

Hereditary Tyrosinemia and the Heme Biosynthetic Pathway

PROFOUND INHIBITION OF δ -AMINOLEVULINIC ACID DEHYDRATASE ACTIVITY BY SUCCINYLACETONE

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ABSTRACT Succinylacetone (4,6-dioxoheptanoic acid) is an abnormal metabolite produced in patients with hereditary tyrosinemia as a consequence of an inherited deficiency of fumarylacetoacetate hydrolase. It is known that patients with this hereditary disease excrete excessive amounts of δ -aminolevulinic acid (ALA) in urine and that certain patients have an accompanying clinical syndrome resembling that of acute intermittent porphyria (AIP). In order to elucidate the relation of succinylacetone to the heme biosynthetic pathway, we have examined the effects of this metabolite on the cellular heme content of cultured avian hepatocytes and on the activity of purified ALA dehydratase from normal human erythrocytes and from mouse and bovine liver. Our data indicate that succinylacetone is an extremely potent competitive inhibitor of ALA dehydratase in human as well as in animal tissues. By using purified preparations of the enzyme from human erythrocytes and mouse and bovine liver, an inhibitor constant ranging from 2×10^{-7} M to 3×10^{-7} M was obtained. In cultured hepatocytes, succinylacetone also inhibited ALA dehydratase activity, decreased the cellular content of heme and cytochrome P-450, and greatly potentiated the induction response of ALA synthase to drugs such as phenobarbital, chemicals such as allylisopropylacetamide and 3,5-dicarbethoxy-1,4-dihydrocollidine, and natural steroids such as etiocholanolone. Four patients with hereditary tyrosinemia have been studied and all were found to have greatly depressed levels of erythrocyte ALA dehydratase activity and elevated concentrations of this inhibitor in urine. These findings indicate that tyrosinemia is a disorder of special pharmacogenetic interest because succinylacetone, an abnormal product of the tyrosine metabolic pathway,

resulting from the primary gene defect of the disease, profoundly inhibits heme biosynthesis in normal cells through a blockade at the ALA dehydratase level, leading to clinical and metabolic consequences that mimic another genetic disease, AIP.

INTRODUCTION

Hereditary tyrosinemia is an inborn error of tyrosine metabolism transmitted in an autosomal recessive fashion (1). Patients with this disease excrete excessive amounts of urinary δ -aminolevulinic acid (ALA)¹ (2-5) and have low ALA dehydratase [EC 4.2.1.24] activity in erythrocytes (6, 7) and in liver (7). Acute neurological symptoms resembling those of acute intermittent porphyria (AIP) have also been reported in this disorder (2, 3, 5, 6). Low ALA dehydratase activity is thought to result from enzyme inhibition by accumulation of succinylacetone (4,6-dioxoheptanoic acid) in plasma resulting from the deficiency of 4-fumarylacetoacetate hydrolase [EC 3.7.1.2] in this disease (7).

We have studied the effects of urine from four patients with hereditary tyrosinemia, and of succinylacetone itself, on the activity of ALA dehydratase purified from human erythrocytes, and mouse and bovine liver. In addition we have examined the effects of succinylacetone on the levels of cytochrome P-450 and cellular heme and on the synthesis of ALA synthase, the rate-limiting enzyme for heme formation, in cultured avian embryonic liver cells. Our data indicate that succinylacetone is an extremely potent inhibitor of ALA dehydratase and heme formation in human as well as in animal tissues. The effects of succinylacetone on the

¹ *Abbreviations used in this paper:* AIA, 2-allyl-2-isopropylacetamide; AIP, acute intermittent porphyria; ALA δ -aminolevulinic acid; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine; PBG, porphobilinogen; PIA, propylisopropylacetamide; RBC, erythrocytes.

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heme biosynthetic sequence produce a mimicry of the biochemical circumstances characterizing acute hepatic porphyrias in that hepatic ALA synthase becomes highly susceptible to induction by porphyrogenic chemicals.

METHODS

Succinylacetone (4,6-dioxoheptanoic acid) was purchased from Calbiochem-Behring Corp., American Hoechst Corp. San Diego, CA. ALA, etiocholanolone (5 β -androstan-3 α -ol-17-one), and sodium phenobarbital were products of Sigma Chemical Co. (St. Louis, MO). 3,5-Dicarbethoxy-1,4-dihydrocollidine (DDC) and reagents for polyacrylamide gel electrophoresis were obtained from Eastman Organic Chemicals (Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY). Standard proteins used for molecular weight determinations were those included in the molecular weight calibration kit of Pharmacia Fine Chemicals, Uppsala, Sweden. 2-Allyl-2-isopropylacetamide (AIA) was a gift from Hoffman-La Roche, Inc. (Nutley, NJ). All reagents used were of analytical grade quality.

Serum-free culture of chick embryo liver cells. Cell suspensions of the chick embryo liver were prepared from 17-d-old embryos in a serum-free modified F12 medium supplemented with bovine pancreas insulin (1 μ g/ml), cortisol (0.05 μ g/ml), and triiodothyronine (1 μ g/ml) as described previously (8). Contaminating erythrocytes and hemoglobin were nearly completely eliminated by hemolysis with ammonium chloride in potassium bicarbonate solution followed by washing (8). For the assay of ALA synthase activity, 5 ml of 200-fold diluted cell suspension was added to tissue culture dishes (60 \times 15 mm) (Costar 3060; Costar, Data Packaging, Cambridge, MA) and, for the assay of heme oxygenase and cytochrome P-450, 10 ml of 75-fold diluted cell suspension was added per dish (100 \times 20 mm) (Costar 3003) and incubated in a humidified CO₂ incubator at 37°C with 5% CO₂ and 95% air. After 24 h of incubation, the medium was replaced by fresh medium and the addition of test chemicals was made at this time. Cultures were incubated for 24 h after the addition of chemicals. Assays of ALA synthase (8), ALA dehydratase (9), heme (8), protein content (8), heme oxygenase (10), and cytochrome P-450 (11) in cultured liver cells were carried out as described previously.

Hereditary tyrosinemia. Four patients with hereditary tyrosinemia were studied. The diagnosis was made by re-

ferring physicians (Dr. H. Levy of The Children's Hospital Medical Center, Harvard Medical School [Boston] and Dr. E. Stoner, Dr. L. S. Levine, and Dr. M. I. New of The New York Hospital-Cornell Medical Center [New York]) and was based on hepatosplenomegaly, anemia, elevated serum tyrosine and methionine, and aminoaciduria, all of which are characteristic of this inherited disorder.

Purification of ALA dehydratase. (a) ALA dehydratase was partially purified from bovine liver by using heat treatment followed by ammonium sulfate fractionation (35–50% ammonium sulfate fractions). The partially purified bovine liver enzyme was dissolved in 20 mM Tris-Cl (pH 7.4), 5 mM 2-mercaptoethanol, and 1 mM ZnCl₂-15% glycerol and stored in liquid nitrogen. The enzyme preparation had 0.5 U/mg protein sp act and was stable for at least 6 mo. ALA dehydratase assays were carried out according to our method described previously (9). One unit of the enzyme activity was defined as 1 μ mol porphobilinogen (PBG) produced/h at 37°C.

(b) ALA dehydratase was also purified to homogeneity from 2.3 liters of outdated human erythrocytes and 130 g of liver from mice (BALB/c strain) according to the method of Anderson and Desnick (12) with some modifications. Our purification procedure included anion exchange chromatography on DEAE-cellulose, ammonium sulfate fractionation, hydrophobic interaction chromatography with phenyl Sepharose, and gel filtration with Sephacryl S-300 instead of Bio-Gel A-1.5 m (12). The results of the enzyme purification from human erythrocytes are summarized in Table I. The purification procedure for the mouse liver enzyme was similar except that the eluate from DEAE-cellulose chromatography was treated at 68°C for 10 min. All other purification procedures were carried out at 4°C.

Analytical sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Samples of denatured ALA dehydratase were electrophoresed according to the method of Weber and Osborn (13) with minor modifications: 10% (wt/vol) polyacrylamide gel contained 25% (vol/vol) ethylene glycol, and 0.4 M Tris-HCl (pH 9.2). A 2-cm stacking gel (5.6%) containing 0.06 M Tris-HCl (pH 6.8) and 0.1% (vol/vol) SDS was used on top of the 10-cm separating gel. The electrode buffer (pH 8.3) contained 0.005 M Tris-HCl, 0.4 M glycine, and 0.1% SDS. Proteins were stained by Coomassie Brilliant Blue R-250 (2.5% in 50% methanol/7% acetic acid/water, vol/vol) for 1.5 h at 37°C and excess dye was removed by a solution containing 10% isopropanol/7% acetic acid/water, vol/vol.

Purity. ALA dehydratase purified from human eryth-

TABLE I
Purification of ALA Dehydratase from Human Erythrocytes

Step	Volume	Total activity	Specific activity	Yield	Purification
	ml	U	U/mg	%	fold
Erythrocyte lysates	2,300	711	0.0033	100	1
DEAE-cellulose	415	357	0.257	50	78
Ammonium sulfate	38	166	0.681	23	206
Phenyl-sepharose	62	147	7.81	21	2,370
Sephacryl S-300	8	132	22.3	19	6,760

The results represent data on a typical purification of human erythrocyte ALA dehydratase.

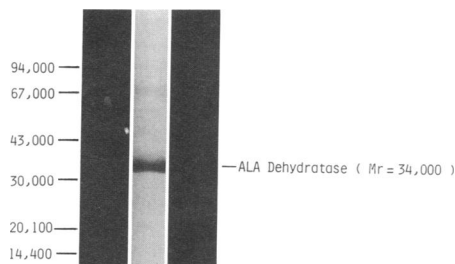


FIGURE 1 Analytical SDS polyacrylamide gel electrophoresis of human erythrocyte ALA dehydratase. Approximately 10 μ g of enzyme was applied to the gel and the electrophoresis was carried out at pH 8.3 as described in Methods. The ALA dehydratase had a $M_r = 34,000$. Molecular weight standards used were: (a) phosphorylase b ($M_r = 94,000$), (b) bovine serum albumin ($M_r = 67,000$), (c) ovalbumin ($M_r = 43,000$), (d) carbonic anhydrase ($M_r = 30,000$), (e) soybean trypsin inhibitor ($M_r = 20,100$), and (f) α -lactalbumin ($M_r = 14,400$)

rocytes appeared to be homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 1). The specific activity (22.3 U/mg protein) of the enzyme and the molecular weight of its subunit (34,000 D) were also in good agreement with purified enzyme preparations from other sources (14-17).

Preparation of rabbit immunoglobulin (Ig) G against human erythrocyte ALA dehydratase. Two New Zealand rabbits were injected intradermally and intramuscularly with ~ 1 mg of homogeneous human erythrocyte ALA dehydratase mixed with an equivalent volume of Freund's complete adjuvant. Booster injections of 0.5 mg and 0.5 mg were given on the 5th and the 7th wk, respectively. The titers of rabbit anti-human ALA dehydratase were determined by immunoprecipitation of the enzyme activity. The IgG fraction from rabbit sera was prepared according to the method of Masters et al. (18). The antibody (IgG) was precipitated from pooled sera (160 ml) by the addition of ammonium sulfate (a final concentration of 1.75 M). After stirring 30 min at 4°C, the mixture was centrifuged at 27,000 g for 10 min, and the precipitate was dissolved in 42 ml of 10 mM potassium phosphate buffer (pH 7.7). Then the solution was dialyzed against 1.2 liter of the same buffer twice for 6 h each time. The dialyzed solution was loaded onto a column of DEAE-cellulose ($\phi 6.2$ cm \times 40 cm) equilibrated with the same buffer solution. Elution of IgG was monitored by absorbance at 280 nm while washing the column with the same buffer. Fractions containing the major protein peak (a total volume of 400 ml) were collected as IgG and concentrated to 32 ml with an Amicon YM-10 filter membrane.

Rocket immunoelectrophoresis of human erythrocyte ALA dehydratase. Rocket immunoelectrophoresis (19) of human erythrocyte ALA dehydratase was performed according to the method described by Grieninger et al. (20). 300 μ l of the purified IgG fraction was mixed with 6 ml of 1% agarose gel solution and cast onto a rectangular sheet (70 \times 100 mm) of polyester film cut from a roll (Cronar unperforated 40E leader, 70 mm \times 1,000 ft, Dupont Instruments, Wilmington, DE). 3- μ l samples were added to each well (2.4 mm in diam) and electrophoresis was performed at room temperature at 10 V/cm for 90 min. After electrophoresis, gels were fixed in a solution of 0.5% tannic acid/1% acetic acid in water for visualization of the immunoprecipitates.

RESULTS

Competitive inhibition of ALA dehydratase activity by succinylacetone. Fig. 2 shows the effect of succinylacetone on the activity of homogenously purified ALA dehydratase from human erythrocytes. An increase in succinylacetone concentration at constant substrate concentration increased the degree of inhibition, and an increase in substrate concentration at a constant level of succinylacetone decreased the degree of inhibition. Thus it is clear that succinylacetone inhibits the activity of ALA dehydratase in a competitive manner. The inhibitor constant (K_i) of the enzyme for this inhibitor was in the range of $2-3 \times 10^{-7}$ M for all enzyme preparations examined (Table II). The Michaelis constants (K_m) for these enzyme preparations were found to be in the range of $1.5-2.2 \times 10^{-4}$ M (Table II). Purified ALA dehydratase preparations from other sources are also known to have a K_m in the range of 2×10^{-4} M and 5×10^{-4} M (14-17). These data indicate that succinylacetone has an $\sim 1,000$ -fold greater affinity for the catalytic site on the enzyme than the natural substrate.

Effect of succinylacetone on hepatic heme metabolism in cultured chick embryo liver cells. Because succinylacetone was found to be a potent inhibitor of ALA dehydratase, we examined its effects on hepatic heme metabolism in isolated cultured hepatocytes from chick embryos. Treatment of liver cultures with succinylacetone up to a concentration of 3×10^{-2} M did not cause significant morphological changes. Succinylacetone added in vitro to the homogenate of cultured chick embryo liver cells showed a dose-dependent inhibition of ALA dehydratase activity (Fig. 3).

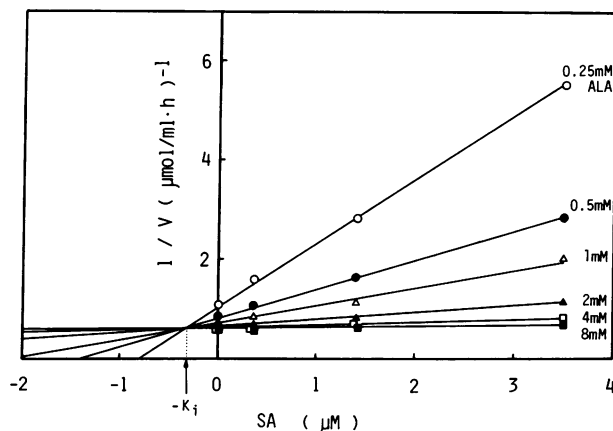


FIGURE 2 Dixon plot of purified human erythrocyte ALA dehydratase activity as a function of succinylacetone concentration. Enzyme assays were carried out as described previously (9). The K_i for succinylacetone was determined to be 3.0×10^{-7} M at the intersection point as shown.

TABLE II
K_i for Succinylacetone and the *K_m* of ALA Dehydratase in
 Various Tissue Preparations

Source of enzyme	<i>K_m</i> ×10 ⁻⁴ M	<i>K_i</i> ×10 ⁻⁷ M
Human erythrocyte ALA dehydratase (homogeneously purified)	2.1	3.0
Mouse liver ALA dehydratase (homogeneously purified)	2.0	2.5
Bovine liver ALA dehydratase (partially purified)	1.5	2.0
Chick embryo liver homogenates	2.2	2.0

Enzyme assays were carried out using 4–8 mU enzyme per assay in a final volume of 50 μ l at pH 6.2 as described earlier (9). The enzyme preparations used were homogeneously purified ALA dehydratases from human erythrocytes (22.3 U/mg protein) and mouse liver (23.2 U/mg protein) and partially purified ALA dehydratase from bovine liver (0.5 U/mg protein). Chick embryo liver homogenates were used for the enzyme assays without purification.

Cell cultures incubated with succinylacetone for 24 h exhibited a curve of inhibition of ALA dehydratase activity superimposable on that of succinylacetone added in vitro to the homogenates (Fig. 3). These data thus indicate that intracellular concentrations of succinylacetone in cultured liver cells equilibrate with

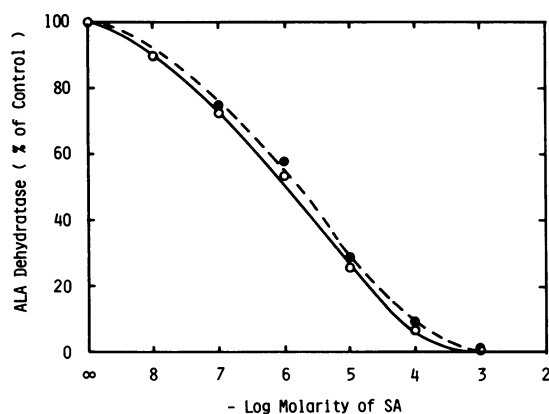


FIGURE 3 Effects of succinylacetone (SA) on ALA dehydratase activity in cultured chick embryo liver cells. Liver cells were prepared and incubations were carried out as described in Methods. Succinylacetone was added at the time of change of medium 24 h after incubation and cells were further incubated with succinylacetone for 24 h (○—○), or the chemical was added to homogenates of liver cells obtained after 48 h incubation in culture (●---●). Control enzyme activity in cultured liver cells was 3.9 nmol/mg protein · h and in homogenates was 3.7 nmol/mg protein · h. Data represent the mean of duplicate determinations.

those added to the culture medium, and they imply that cell cultures incubated with succinylacetone for 24 h display no toxicity from this compound because the activity of ALA dehydratase is dependent on the integrity of the cells that is essential for the regeneration of reduced glutathione, an essential cofactor for this enzyme; the loss of this capacity would be a sensitive index of cellular damage (21). In contrast to the highly inhibitory effect of succinylacetone on ALA dehydratase activity, the treated liver cells in culture did not show appreciable changes in heme content until the succinylacetone concentration reached a level of $\sim 10^{-3}$ M. Cytochrome P-450 content was decreased >50% at a 10^{-3} M succinylacetone concentration (Fig. 4). Total heme content was also reduced from 155 ± 8 pmol/mg protein for control cultures to 112 ± 3 pmol/mg protein for cultures treated with 10^{-3} M succinylacetone (data not shown).

In contrast with the decline in cytochrome P-450 content produced by succinylacetone, the activity of microsomal heme oxygenase was not affected by treatment with the chemical at comparable concentrations (Fig. 4). These findings suggest that the succinylacetone-mediated decreases in heme and cytochrome P-450 levels are due to inhibition of heme biosynthesis and that such changes are not due to an accelerated metabolism of heme by microsomal heme oxygenase.

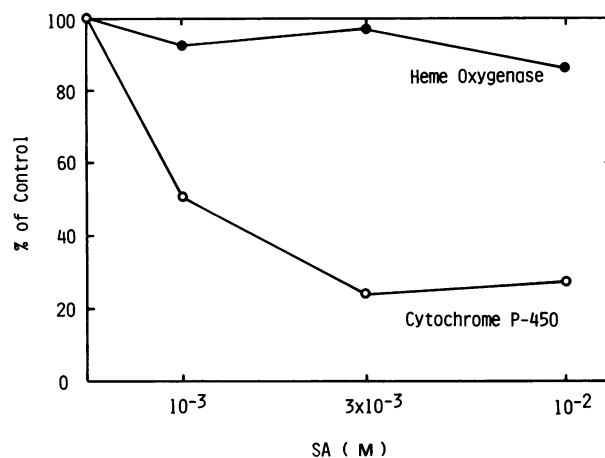


FIGURE 4 Effects of succinylacetone (SA) on cytochrome P-450 content and heme oxygenase activity in cultured chick embryo liver cells. Liver cells were prepared and incubations were carried out as described in Methods. Succinylacetone was added at the time of change of medium and cells were incubated for 24 h. Determinations of cytochrome P-450 content (11) and heme oxygenase activity (10) were carried out as described previously by using ~ 4 mg cellular protein from a 10-cm petri dish. Cytochrome P-450 content in control cultures was 14.5 ± 2.0 (mean \pm SE) pmol/mg protein and heme oxygenase activity in control cultures was 1.4 ± 0.1 (mean \pm SE) pmol bilirubin formed/mg protein · h, for triplicate determinations.

Potentialization of chemical induction of ALA synthase by succinylacetone. The question of whether the decreases of heme and cytochrome P-450 content produced by succinylacetone in the cultured liver cells are due to specific inhibition of ALA dehydratase, or due to toxic effects of the compounds on these cells can also be critically examined by determining the cellular induction responses of ALA synthase, the rate-limiting enzyme of heme synthesis, to chemicals, as the induction of this enzyme requires intact RNA and protein synthetic mechanisms (22). Succinylacetone alone, up to a concentration of 10^{-2} M, had no appreciable effect on the basal level of ALA synthase in the cultured liver cells (Table III). In contrast, the addition of succinylacetone (10^{-3} M) considerably potentiated the induction of ALA synthase produced by low concentrations of porphyrigenic agents such as AIA, DDC, phenobarbital, and etiocholanolone (Table III). The magnitude of potentiation of ALA synthase induction by succinylacetone ranged from ~3- to 16-fold, depending on the chemical nature of the inducer.

These findings support the idea that the regulatory free heme pool for ALA synthase in liver can be depleted by succinylacetone through the inhibition of ALA dehydratase, but that heme depletion alone cannot derepress ALA synthase formation sufficiently to evoke a significant rebound induction of the enzyme as has been proposed (23). However, the regulatory free heme pool for the enzyme is clearly diminished by succinylacetone because this chemical treatment leads to a marked increase in the susceptibility of hepatic ALA synthase to induction by porphyrigenic

TABLE III
Potentiating Action of Succinylacetone on the Induction of ALA Synthase by Various Chemicals

Treatment	ALA Synthase unit/mg protein · h
None	≤1.0
Succinylacetone, 10^{-4} – 10^{-2} M	≤1.0
AIA, 2 μg/ml	11.3±1.0
AIA, 2 μg/ml + succinylacetone 10^{-3} M	29.5±1.3
DDC, 0.5 μg/ml	≤1.0
DDC, 0.5 μg/ml + succinylacetone 10^{-3} M	15.5±2.5
Phenobarbital, 25 μg/ml	1.4±0.7
Phenobarbital, 25 μg/ml + succinylacetone, 10^{-3} M	7.9±1.2
Etiocholanolone, 0.5 μg/ml	1.8±0.1
Etiocholanolone, 0.5 μg/ml + succinylacetone, 10^{-3} M	4.7±0.8

The assays of ALA synthase were carried out as described previously (8). Data represent the mean±SE for triplicate determinations.

chemicals. These data also suggest that the combined treatment of liver cells with succinylacetone and a chemical inducer of ALA synthase might decrease the lower limit of detection of an induction response to chemicals suspected of having porphyrigenic properties. Data relating to such an experiment are shown in Fig. 5. In this experiment, treatment with AIA alone induced ALA synthase activity only at concentrations >0.5 μg/ml. In contrast, treatment with a combination of AIA and succinylacetone greatly increased the induction response of the enzyme to AIA and the lowest detection level of ALA synthase induction became 0.05 μg AIA/ml; i.e., a 10-fold increase in sensitivity (Fig. 5). These findings confirm that a partial block of heme synthesis alone does not in itself lead to a major compensatory increase in ALA synthase production but that such a block does sensitize the regulatory system for ALA synthase production to the effects of inducers of the enzyme.

ALA dehydratase activity in patients with hereditary tyrosinemia. The activity of erythrocyte ALA dehydratase in four patients with hereditary tyrosinemia is summarized in Table IV. The activity of the enzyme ranged from 0.4 to 38% of control incubations with normal erythrocytes (RBC). In case 4, the enzyme activity was below the detection level of 10 nmol PBG/ml RBC · h.

Urine from patients with hereditary tyrosinemia was

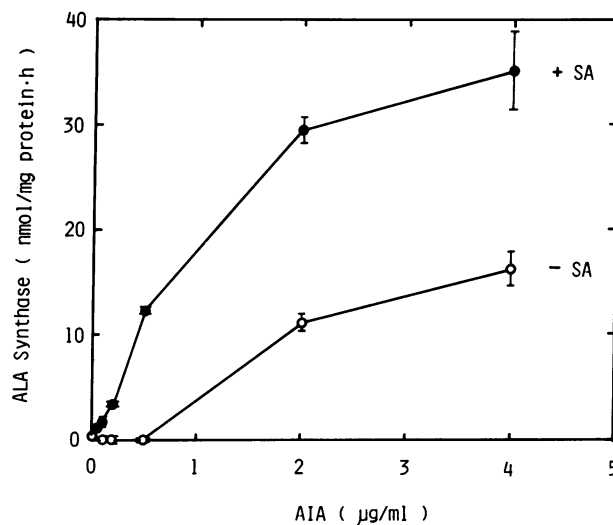


FIGURE 5 Potentiation of AIA-induction of ALA synthase by succinylacetone (SA) in cultured chick embryo liver cells. Conditions of cell cultures were the same as in Fig. 4. Addition of chemicals was made at the time of change of medium after 24 h incubation and cells were then incubated for an additional 24 h. ALA synthase assays were carried out as described previously (8). Data represent the mean±SE for triplicate determinations.

TABLE IV
Erythrocyte ALA Dehydratase Activity and Urinary Inhibitor
Concentration in Hereditary Tyrosinemia

Patient	Age	Sex	Erythrocyte	ALA dehydratase*	Inhibition of ALA dehydratase†
			nmol/ml RBC·h	% of control incubations	% of control incubations
1	5 mo	♂	126	5	24
2	4½ yr	♀	412	16	21
3	20 yr	♀	960	38	72
4	14 mo	♀	<10	0.4	24

* ALA dehydratase assays were performed as described by Sassa et al. (9) in the presence of 20 mM dithiothreitol to maximally activate the enzyme activity.

† To each incubation was added 2 µl urine and 5 µl of normal erythrocyte lysates as the source of ALA dehydratase in a final volume of 50 µl reaction mixture. The mixture was incubated for 60 min at 37°C, and PBG was quantitated as described earlier (9).

added to an assay for ALA dehydratase containing normal erythrocyte lysates as the source of the enzyme in order to approximately quantitate the level of the urinary inhibitor of the enzyme activity. 2 µl of urine from each patient added to the assay mixture, having a final volume of 50 µl, caused inhibition of the enzyme by 28–79% (Table IV). Urine from both parents of case 4, as well as urine from one patient with tyrosinosis and from four normal controls did not inhibit the activity of ALA dehydratase (data not shown). In one set of assays, urine from case 4 was incubated either with normal erythrocyte lysates or with the purified preparation of human erythrocyte ALA dehydratase, and it was found that inhibition of the enzyme activity by urine was similar for both enzyme sources (data not shown). ALA dehydratase activities in erythrocytes and in a biopsied liver specimen from this patient were below the detection limit of the assay, i.e., <2% of normal controls at the time of hospital admission. The patient excreted elevated levels of ALA in urine but not PBG or other porphyrins. The enzymatic activity increased substantially in erythrocytes (not detectable → 601 nmol PBG/ml RBC·h, 37°C) and in liver (not detectable → 108 nmol PBG/g liver·h, 37°C) after treatment with diet formula having a low tyrosine and phenylalanine content.²

Laurel's electroimmunoassay (19) was used for identification and quantitation of erythrocyte ALA dehydratase protein in the cell lysates of this patient. In the assay, the length of the rockets (immunoprecipitates) that form are proportional to the amount of antigen. The rockets from a normal lysate and that from case 4 produced equivalent amounts of precipitable ALA dehydratase protein (data not shown) despite the fact

that the activity of ALA dehydratase in the tyrosinemia patient was <0.4% of normal.

DISCUSSION

The results of this study demonstrate that succinylacetone, an abnormal metabolic derivative produced in large amounts as a consequence of the enzyme defect in hereditary tyrosinemia, is a very potent inhibitor of ALA dehydratase activity in human erythrocytes and in several animal tissues, and significantly decreases heme concentrations in avian embryonic liver cells grown in culture. The inhibition of enzyme activity produced by this compound is competitive in nature and was observed with a purified ALA dehydratase from human erythrocytes (Fig. 2) and mouse liver (Table II); a partially purified ALA dehydratase from bovine liver (Table II); and with the enzyme in chick embryo liver homogenates (Table II), human hemolysates, cultured human fibroblasts (unpublished results), and cultured chick embryo liver cells (Fig. 3). By using purified ALA dehydratase from human, mouse, and bovine sources and the enzyme in chick embryo liver homogenates, the K_i for succinylacetone was found to be in the range of $2-3 \times 10^{-7}$ M. Because the K_m of the enzyme for the substrate, ALA, is $1.5-2.2 \times 10^{-4}$ M (Table II), the inhibitor has an ~1,000 times greater affinity for the enzyme than its natural substrate. The competitive nature of the inhibition of ALA dehydratase by succinylacetone is presumably due to its structural similarity to the substrate, ALA (Fig. 6). Because the enzyme binds two molecules of ALA in a sequential manner to form PBG (24), succinylacetone could interfere with ALA binding at either one or both binding sites (Fig. 6). It has been reported that succinylacetone forms a pyrrole with ALA by a Knorr-type condensation (25), which may

² H. Starkman et al. Submitted for publication.

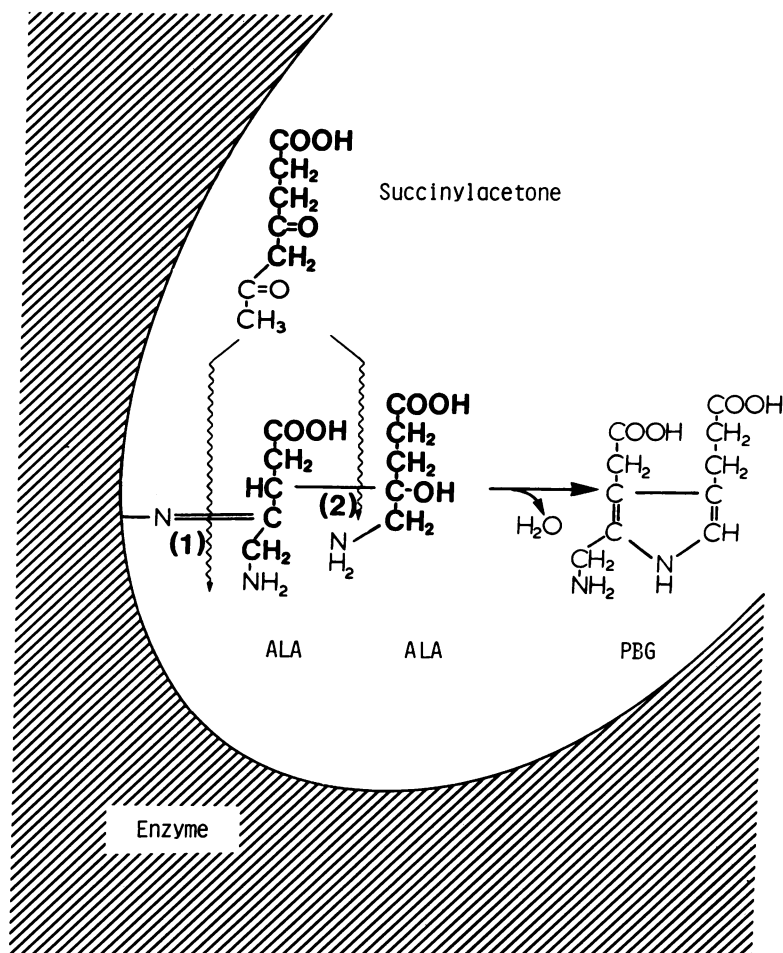


FIGURE 6 Possible mechanism of the inhibition of ALA dehydratase by succinylacetone. In the ALA dehydratase reaction, the first molecule of ALA is bound covalently to the enzyme through the formation of a Schiff base between the ϵ -amino group of a lysine residue and the keto group of ALA (step 1). This stabilized carbanion then participates in an aldol condensation with a second ALA molecule (step 2) which, upon the removal of one molecule of water, leads to the formation of PBG (24). Succinylacetone shares a common five-carbon structure with ALA (shown in bold letters), and thus could compete with ALA binding at step 1, or step 2, or both.

be a process analogous to the binding of the second ALA molecule to the first one (Fig. 6) except that the succinylacetone-ALA pyrrole would inhibit the formation of PBG.

Lindblad et al. (7) first reported that the high urinary excretion of ALA in patients with hereditary tyrosinemia is due to inhibition of ALA dehydratase by succinylacetone and their studies indicated that the primary enzymatic defect in hereditary tyrosinemia might be a decreased activity of fumarylacetoacetate hydrolase, an enzyme deficiency that was later demonstrated in liver biopsy specimen of nine patients (26).

Ebert et al. (27) reported that treatment of murine

erythroleukemia cells with succinylacetone inhibits ALA dehydratase activity and lowers the heme concentration in the cells as well. Succinylacetone treatment also caused inhibition of growth of murine erythroleukemia cells presumably by inducing a heme deficiency (28). More recently, Tschudy et al. (29) reported that succinylacetone inhibits ALA dehydratase activity in rat liver and concluded that the enzyme inhibition is irreversible. Our data also clearly indicate that succinylacetone is a potent inhibitor of ALA dehydratase from human, bovine, and avian sources but indicate that its mode of inhibition is competitive in nature (Fig. 2).

Consistent with the profound inhibition of ALA de-

hydratase activity, succinylacetone significantly decreased the cellular heme content and the cytochrome P-450 concentration in cultured chick embryo liver cells. The regulatory free heme pool was apparently also decreased because the induction response of hepatic ALA synthase to porphyrinogenic chemicals was markedly augmented. Considerably higher concentrations of succinylacetone were required for eliciting these effects than the concentrations required to produce inhibition of ALA dehydratase *in vitro*. The apparent discrepancy between the concentration of succinylacetone required for the inhibition of ALA dehydratase *in vitro* and that which brings about a decline of cytochrome P-450 and cellular heme content in the cell culture could suggest, among other possibilities, that the compound does not readily enter the cultured liver cells or that hepatic ALA dehydratase activity must be maximally inhibited in order to interfere with the formation of heme in liver. The data shown in Fig. 3 indicate that succinylacetone does enter cultured liver cells readily and reaches an equilibrium intracellularly with its concentration in the medium. Thus, it can be inferred that heme synthesis in cultured liver cells is blocked only when ALA dehydratase activity is almost completely inhibited. Despite this conclusion, it should be noted that succinylacetone was not cytotoxic up to a concentration of 30 mM, whereas marked inhibition of heme synthesis in the liver could be demonstrated at concentrations around 1 mM.

Potential of the induction response of ALA synthase to drugs, steroids, and other chemicals (Table III, Fig. 5) suggests that the concentration of the regulatory free heme pool for the enzyme in liver cells is substantially decreased by succinylacetone treatment. Because the induction of ALA synthase in cultured chick embryo liver cells has been shown to be the result of *de novo* synthesis of the enzyme (30), the potentiation of ALA synthase induction by succinylacetone affirms that the transcriptional and translational events leading to the synthesis of the enzyme take place normally and that decreases in heme content or cytochrome P-450 levels in succinylacetone-treated cells are not due to toxicity of the compound for the cells.

Recently, Lim et al. (23) reported that treatment of chick embryos with AIA and propylisopropylacetamide (PIA) led to the induction of ALA synthase (~16-fold) and decreased cytochrome P-450 (by ~30–50%). On the basis of these findings, they proposed that all chemicals act to induce synthesis of ALA synthase by producing an initial depletion of cytochrome P-450 heme. In contrast, Krupa et al. (31) and Sinclair et al. (32) reported that the allyl-containing acetamide AIA, but not the nonallyl acetamide, PIA, decreased

cytochrome P-450 heme, whereas hepatic ALA synthase could be induced equally by both acetamides (32).

Our data also indicate that depletion of cellular heme alone, by succinylacetone treatment, is not associated with measurable increases of ALA synthase in the cultured liver cells. For example, cultures treated with 10^{-3} M succinylacetone showed an ~30% reduction in heme content and a 60% decrease in cytochrome P-450 content without accompanying measurable increases in ALA synthase activity. Tschudy et al. (29) reported slight increase of ALA synthase in the rat after injection of succinylacetone, but these were much smaller than those produced by treatment with porphyrinogenic chemicals such as AIA (33). Recently, Badawy (34) summarized the evidence from a number of laboratories for and against the hypothesis that cytochrome P-450 heme controls the synthesis of hepatic ALA synthase and concluded that the regulatory heme pool for the enzyme in the liver is not closely related to the heme moiety of this cytochrome.

Our data demonstrate that liver cell cultures treated with succinylacetone become extremely sensitive to the induction of ALA synthase by a variety of porphyrinogenic chemicals. By using AIA, we showed that the lowest detection level of induction response decreased by ~10-fold after treatment with succinylacetone and comparable sensitization of induction responses to other chemicals were also observed (Table III). These findings clearly indicate that ALA synthase activity is not increased substantially simply by decreasing the heme concentration in liver cells, but that a decrease in cellular heme concentration does on the other hand greatly augment the response of liver cells to the porphyrinogenic action of endogenous or exogenous chemicals.

In this regard, it should also be noted that the great majority (~90%) of individuals with inherited PBG deaminase deficiency do not develop the clinical syndrome of AIP. It has also been established that the levels of this enzyme activity in clinically manifest AIP patients and in clinically latent gene carriers are equally diminished (~50%) from normal (35), indicating that the PBG deaminase defect alone does not determine the induction of hepatic ALA synthase that occurs in clinically manifest AIP (35). However, the PBG deaminase defect may sensitize the response of the liver to the ALA synthase-inducing action of drugs, hormones, and other chemicals and may in this manner produce biochemical and clinical exacerbation of AIP in certain gene carriers of the disease. Sensitization of the induction response of ALA synthase to these porphyrinogenic chemicals as a result of partial depletion of the regulatory free heme pool for the enzyme would also explain why such chemicals provoke the AIP syn-

drome only in PBG deaminase-deficient individuals, but not in normal subjects. The reason why only a minority (~10%) of AIP gene carriers develop the clinical syndrome, however, remains an unresolved matter (36-38).

Studies on one of the four patients with hereditary tyrosinemia reported here demonstrated that ALA dehydratase activity in erythrocytes and in a liver biopsy specimen was <2% of normal before dietary treatment, and that enzyme activity improved significantly on a low tyrosine and low phenylalanine diet. The patient's urine also contained high concentrations of succinylacetone on admission, which significantly decreased after treatment (data not shown). Rocket immunoelectrophoresis of erythrocyte lysates indicated that the patient's erythrocytes contained a normal amount of ALA dehydratase protein. These data are consistent with an inhibition of ALA dehydratase by succinylacetone and suggest that the synthesis of enzyme protein is not affected in this disorder nor is an abnormal enzyme apparently synthesized.

Some individuals with hereditary tyrosinemia have been reported to display neurological symptoms similar to those observed in patients with AIP (2, 3, 5, 6). The basis of the neurological symptoms in AIP (or in hereditary tyrosinemia) has not been clarified. ALA (39) or porphobilin (40), an oxidation product of PBG, have been proposed to be the neurotoxic substances produced in AIP. The occurrence of AIP-like neurological symptoms in certain hereditary tyrosinemia patients suggests, however, that PBG or its derivative porphobilin are not likely to be the cause of the neurotoxicity in these diseases because ALA dehydratase in the tissues of hereditary tyrosinemia is profoundly inhibited and patients excrete excessive amounts of ALA, rather than PBG. In this respect it is also of considerable interest that an AIP-like syndrome has been recently described in a family with a hereditary deficiency of ALA dehydratase (41). These observations suggest that the neurovisceral manifestations of AIP, and of hereditary tyrosinemia, may all be related to ALA, or perhaps some metabolic derivative of this compound, rather than to other sequential intermediates, i.e., PBG or porphyrins, in the heme pathway.

Hereditary tyrosinemia appears to be a disease of special pharmacogenetic interest because an abnormal compound produced as a consequence of the primary enzyme deficiency in the tyrosine metabolic pathway profoundly inhibits ALA dehydratase, an enzyme in the heme biosynthetic pathway; this inhibition results in some patients in clinical symptomatology and certain biochemical findings resembling those characterizing another genetic disease, AIP. This interrelationship of two distinct metabolic pathways may be a paradigm of the interaction of heritable and acquired

factors in regulating the expression of other normal and abnormal physiological processes in man.

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REFERENCES

1. LaDu, B. N., and L. R. Gjessing. 1978. Tyrosinosis and tyrosinemia. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, New York. 4th edition. 256-267.
2. Gentz, J., B. Lindblad, S. Lindstedt, L. Levy, W. Shashteen, and R. Zetterström. 1967. Dietary treatment in tyrosinemia (tyrosinosis). *Am. J. Dis. Child.* 113: 31-37.
3. Gentz, J., S. Johansson, B. Lindblad, S. Lindstedt, and R. Zetterström. 1969. Excretion of δ -aminolevulinic acid in hereditary tyrosinemia. *Clin. Chim. Acta.* 23: 257-263.
4. Gaull, G. E., D. K. Rassin, G. E. Solomon, R. C. Harris, and J. A. Sturman. 1970. Biochemical observations on so-called hereditary tyrosinemia. *Pediatr. Res.* 4: 337-344.
5. Kang, E. S., and P. S. Gerald. 1970. Hereditary tyrosinemia and abnormal pyrrole metabolism. *J. Pediatr.* 77: 397-406.
6. Strife, C. F., E. L. Zuroweste, and E. A. Emmett. 1977. Tyrosinemia with acute intermittent porphyria: aminolevulinic acid dehydratase deficiency related to elevated urinary aminolevulinic acid levels. *J. Pediatr.* 90: 400-404.
7. Lindblad, B., S. Lindstedt, and G. Steen. 1977. On the enzymic defects in hereditary tyrosinemia. *Proc. Natl. Acad. Sci. USA.* 74: 4641-4645.
8. Sassa, S., and A. Kappas. 1977. Induction of δ -aminolevulinic synthase and porphyrins in cultured liver cells maintained in chemically defined medium. Permissive effects of hormones on the induction process. *J. Biol. Chem.* 252: 2428-2436.
9. Sassa, S., S. Granick, D. R. Bickers, R. D. Levere, and A. Kappas. Studies on the inheritance of human erythrocyte δ -aminolevulinic dehydratase and uroporphyrinogen synthetase. *Enzyme (Basel).* 16: 325-333.
10. Maines, M. D., and A. Kappas. 1975. Cobalt stimulation of heme degradation in the liver. Dissociation of microsomal oxidation of heme for cytochrome P-450. *J. Biol. Chem.* 250: 4171-4177.
11. Sinclair, J. F., P. R. Sinclair, and H. L. Bonkowsky. 1979. Hormonal requirement for the induction of cytochrome P-450 in hepatocytes cultured in serum-free medium. *Biochem. Biophys. Res. Commun.* 86: 710-717.

12. Anderson, P. M., and R. J. Desnick. 1979. Purification and properties of δ -aminolevulinic acid dehydratase from human erythrocytes. *J. Biol. Chem.* **254**: 6924-6930.
13. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406-4412.
14. Gurba, P. E., R. E. Sennett, and R. D. Kobes. 1972. Studies on the mechanism of action of δ -aminolevulinic acid dehydratase from bovine and rat liver. *Arch. Biochem. Biophys.* **150**: 130-136.
15. Wu, W. H., D. Shemin, K. E. Richards, and R. C. Williams. 1974. The quaternary structure of δ -aminolevulinic acid dehydratase from bovine liver. *Proc. Natl. Acad. Sci. USA.* **71**: 1767-1770.
16. Despau, N., E. Comoy, C. Bohuon, and C. Boudene. 1979. Purification and properties of human erythrocyte δ -aminolevulinic acid dehydratase (EC 4-2-1-24). *Biochimie (Paris)*. **61**: 1021-1028.
17. Fujita, H., Y. Orii, and S. Sano. 1981. Evidence of increased synthesis of δ -aminolevulinic acid dehydratase in experimental lead poisoned rats. *Biochim. Biophys. Acta.* **678**: 39-50.
18. Masters, B. S. S., J. Baron, W. E. Taylor, E. L. Isaacson, and J. LoSpalluto. 1971. Immunochemical studies on electron transport chains involving cytochrome P-450. I. Effects of antibodies to pig liver microsomal reduced triphosphopyridine nucleotide-cytochrome c reductase and the non-heme protein from bovine adrenocortical mitochondria. *J. Biol. Chem.* **246**: 4143-4150.
19. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* **15**: 45-52.
20. Grieninger, G. E., J. Pindyck, K. M. Hertzberg, and M. W. Mosesson. 1979. Application of electroimmunoassay to the study of plasma protein synthesis in cultured hepatocytes. *Ann. Clin. Lab. Sci.* **9**: 511-517.
21. Granick, J. L., S. Sassa, S. Granick, R. D. Levere, and A. Kappas. 1973. Studies in lead poisoning. II. Correlation between the ratio of activated to inactivated δ -aminolevulinic acid dehydratase of whole blood and the blood lead level. *Biochem. Med.* **8**: 149-159.
22. Sassa, S., and S. Granick. 1970. Induction of δ -aminolevulinic acid synthetase in chick embryo liver cells in culture. *Proc. Natl. Acad. Sci. USA.* **67**: 517-522.
23. Lim, L.-K., G. Srivastava, J. D. Brooker, B. K. May, and W. H. Elliott. 1980. Evidence that in chick embryos destruction of hepatic microsomal cytochrome P-450 haem is a general mechanism of induction of δ -aminolevulinic acid synthase by porphyria-causing drugs. *Biochem. J.* **190**: 519-526.
24. Nandi, D. L., and D. Shemin. 1968. δ -Aminolevulinic acid dehydratase of *Rhodospseudomonas spheroides* III. Mechanism of porphobilinogen synthesis. *J. Biol. Chem.* **243**: 1236-1242.
25. Brumm, P. J. and H. C. Friedmann. 1981. Succinylacetone pyrrole, a powerful inhibitor of vitamin B₁₂ biosynthesis: effect on δ -aminolevulinic acid dehydratase. *Biochem. Biophys. Res. Commun.* **102**: 854-859.
26. Lindblad, B., S. Lindstedt, and G. Steen. 1979. Deficiency of fumarylacetoacetase in hereditary tyrosinemia. *XIth Int. Congr. Biochem.*, Toronto, Canada. 048a. (Abstr.)
27. Ebert, P. S., R. A. Hess, B. C. Frykholm, and D. P. Tschudy. 1979. Succinylacetone, a potent inhibitor of heme biosynthesis: effect on cell growth, heme content and δ -aminolevulinic acid dehydratase activity of malignant murine erythroleukemia cells. *Biochem. Biophys. Res. Commun.* **88**: 1382-1390.
28. Tschudy, D. P., P. S. Ebert, R. A. Hess, B. C. Frykholm, and E. C. Weinbach. 1980. Effect of heme depletion on growth, protein synthesis and respiration of murine erythroleukemia cells. *Biochem. Pharmacol.* **29**: 1825-1831.
29. Tschudy, D. P., R. A. Hess, and B. C. Frykholm. 1981. Inhibition of δ -aminolevulinic acid dehydratase by 4,6-dioxoheptanoic acid. *J. Biol. Chem.* **256**: 9915-9923.
30. Whiting, M. J., and S. Granick. 1976. δ -Aminolevulinic acid synthase from chick embryo liver mitochondria. II. Immunochemical correlation between synthesis and activity in induction and repression. *J. Biol. Chem.* **251**: 1347-1353.
31. Krupa, V., J. C. Creighton, M. Freeman, and G. S. Marks. 1974. Drug-induced porphyrin biosynthesis—XII. Levels of cytochrome P-450 in chick embryo liver following administration of allylisopropylacetamide and propylisopropylacetamide. *Can. J. Physiol. Pharmacol.* **52**: 891-895.
32. Sinclair, P. R., J. F. Healey, J. F. Sinclair, and H. L. Bonkowsky. 1981. Induction of 5-aminolevulinic acid synthase in chick embryo liver by propylisopropylacetamide does not involve destruction of cytochrome P-450. *Biochem. J.* **200**: 709-710.
33. Marver, H. S., A. Collins, A. D. P. Tschudy, and M. Rechcigl, Jr. 1966. δ -Aminolevulinic acid synthetase. II. Induction in rat liver. *J. Biol. Chem.* **241**: 4323-4329.
34. Badawy, A. A.-B. 1982. Evidence against involvement of cytochrome P-450 haem in the regulation of synthesis of mammalian liver δ -aminolevulinic acid synthase. *Biochem. J.* **202**: 807-808.
35. Sassa, S., S. Granick, D. R. Bickers, H. L. Bradlow, and A. Kappas. 1974. A microassay for uroporphyrinogen I synthase, one of three abnormal enzyme activities in acute intermittent porphyria, and its application to the study of the genetics of this disease. *Proc. Natl. Acad. Sci. USA.* **71**: 732-736.
36. Anderson, K. E., H. L. Bradlow, S. Sassa, and A. Kappas. 1979. Studies in porphyria III. Relationship of the 5 α -reductive metabolism of steroid hormones to clinical expression of the genetic defect in acute intermittent porphyria. *Am. J. Med.* **66**: 644-650.
37. Sassa, S., and A. Kappas. 1981. Genetic, metabolic, and biochemical aspects of the porphyrias. In *Advances in Human Genetics*. H. Harris and K. Hirschhorn, editors. Plenum Publishing Corporation, New York. **11**: 121-231.
38. Kappas, A., S. Sassa, and K. E. Anderson. 1982. The porphyrias. In *The Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, D. S. Frederickson, J. S. Goldstein, and M. S. Brown, editors. McGraw-Hill, New York, 5th edition. 1301-1384.
39. Müller, W. E., and S. H. Snyder. 1977. δ -Aminolevulinic acid. Influences on synaptic GABA receptor binding may explain CNS symptoms of porphyria. *Ann. Neurol.* **2**: 340-342.
40. Feldman, D. S., R. D. Levere, J. S. Lieberman, R. A. Cardinal, and C. J. Watson. 1971. Presynaptic neuromuscular inhibition by porphobilinogen and porphobilin. *Proc. Natl. Acad. Sci. USA.* **68**: 383-386.
41. Doss, M., R. Von Tiepermann, J. Schneider, and H. Schmid. 1977. New type of hepatic porphyria with porphobilinogen synthase defect and intermittent acute clinical manifestation. *Klin. Wochenschr.* **57**: 1123-1127.