Porphobilinogen Oxygenase from Rat Liver: Induction, Isolation, and Properties†

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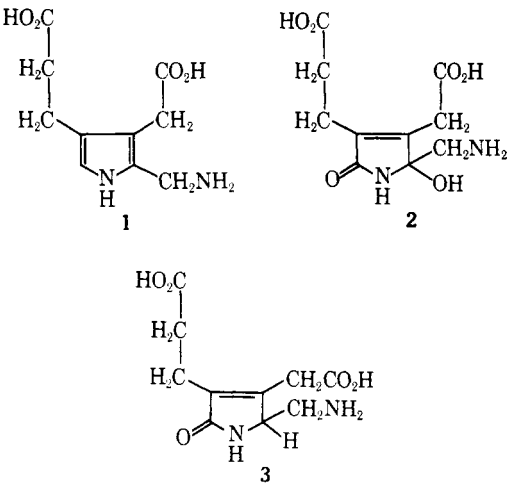
ABSTRACT: Porphobilinogen oxygenase, the porphobilinogen-oxidizing enzyme, was isolated from rat liver. It was located in the microsomes, from which it was liberated by disruption of the former with digitonin or deoxycholate. The activity of the microsomal enzyme was usually very low, owing to the presence of a powerful inhibitor. The inhibitor was partially separated by an exchange on DEAE-cellulose of the disrupted microsomal preparation. The enzymatic activity in the crude microsomal preparations could be induced by previous administration to the rats of a mixture of phenobarbital plus porphobilinogen, or of the steroids pregnenolone, progesterone, and 11-deoxycorticosterone. The induction of the enzymatic activity was coincident with a decrease of the inhibitors activity. It was a metalloenzyme containing nonheminic iron. It existed in multiple active forms of different molecular weights. It had an allosteric kinetics and was desensitized by the addition of Mg^{2+} or by freezing. Tryptophan was an inhibitor of the enzyme, as well as different divalent metals. The oxidation products formed by the oxygenase were identical with those formed by the oxygenase isolated from wheat germ, namely, 2-hydroxy-5-oxoporphobilinogen and 5-oxoporphobilinogen.

The presence of an enzyme in wheat germ extracts which oxidizes porphobilinogen **1** was described (Frydman *et al.*, 1973). The same enzyme, for which the name of porphobilinogen oxygenase was proposed, was also detected in rat liver and rat brain preparations (Frydman *et al.*, 1972a). Since porphobilinogen is the universal precursor of chlorins, prophyrins, and the corrin nucleus (Lascelles, 1964), its oxidation to non-porphyrin-forming substances could be of importance for tetrapyrrole biosynthesis. The physiological concentrations of porphobilinogen are very low and are known to be regulated by the activity of δ-aminolevulinic acid synthetase (De Matteis, 1967). Its removal by oxidation from the porphyrin metabolic pathway should then be strictly controlled. Porphobilinogen oxygenase activity in crude extracts of plant sources was inhibited by the presence of a powerful proteic inhibitor (Frydman *et al.*, 1973). When the inhibitor was separated by simple fractionation procedures, a completely active enzyme was obtained (Frydman *et al.*, 1973). Although the physiological function of the inhibiting protein was not clarified yet, it undoubtedly plays a major role in avoiding the enzymatic oxidation of porphobilinogen under normal conditions. It was also demonstrated that the wheat germ enzyme had allosteric kinetics (Frydman *et al.*, 1973), which again pointed in the general direction of avoiding the enzymatic oxidation of porphobilinogen when it is present in low concentrations.

Those properties made the study of the mammalian enzyme all the more interesting. The mammalian enzyme present in the microsomes of rat liver had a very low activity. It was sharply increased when several drugs were administered to the rats before the isolation procedure. Phenobarbital, phenobarbital plus porphobilinogen, and the steroids pregnenolone, progesterone, and 11-deoxycorticosterone had a powerful inducing effect on the activity of porphobilinogen oxygenase. The properties of both the noninduced and induced enzymes were then studied. Both gave origin to the same oxidation products as those produced by the plant enzyme, *i.e.*, the major product was the 2-hydroxypyrrolinone (**2**), and the minor product was the pyrrolinone (**3**). The structure of both



products was established as already described (Frydman *et al.*, 1973).

Materials and Methods

Porphobilinogen was obtained by synthesis (Frydman *et al.*, 1969). NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide, sodium dithionite, phenobarbital, pregnenolone, progesterone, 11-deoxicorticosterone, and hydrocortisone acetate were commercial products of analytical grade. Wistar albino female rats weighing 150–200 g were used. When the enzymatic induction was studied, the rats were treated with a mixture of phenobarbital (40 mg/kg) and/or porphobilinogen (2 mg/kg) dissolved in a saline solution. The animals were injected subcutaneously during 4 days and killed by decapitation on the fifth day. Control experiments were run by injecting only saline solution. When pregnenolone, progesterone, or hydrocortisone was injected, 2 or 20 mg per kg was dissolved in corn oil and administered

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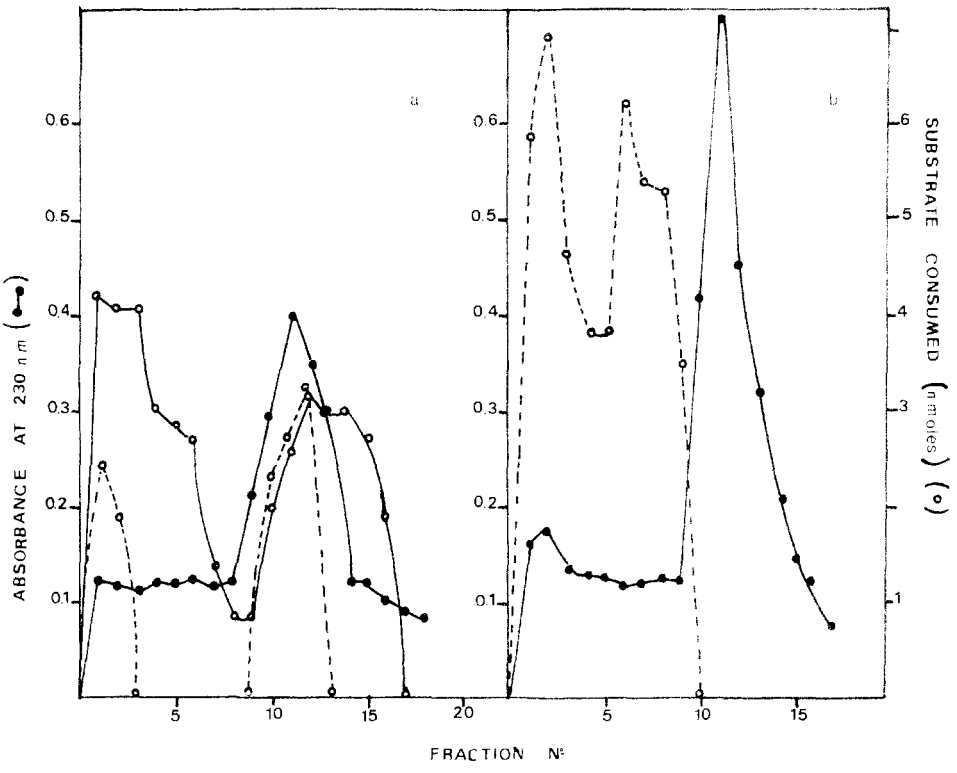

FIGURE 1: DEAE-cellulose elution profile of the 105,000*g* supernatant of deoxycholate disrupted microsomes: (a) control rats; (b) phenobarbital plus porphobilinogen-treated rats. The incubation mixtures and the conditions were the described under Methods: (- - -) assayed the same day; (—) assayed after 48 hr.

subcutaneously during 4 days. The rats were killed on the fifth day by decapitation. 11-Deoxycorticosterone was injected dissolved in sesame oil. Corresponding controls were performed.

Preparation and Purification of Porphobilinogen Oxygenase. Livers were excised and washed with an isotonic saline solution. They were then minced and homogenized in a Potter–Elvehjem in eight volumes of 0.25 M sucrose (w/v). The suspension was then centrifuged at 20,000*g* for 15 min, and the supernatant was centrifuged for 60 min in a Spinco centrifuge at 105,000*g*. The microsomal pellet was suspended in 4 volumes of a phosphate buffer (0.05 M) solution (pH 7.4) containing potassium chloride (0.15 M), and again centrifuged at 105,000*g* for 30 min. The pellet was resuspended in the same buffer solution with no potassium chloride addition, so as to obtain a 10–15-mg/ml of protein concentration. The preparation thus obtained will be referred to as "microsomes." During the subsequent purification steps, the microsomes were disrupted either by treatment with digitonin (0.1%) or with deoxycholate (0.1%). The resulting suspension was then centrifuged at 105,000*g* for 60 min, and the supernatant containing almost all the activity was used during the subsequent work-up.

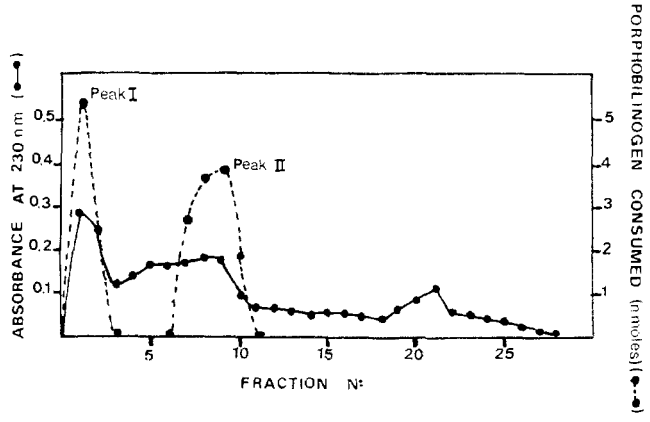

FIGURE 2: Sephadex G-75 elution profile of porphobilinogen oxygenase from rat liver. The elution conditions were the described in the text. Incubations conditions were described in Methods. Incubation time was 30 min.

One milliliter of the microsomal supernatant was applied to a DEAE-cellulose column (1 × 20 cm) equilibrated with phosphate buffer (0.05 M, pH 7.4) and eluted with the same buffer. Fractions of 1.5 ml were collected. A typical elution profile is depicted in Figure 1a. The active fractions were pooled, concentrated with Carbowax, and applied (300 μl) on a Sephadex G-75 column (1 × 30 cm) equilibrated with phosphate buffer (0.05 M, pH 7.4), which was also used for elution. Fractions of 1.5 ml were collected (Figure 2).

An identical purification sequence was used when microsomes from either phenobarbital or phenobarbital plus porphobilinogen-treated rats were prepared. The purification results obtained in each sequence were summarized in Table I. The microsomal preparations and the supernatants of the disrupted microsomes were stored at −15°, while the purified enzymes were stored at 0–4°.

Assay of Porphobilinogen Oxygenase. The standard incubation mixtures contained, unless otherwise stated: phosphate buffer (10 μmol, pH 7.4), porphobilinogen (13 nmol), 50 μl of the microsomal suspension or the purified enzymes, and either sodium dithionite (100 nmol) or NADPH (40 nmol) plus the NADPH-regenerating system (nicotinamide (5 μmol), glucose 6-phosphate (0.5 μmol), and magnesium chloride (1 μmol)).

Incubations were performed at 37° either during 30 or 60 min. Porphobilinogen consumption was estimated with Ehrlich's reagent as described (Frydman *et al.*, 1973).

TABLE I: Purification of Porphobilinogen Oxygenase from Rat Liver Microsomes.

	Control Animals				Phenobarbital + Porphobilinogen-Treated Animals			
Step	Total Act.[a] (Units)[b]	Total Protein (mg)	Sp Act.	Yield (%)	Total Act. (Units)[b]	Total Protein (mg)	Sp Act.	Yield (%)
Microsomes	0.130	40	0.0032		0.390	40	0.0097	
DEAE-cellulose	0.196	4	0.049	150	2.72	3.6	0.75	692
	2.572[c]	4	0.64	1,976				
Sephadex G-75	14	1	14	10,000	17	1	17	4300

[a] Referred to as 1 g of liver tissue. [b] One unit of activity was defined as 1 μmol of substrate consumed in 1 hr/ml of enzyme under the standard incubation conditions. [c] The above DEAE-cellulose enzyme was assayed 48 hr latter.

Results

Purification of Porphobilinogen Oxygenase. When porphobilinogen oxygenase was isolated from rat liver microsomes it was free from porphobilinogen deaminase. This simplified the problem which existed during the purification of the enzyme from plant material. The main problem here was the very low activity of the oxygenase in the isolated microsomes. As a matter of fact, sometimes the microsomal preparations, assayed as such or disrupted with digitonin, were entirely devoid of enzymatic activity. The latter started to be consistently detected only after the DEAE-cellulose purification stage. We already described a similar effect for the wheat germ enzyme and demonstrated that it was due to the presence of an inhibitor in the crude enzymatic preparation (Frydman *et al.*, 1973). This was also the case with these microsomal preparations. When the DEAE-cellulose enzyme was assayed after 48 hr, a large increase in the enzymatic activity took place (Table I, controls; and Figure 1a), a phenomenon which was due to the inactivation of an inhibitor present in the enzymatic fractions.

Porphobilinogen Oxygenase Induction. As described above porphobilinogen oxygenase activity in rat liver microsomes was very low. It was found that it could be considerably increased by treatment of the rats with a number of different chemicals. Subcutaneous injection of phenobarbital produced an increase in the enzymatic activity of the crude microsomes, but the results were not always reproducible. A more consistent effect was achieved by subcutaneous administration of a mixture of phenobarbital plus porphobilinogen (Table I and Figure 1b). Good results were also obtained when steroids were used. Subcutaneous injection of pregnenolone, progesterone, or deoxicorticosterone resulted in a consistent increase of porphobilinogen oxygenase activity in the liver microsomes. Pregnenolone was the best inducing agent (Figure 3), when four doses of 2 mg/kg were administered every 24 hr. With larger amounts of drug, the inducing effect was less noticeable (Figure 3). The inducing effect was also diminished when the drug was administered during shorter or longer periods of time. Administration of hydrocortisone (2.5 mg/100 g) followed by liver extraction after 5 hr did not induce any porphobilinogen oxygenase activity.

The induction phenomenon resulted in an increase of the activity of porphobilinogen oxygenase very likely due to the partial decrease of the amount of inhibitor present. As can be seen in Figure 1a,b, the noninduced preparations were activated after 48 hr, while the activity of the preparations from induced rats, remained essentially unaltered after the same period of time. Nevertheless, the enzyme preparations from induced rats still contained a considerable amount of inhibitor, which was removed during the purification procedures (Table I).

Properties of the Enzyme. Porphobilinogen oxygenase from rat liver had an absolute requirement for oxygen and a reducing agent. As was the case with other pyrrolooxygenases (Frydman *et al.*, 1972b), sodium dithionite was the best reducing agent. Porphobilinogen (1 mol), oxygen (1 mol), and dithionite (1 mol) were consumed during the oxidation (Frydman *et al.*, 1972a). When whole or disrupted microsomes were used, NADPH efficiently replaced sodium dithionite as the reducing source. The DEAE-cellulose enzyme was active only

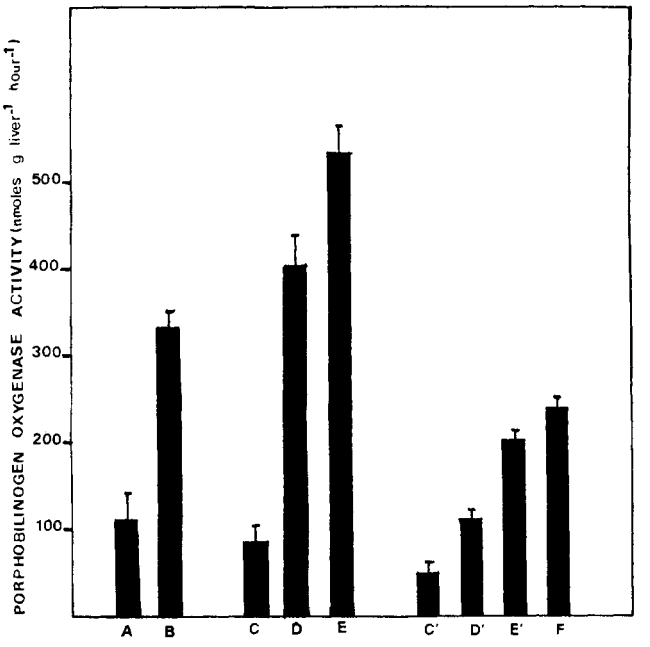

FIGURE 3: Induction of porphobilinogen oxygenase activity in rat liver by the action of different drugs. Female rats were treated as described under Methods. Each bar represents an average of three experiments (three animals each). In the case of pregnenolone and progesterone (2 mg/kg) the bars were an average of six experiments (three animals each): (A) saline control; (B) rats given phenobarbital plus porphobilinogen; (C) control injected with oil; (D) progesterone (2 mg/kg per day); (E) pregnenolone (2 mg/kg per day); (C', D', and E') the same as in C, D, and E except that a dose of 20 mg/kg was administered each day; (F) 11-deoxycorticosterone (20 mg/kg per day). Each bar represents an average of three experiments (three animals each). Activity was assayed by incubating microsomes, prepared as described in the text, during 30 min.

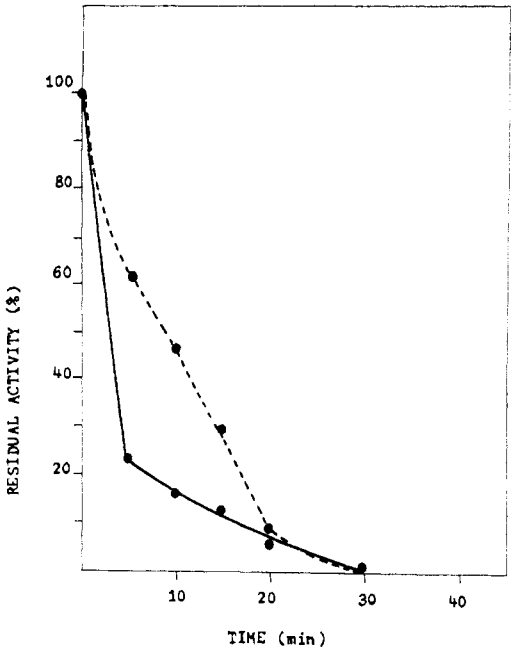

FIGURE 4: Time-course inactivation with temperature. The enzyme (50 μl) plus phosphate buffer (10 μmol) was preincubated at the indicated times at: (—) 65°; (- -) 60°. The incubation mixtures were then completed as described in Methods and incubated at 37° during 60 min.

in the presence of dithionite, or in the presence of NADPH if microsomes were added. The microsomal enzyme was stable at −15° during several months. The purified enzyme was usually kept at 0–4° during several weeks.

HEAT STABILITY. The purified microsomal enzyme (induced or not induced) was less stable to temperature increases than the enzyme from wheat germ. Thermal inactivation with time of the DEAE-cellulose purified and dialyzed enzyme at 60 and 65° is shown in Figure 4. The optimum temperature for the enzyme from rat liver was found to be 37°, while for the wheat germ enzyme it was 45°. At 60° the purified enzyme had

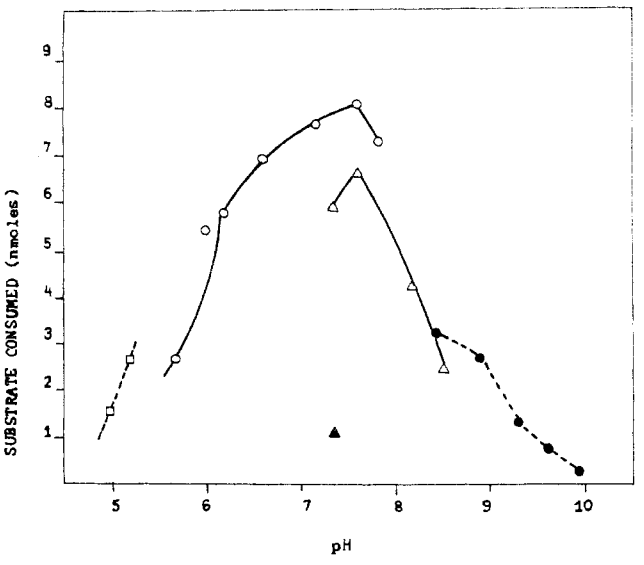

FIGURE 5: Effect of pH on the reaction rates. The incubations were carried out with 10 μmol of the indicated buffers at the indicated pH values. The incubation mixtures and conditions were the indicated in Methods. Incubation time was 60 min and the DEAE-cellulose enzyme was used: (□) citrate buffer; (○) phosphate buffer; (●) glycine buffer; (△) Tris-HCl buffer; (▲) Tris-maleate buffer.

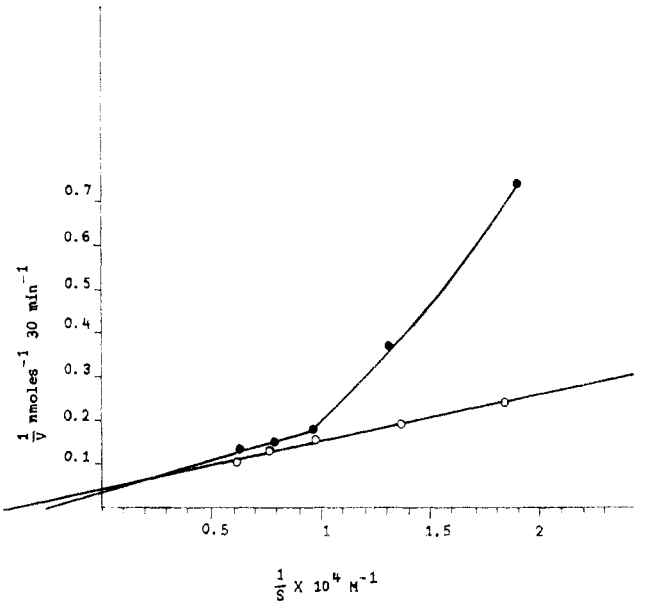

FIGURE 6: Effect of substrate concentration on the reaction rate of the digitonin-disrupted microsomes of pregnenolone-treated rats. The incubation mixtures were those indicated in Methods and incubations were performed during 30 min at 37°: (●) incubated without additions; (○) incubated in the presence of Mg^{2+} (0.12 mM).

only 15% of its activity at 37°. At 25° the activity was of 55% the activity at the optimum temperature.

pH OPTIMUM. The curve profile showed a maximum between pH 7 and 8 (Figure 5). High phosphate concentrations were found to be inhibitory of the oxygenase activity.

KINETICS. The effect of enzyme concentration on the enzymatic activity indicated a decrease in the activity with higher enzyme concentrations which could be attributed to an increased interaction with the inhibitor present, or to the formation of less active aggregation forms. The time course of the reaction with purified enzymes was linear up to 30 min.

The effect of substrate concentration on the activity of enzymes obtained from induced or noninduced animals showed an allosteric behavior. It is shown in Figure 6 using a supernatant obtained from digitonin disrupted microsomes of a pregnenolone-treated rat. Addition of Mg^{2+} (0.12 mM) transformed the allosteric kinetics into a Michaelis–Menten one. The V_{max} found in the absence or presence of Mg^{2+} was 0.32 μmol/min per mg of protein, and a $K_{m,app}$ of 0.3 mM for the Mg^{2+}-treated enzyme was found. A Hill number of n = 2.2 for the enzyme in the absence of Mg^{2+} was calculated, while a n = 1.0 was determined in the presence of Mg^{2+}. By freezing the disrupted microsomes at −15° during 10 days, the enzyme was desensitized and the kinetics became Michaellian with $K_{m,app}$ = 0.2 mM.

The enzyme showed different molecular forms when filtered through a Sephadex G-75 column (Figure 2). The form with the highest molecular weight (peak I) showed a pronounced allosteric kinetics with a V_{max} = 0.14 μmol/min per mg of protein, and a Hill number of n = 1.6 was calculated. The fraction of lower molecular weight (peak II) had n = 1.25, indicating a more Michaelian kinetic (Figure 7). The allosteric behavior seems thus related to the polymeric forms of the enzyme.

Effect of Metals and Inhibitors. The mammalian enzyme was a metalloenzyme containing Fe^{2+} as was the case with the wheat germ enzyme. Its activity was completely abolished by the addition of α,α′-dipyridyl (5 mM) while addition of EDTA

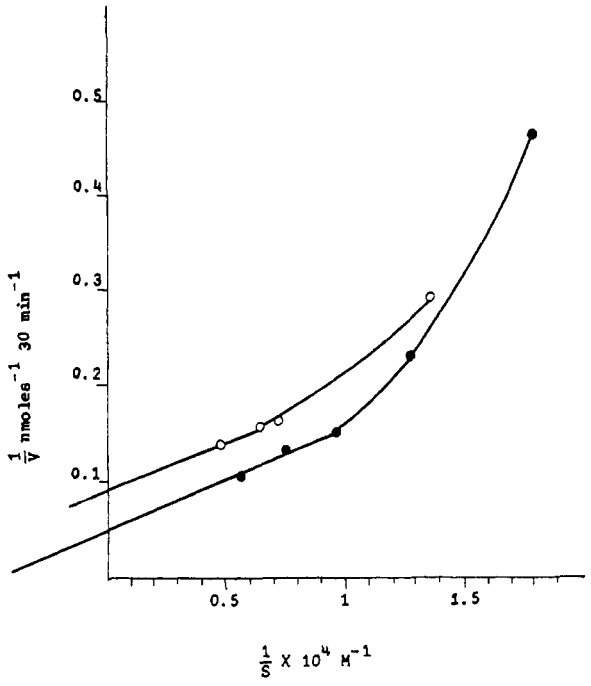

FIGURE 7: Effect of substrate concentration on the reaction rate of the eluates of a Sephadex G-75 column. Incubations were performed during 30 min at 37°.

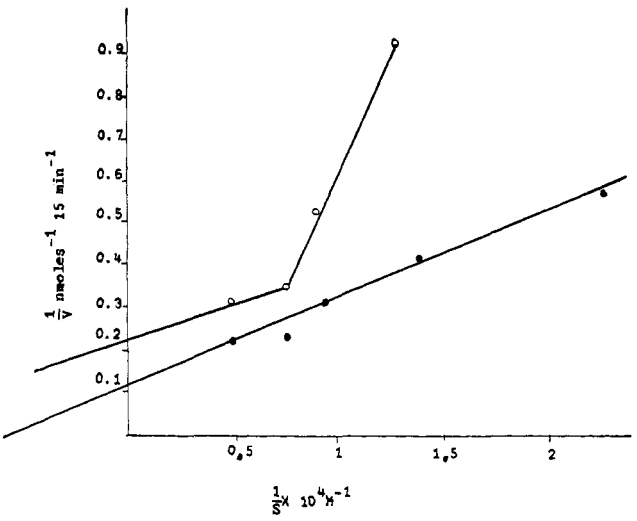

FIGURE 8: Effect of tryptophan addition on the reaction rate of a desensitized enzyme as a function of substrate concentration. (●) The incubation mixture was that indicated in Methods and a frozen microsomal pregnenolone-induced enzyme was used. (○) Tryptophan (0.5 mM) was added to the mixture. Incubation time was 15 min.

(5 mM) to the DEAE-cellulose enzyme left a remanent of 30% of the enzymatic activity. Addition of potassium cyanide (25 mM) had no effect on the activity of the enzyme. Cd^{2+}, Cu^{2+}, Ca^{2+}, and Pb^{2+} were strong inhibitors of the enzyme (Table II). An activating effect on the activity of enzyme was exerted by Co^{2+} and Fe^{2+} salts. Previous dialysis was necessary to eliminate the phosphate salts which had a chelating effect on the metal ions.

Tryptophan had a strong inhibitory effect on this enzyme. When measured on a frozen microsomal preparation from pregnenolone-treated rats, the Michaelian kinetics was transformed into an allosteric one in the presence of tryptophan (Figure 8). The Hill plot was used to calculate $n = 2.25$ in the presence of tryptophan and $n = 1.0$ in its absence.

Discussion

Since the physiological concentration of porphobilinogen available for heme biosynthesis is of crucial importance, its oxidation to non-porphyrin-forming substances must be strictly regulated. Porphobilinogen oxygenase is accordingly present in an inhibited form in extracts from rat liver microsomes (Table I). The inhibition is produced by the presence of an inhibitor which can be separated by the usual fractionation procedures. The release of the activity of the oxygenase by administration of phenobarbital and porphobilinogen was due to a decrease in the amount of inhibitor present (Figure 1). From the total activity data (Table I) it cannot be concluded if this increase in the enzymatic activity was also due to an induction of the *de novo* synthesis of the enzyme, or is exclusively related to the detected decrease in the inhibitor concentration. It should also be noted that phenobarbital prevented the induction of the so-called chemical porphyria, where the amount of porphobilinogen was strongly increased (Kaufman *et al.*, 1970).

Progesterone at very high concentrations (300 mg/kg) induced the activity of δ-aminolevulinic acid synthetase (Kaufman *et al.*, 1970), the rate-limiting enzyme in porphyrin metabolism (De Matteis, 1967). At much lower concentrations (Figure 3) it increased strongly porphobilinogen oxygenase activity. The regulatory implicance of this effect is obvious if a role for the oxygenase is accepted as a metabolic regulator of the amount of porphobilinogen available for porphyrin biosynthesis.

The oxygenase existed in different aggregation forms (Figure 2). This effect was already described for the plant enzyme, and it was demonstrated that the different molecular forms aggregate and disaggregate freely. It is yet impossible to determine exactly their relative contribution to the enzymatic activity and properties. It remains yet to be demonstrated that porphobilinogen oxygenase has a physiological role in heme biosynthesis.

TABLE II: Effect of Metals on Porphobilinogen Oxygenase Activity from Rat Liver Microsomes.[a]

Metal Ions	Concn (mM)	Remanent Porphobilinogen Oxygenase Act. (%)
$CaCl_2$	25	23
$MnSO_4$	25	0
$CuCl_2$	25	23
$CdCl_2$	2.5	44
$MgCl_2$	5	65
$PbCl_2$	2.5	27
$NiCl_2$	10	104
$CoCl_2$	5	130
$FeCl_2$	25	163
$FeSO_4$	25	163

[a] The metals were added to the standard assay mixture. Glycine–sodium hydroxide buffer (pH 8.4) was used. The activity of a DEAE-cellulose-purified and -dialyzed enzyme (9 μg of protein) was considered to be 100%. Incubations were carried out for 15 min at 37°.

Acknowledgment

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Effect of Ionic Strength on the Activity of Carnitine Palmityltransferase I†

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ABSTRACT: Changes in ionic strength at a constant osmolarity produced increases in carnitine palmityltransferase I activity in intact mitochondria. This enzyme catalyzes the reaction: palmityl-CoA + carnitine ⇄ palmitylcarnitine. The linearity of the effect of ionic strength on palmitylcarnitine formation was present only at ionic strengths below 0.060 M. The use of the Brönsted–Bjerrum equation for the effect of ionic strength on ion–ion interactions yielded a positive slope of 3 that was pH sensitive. A decrease in pH lowered the slope of the equation, suggesting involvement of the negative charges 3 and 1 (Z_A,Z_B) in the effect of ionic strength on the reaction rate. Similar increases in ionic strength increased linearly the association of palmityl-CoA with mitochondria incubated in the presence of antimycin A. A positive slope of 3 was obtained when the Brönsted–Bjerrum equation was applied. A positive correlation between increases in palmitylcarnitine formation and palmityl-CoA bound to the mitochondria at different ion concentrations was observed. Carnitine palmityltransferase activity released with digitonin was not affected by changes in ionic strength. Addition of a mitochondrial outer membrane fraction to the released enzyme activity restored the ionic strength dependency. This effect was proportional to increased association of palmityl-CoA with the outer membrane fraction. The augmentation of carnitine palmityltransferase activity with increased ionic strength was postulated to be related to the increased concentration of palmityl-CoA in the vicinity of the enzyme active site.

The partial latency of carnitine palmityltransferase has been described (Yates and Garland, 1970). It was originally proposed by Fritz and Yue (1963) that the mitochondria contain two carnitine palmityltransferase activities (transferases I and II) that catalyze reactions 1 and 2. In unbroken mitochondria, type-I transferase is accessible to the inhibitor

$$\text{palmityl-CoA} + \text{carnitine} \rightleftharpoons \text{palmitylcarnitine} + \text{CoA}^{1} \quad (1)$$
(transferase I)

$$\text{palmitylcarnitine} + \text{CoA} \rightleftharpoons \text{carnitine} + \text{palmityl-CoA} \quad (2)$$
(transferase II)

2-bromostearyl-CoA, whereas the type-II ("latent") transferase is inaccessible (Yates and Garland, 1970). The type-I transferase ("external") is located on the external portion of the inner membrane of mitochondria, whereas the type-II transferase is bound within the inner mitochondrial membrane (Yates and Garland, 1970; Hoppel and Tomec, 1972; Brosnan *et al.*, 1973).

Brosnan and Fritz (1971) examined factors that might influence the expression of the "external" carnitine palmityltransferase. Changes in the ability of intact mitochondria to oxidize palmityl-CoA were shown to depend on the ionic strength of the assay medium. Under the appropriate conditions, the "latency" or hypothesized deficiency of fetal heart mitochondria for the external transferase (Wittels and Bressler, 1965; Warshaw and Terry, 1970) was expressed in intact mitochondria by increased ionic strength or by storage in ice (Brosnan and Fritz, 1971).

The apparent "masked" activity of the external enzyme, carnitine palmityltransferase I, in media of low ionic strengths was examined in the present study. Maximal expression of carnitine palmityltransferase I was seen to be directly related to increased association of palmityl-CoA with the mitochondrion.

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[1] Abbreviations used are: CoA, coenzyme A; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; μ, ionic strength; mosm, milliosmolar.