function oxidase activity by its effect on microsomal cytochrome P-450 content. There is some evidence to suggest that AA may be essential for normal synthesis of the heme component of cytochrome P-450 [10]. It was found that cytochrome P-450 levels returned to normal when scorbutic guinea pigs were injected with ALA, a key substrate for heme biosynthesis. Recent investigations [11–13] into the initial and rate-limiting steps in heme synthesis indicated no significant differences in ALA synthetase activity in AA-deficient liver homogenates or sonicated mitochondria. There were no substantial differences in ALA dehydratase or ferrochelatase activities. Consequently, AA deficiency does not appear to affect the activities of the key enzymes involved in heme synthesis. In addition, we failed to find any accumulation of porphyrins or porphyrin precursors in the urinary excretions of AA-depleted guinea pigs (Table 1). Further supporting evidence for a lack of AA effect on heme synthesis was found in this study when previous cited results could not be duplicated [10]. We did not find that either the administration of 5 mg ALA every 12 hr for 48 hr or the administration of 5 mg ALA once a day for 10 days would return cytochrome P-450 or drug metabolism to normal levels or activities. Administration of ALA once a day for 10 days actually lowered cytochrome P-450 and drug metabolism below that found in AA-depleted animals. This finding is of interest since similar treatment in normal rats results in no change of cytochromes; however, it does increase the "free" microsomal heme pool [36]. The increased "free" heme pool in AA-deficient guinea pigs might result in feedback inhibition of ALA synthetase with subsequent further decreases in heme synthesis [37].

Table 5. Effect of AA and ALA on blood catalase activity

Treatment	N	Catalase activity (units/g Hgb)
−AA	18	0.452 ± 0.068
+AA	25	0.320 ± 0.030
−AA, +ALA (10 days)	8	0.506 ± 0.093

* Values are expressed as means ± S.E.M.

It is possible that if "free" microsomal heme pools exist in livers of AA-deficient animals with depressed cytochrome P-450, then the assembly of holoenzyme from apoprotein and heme might be defective. This appears to be supported by our findings where the incorporation of injected [^{3}H]ALA into AA-deficient animals is less than that found in AA-supplemented guinea pigs. Alternatively, AA depletion might cause an increase in the degradation of heme or of the apocytochrome. Previous studies in this laboratory did not reveal evidence of increased degradation via microsomal heme oxygenase [7] or any general problem in the turnover of hepatic cytochrome CO-binding particles [14]. Others have not found an increase in lipid peroxidation [4]. Multiple forms of cytochrome P-450 exist in guinea pig microsomes. Recent studies by Rikans *et al.* [13] have provided separation of 44,000–60,000 dalton polypeptides by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, which revealed quantitative differences between the polypeptides obtained from AA-deficient animals and those obtained from controls. Three polypeptide bands (mol. wt 44,000, 52,000 and 57,000) were reduced and two polypeptide bands (mol. wt 54,000 and 55,000) were increased in AA-deficient microsomes compared to normal microsomes, thereby suggesting that AA deficiency may cause some error in P-450 apocytochrome formation. Degradation of apocytochrome might be mediated by lysosomal enzymal enzymes. There is one report [38] demonstrating elevated hepatic lysosomal hydrolases in AA-depleted animals. Based on these reports, the synthesis and degradation of P-450 apocytochrome should be investigated further.

The present study also points out that AA depletion results in depressed blood heme parameters. This anemia is apparently not of the macrocytic type which is associated with vitamin B_{12} or folic acid deficiency. We did not, however, find any influence of AA status on blood catalase activity which may be indicative of the turnover of that particular hemoprotein.

In conclusion, the results in this study are in contrast to previous reports showing that exogenously administered ALA to AA-deficient guinea pigs increases hepatic microsome drug metabolism and cytochrome P-450 levels [10]. Our evidence is con-

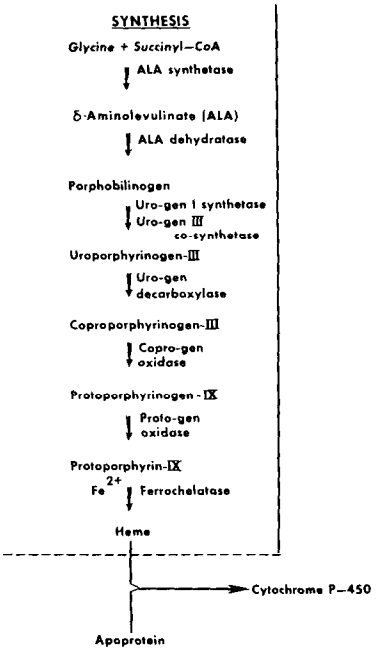

Fig. 2. Synthesis pathway for hepatic cytochrome P-450 heme and blood heme.

sistent with the recent findings that failed to find any error in the key enzymes responsible for heme synthesis [11, 12]. The results demonstrate that AA deficiency in guinea pigs does not interfere with the general synthesis of heme (Fig. 2). Previously [39], we found that heme degradation was not increased in AA-deficient animals, which, by elimination, suggests that consideration should be given to (1) apocytochrome synthesis, (2) assembly of apocytochrome and heme to form active holocytochrome, and (3) perhaps based on some of our recent investigations [40], incorporation of Fe^{2+} in the heme moiety of cytochrome P-450.

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