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Porphobilinogen Oxygenase from Wheat Germ: Isolation, Properties, and Products Formed†

Rosalia B. Frydman, Maria L. Tomaro, Alfredo Wanschelbaum,‡ Elvira M. Andersen,§ Josefina Awruch,¶ and Benjamin Frydman*

ABSTRACT: The enzymatic oxidation of porphobilinogen by a new enzyme isolated from wheat germ is described. The name of porphobilinogen oxygenase was proposed for the enzyme. An exchange on DEAE-cellulose followed by filtration through DEAE-Sephadex A25 allowed to separate the enzyme from a proteic inhibitor present in the crude extracts. Porphobilinogen oxygenase belonged to the family of the pyrrolooxygenases, and required the presence of oxygen and of a reducing agent. The enzyme existed in several molecular aggregation forms. Three multiple weight active forms were obtained which interconverted among them. The oxygenase had an allosteric kinetics and was desensitized by the addition of Co^{2+} or Ni^{2+} or by freezing. It is a metalloenzyme containing iron as ferrous ion, and was inhibited when the metal was removed by exhaustive dialysis against 1 mM EDTA. Activity was restored by addition of various metal ions. Metal-chelating substances inhibited the enzymatic activity. Porphobilinogen oxygenase had a relatively broad specificity for alkylpyrroles containing a free α position. The oxidation of porphobilinogen produced 2-hydroxy-5-oxoporphobilinogen as the major product and 5-oxoporphobilinogen as the minor product.

Porphobilinogen, 2-aminomethyl-3-carboxymethyl-4-carboxyethylpyrrole, is the universal precursor of heme, chlorophylls, porphyrins, and the corrin nucleus (Lascelles, 1964). As such, it is the only known monopyrrole in primary metabolism and has no other biological analogs. It is originated in the self-condensation of two units of δ-aminolevulinic acid. The δ-aminolevulinic acid synthetase, which is the enzyme responsible for the metabolic formation of the latter, is apparently also the one which regulates the amount of porphobilinogen formed (De Matteis, 1967). Porphobilinogen itself is consumed by a complex of two enzymes, porphobilinogen deaminase and uroporphyrinogen III cosynthetase, which transforms it into uroporphyrinogen III, the first cyclic tetrapyrrole intermediate in the biosynthetic sequence leading to the natural porphyrins (Lascelles, 1964).

Of the two enzymes mentioned above, only porphobilinogen deaminase consumes porphobilinogen. The purified enzyme from wheat germ had a K_m of the order of 10^{-5} (Frydman and Frydman, 1970), suggesting that under physiological conditions the concentration of porphobilinogen may be very small. During the metabolic disorders known as hepatic porphyrias a considerable increase in the physiological concentrations of both δ-aminolevulinic acid and porphobilinogen takes place, a phenomenon that also occurs during the induction with drugs of the so-called experimental porphyrias (De Matteis, 1967). An analogous increase in the concentration of porphobilinogen in plant material is not known.

We already described in a preliminary note (Frydman *et al.*, 1972a) the existence in plants and animals of a new enzyme which oxidized porphobilinogen, for which we proposed the name of porphobilinogen oxygenase. The enzyme belonged to the general type of the pyrrolooxygenases, a new group of enzymes recently described (Frydman *et al.*, 1972b). The oxidation of porphobilinogen appears as an alternative pathway for porphobilinogen, diverting it from its well-known function as porphyrin precursor. The properties of the enzyme isolated from wheat germ will be described, as well as the nature of the new products formed by it.

Materials and Methods

Materials

Porphobilinogen (**1**)[1] and porphobilinogen lactam **2** were obtained by synthesis (Frydman *et al.*, 1969). 2-Aminomethyl-3-carboxymethylpyrrole (**3**) and its lactam **4**, 2-aminomethyl-4-methyl-3-carboxymethylpyrrole (**5**) and its lactam **6**, 2-aminomethyl-4-ethyl-3-carboxymethylpyrrole (**7**) and 2-methyl-3-carboxymethyl-4-carboxyethylpyrrole (**8**) were of synthetic origin (Frydman *et al.*, 1973).

Sodium dithionite, *p*-dimethylaminobenzaldehyde, and all other chemical reagents were commercial products of analytical grade. Wheat germ was a gift of Molinos Rio de la

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‡ Fellow of the Instituto Nacional de Farmacología y Bromatología.
§ Fellow of the Universidad de Buenos Aires.
¶ Fellow of the Consejo Nacional de Investigaciones.

[1] Abbreviations used are: PBG, porphobilinogen, Paa, 2-aminomethyl-3-carboxymethylpyrrole; MPaa, 2-aminomethyl-3-carboxymethyl-4-methylpyrrole; PBGL, porphobilinogen lactam; MPaaL, 2-aminomethyl-3-carboxymethyl-4-methylpyrrole lactam.

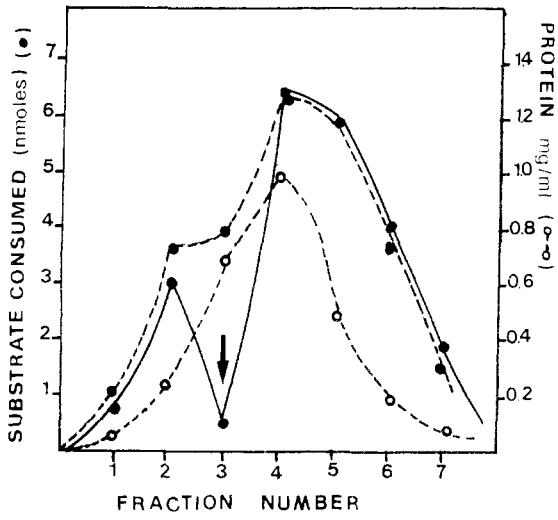


FIGURE 1: DEAE-cellulose elution profile. The elution conditions were described in Methods. Incubations were carried out as described in assay: (—) assayed the same day; (- - -) assayed 48 hr later.

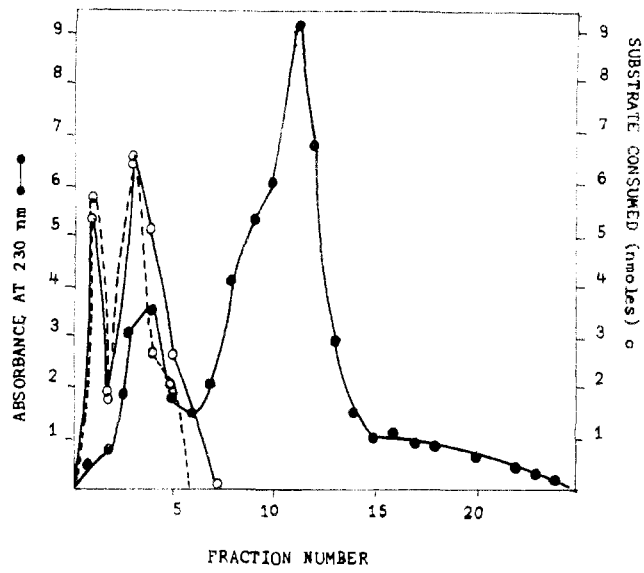


FIGURE 2: DEAE-Sephadex A25 elution profile. Elution conditions and assay of enzyme were performed as described in Methods: (—) assayed the same day; (- - -) assayed 48 hr later.

Plata (Buenos Aires). Sephadex and DEAE-Sephadex were Pharmacia products. All solvents used were previously distilled.

Methods

Preparation and Purification of Porphobilinogen Oxygenase. Porphobilinogen oxygenase was isolated from wheat germ following the general procedure described for the isolation of other pyrrolooxygenases (Frydman *et al.*, 1972b). Wheat germ (100 g) was extracted with 400 ml of water. The preparation was filtered through a nylon cloth and centrifuged at 20,000*g* for 15 min. The supernatant was adjusted to pH 5 with 1 N acetic acid, allowed to stand for 1 hr, and then centrifuged at 20,000*g* for 15 min. The supernatant was fractionated by addition of ammonium sulfate. The fraction precipitating between 30 and 50% (30–50 AS) was dissolved in 25 ml of 0.01 M Tris buffer (pH 7.6) and dialyzed overnight against 4 l. of the same buffer. During all these steps porphobilinogen oxygenase activity went together with porphobilinogen deaminase activity. The dialyzed 30–50 AS fraction was centrifuged in order to remove a precipitate which appeared during the dialysis, and applied to a DEAE-cellulose column (3 × 20 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.6). The oxygenase activity was eluted with 400 ml of the same buffer, and was free of porphobilinogen deaminase activity. Fractions of 40 ml were collected (Figure 1). The obtained elution profile indicated an inhibition of the oxygenase activity in several of the eluted fractions, a peculiar feature which will be discussed later. The most active fractions were pooled and 10 ml of the enzyme solution was applied to a DEAE-Sephadex A25 column (2 × 20 cm), previously equilibrated with 0.03 M Tris-HCl buffer (pH 7.6). The resulting elution profile (Figure 2) again indicated an inhibition of the enzymatic activity in one fraction. An alternative purification step to the DEAE-Sephadex A25 procedure was the filtration through either a Sephadex G-100 or a Sephadex G-75 column. The total purification scheme of porphobilinogen oxygenase was summarized in Table I.

Assay of Porphobilinogen Oxygenase. The standard reaction mixture contained unless otherwise indicated, 10 μmol of phosphate buffer (pH 7.4), 0.10 μmol of sodium dithionite, porphobilinogen (13 nmol), and enzyme (2–20 μg of protein), in a final volume of 100 μl. Incubations were usually run at 37° for 15–30 min. Two blanks were always run simultaneously, omitting either dithionite or enzyme. Porphobilinogen oxygenase activity was assayed by measuring the disappearance of substrate with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid–perchloric acid (84:16, v/v)) at 552 nm after previous addition of Hg^{2+}. Protein was estimated by the method of Lowry or alternatively from the absorption at 260–280 nm. Thin-layer chromatography (tlc) determinations were performed either on cellulose-coated plates (0.25 mm) using as solvent the upper layer of a butanol–acetic acid–water mixture (4:1:5); or on silica gel coated plates (0.25 mm) using as solvents an ethyl acetate–methanol mixture (3:1) (solvent I), or else a chloroform–methanol mixture (95:5) (solvent II). When preparative amounts of the oxidation products of porphobilinogen were needed, the incubation mixtures contained 30 nmol of porphobilinogen and were incubated during 60 min at 37° in a final volume of 50 μl. Two-hundred incubations were pooled, the solution was adjusted to pH 5 with acetic acid, and the mixture was adsorbed on a Dowex 1-X4 resin column (1.5 × 13 cm) in its acid form. The column was washed with 75 ml of water, and then eluted with 0.8 M acetic acid. The eluates containing residual porphobilinogen and the oxidation products were evaporated to dryness and the residue was filtered through a cellulose column (2 × 20 cm), prewashed with a water-saturated 1-butanol solution, and eluted with the same solvent. The fractions containing the oxidation products (located by tlc) were pooled, evaporated to dryness, and stored at −15°. The products were located on the tlc plates by spraying with Ehrlich's reagent followed by heating at 120°, when they developed a yellow-orange to deep orange color depending of the concentration of the product in the spot.

When pyrrole lactams or 2-methyl-3-methoxycarbonylmethyl-4-methoxycarbonylethylpyrrole was used as substrates and the formed products were isolated, the standard incubation mixtures contained 140 nmol of substrate and were incubated at 37° for 60 min in a final volume of 50 μl. One-hundred incubation mixtures were pooled and the solution was adjusted to pH 5 with acetic acid and evaporated to dryness *in vacuo*. When the pyrroles contained acid groups the

TABLE I: Purification of Porphobilinogen Oxygenase from Wheat Germ.

Preparation Stage	Vol (ml)	Total Protein (mg)	Total Units[a]	Sp Act. (Units/mg of Protein)	Yield (%)	Purification Factor
1. Crude extract	255	6375	27.5	0.0043	100	
2. pH supernatant	250	5000	40.3	0.008	146	1.86
3. Ammonium sulfate 30–50%	40	1140	21.6	0.018	76	4.1
4. DEAE-cellulose pooled fractions	160	103	352	3.5	1270	813
5. DEAE-Sephadex A-25[b]	20	2.4	11	4.6	200[c]	1620

[a] One unit of enzyme is defined as: μmoles of substrate consumed per ml of enzyme per hr, under the standard assay conditions. [b] Five milliliters of a DEAE-cellulose fraction (5.4 μmol, specific activity 2.1 units/mg of protein) was applied to the DEAE-Sephadex column). Similar results were obtained when the DEAE-Sephadex step was substituted by a filtration through Sephadex G-100 or G-75, except for the fact that the enzyme was eluted in two or more peaks of different molecular weights (see Results). [c] 200% yield as compared to the DEAE-cellulose fraction used.

residue of the evaporation was dissolved in methanol, treated with an excess of ethereal diazomethane, evaporated again to dryness, and the residue was extracted with hot methanol. When the incubation products were neutral compounds, the methanolic extractions were performed directly on the evaporated incubation mixture. After evaporation of the methanolic extracts the residual products were identified by tlc on silica gel coated plates. They were isolated by dissolving the residues in the solvents used for tlc and adsorbing the mixture on a column (1.5 × 15 cm) of the same silica gel used for tlc prewashed with the corresponding solvent. By applying a slight pressure and by eluting with the same solvents used during the tlc procedures, the products were collected in fractions according to their decreasing R_F on the plates. The method is more simple and more efficient than conventional preparative tlc. The products, which were not located with Ehrlich's reagent, were located on the plates by spraying with Folin and Ciocalteu's reagent (E. Merck Folin-Ciocalteus phenol reagent diluted with two volumes of water), followed by a 16% sodium carbonate solution. The products appeared as blue spots.

Nuclear magnetic resonance spectra were run on a Perkin-Elmer R 12 instrument; infrared spectra were recorded on a Perkin-Elmer 21 instrument; ultraviolet and visible spectra were recorded on a Perkin-Elmer 202 spectrophotometer; mass spectra were determined by the Morgan and Schaffer Corp. (Montreal).

Results

Purification of Porphobilinogen Oxygenase. Two main problems arose during the purification procedures of porphobilinogen oxygenase. The most simple to tackle was the presence of porphobilinogen deaminase together with the oxygenase during the first stages of the process. Both consume porphobilinogen, but the deaminase forms uroporphyrinogen from porphobilinogen in a reaction of known stoichiometry (4 mol of porphobilinogen give 1 mol of uroporphyrinogen), while the oxygenase did not form any porphyrin. Hence, the amount of porphobilinogen consumed by the deaminase present can be easily calculated from the amount of porphyrin formed and deducted in each step. A more serious problem was posed by the presence in the enzymatic preparations of a proteic inhibitor of the oxygenase, which can only be removed during the last stages of the purification process. The inhibitor is however heat and cold labile and can thus be destroyed without affecting the oxygenase activity.

The purification scheme of porphobilinogen oxygenase (Table I) shows a steady increase in the total yield of the enzymatic activity, closely paralleling the results obtained with skatole and tryptophan pyrrolooxygenases (Frydman *et al.*, 1972b). An increase in the total yield could also be obtained by storage at −15°, which leads to an inactivation of the inhibitor. The drop in yield in the 30–50 AS step was due to the fact that part of the oxygenase activity precipitated in the 50–70 AS fraction and was not used in the further work-up. During the DEAE-cellulose purification step the presence of the proteic inhibitor was evident in several fractions. The inhibition disappeared after storage during 48 hr at 4° (Figure 1). When increasing amounts of the fraction containing the largest concentration of inhibitor (Figure 1, marked with an arrow) were added to the pooled active fractions of the DEAE-cellulose column, an increasing inhibitory effect on the oxygenase activity was found, until total inhibition was achieved. When the active fractions were pooled and concentrated either by precipitation with ammonium sulfate, or by Carbowax, much of the enzymatic activity was lost. This could be explained by the simultaneous concentration of both the enzyme and the remaining inhibitor, which resulted in a stronger interaction among them. It could also be attributed to a change in the molecular aggregation forms of the enzyme (see below), where the higher molecular weight forms would have a lower reaction rate than the lower aggregation forms.

Several properties of the inhibitor were summarized in Table II. It was destroyed by aging, by heating at 60° during 30 min and by the action of trypsin. The inhibitor had a molecular weight of less than 25,000, as could be estimated by ultrafiltration through a Sartorius collodion filter bag. When a fraction of the DEAE-cellulose column (*e.g.*, fraction 4 of Figure 1) was filtered through a collodion bag, the concentrated enzyme showed good oxygenase activity which increased with increasing amounts of protein. The filtrate also had oxygenase activity, but showed a decrease in this activity when increasing amounts of enzyme were assayed. This indicated that the proteic inhibitor was present in the filtrates but not in the concentrates. The addition of the filtrates to the concentrates inhibited its activity, and by storing the filtrates during 3 days at 4°, they lost this inhibitory power.

Multiple Active Forms of Porphobilinogen Oxygenase. As mentioned above the oxygenase activity could be detected both in the concentrates and in the filtrates of a Sartorius collodion filter bag. This indicated that the enzyme existed as multiple active forms, an observation already described for skatole pyrrolooxygenase (Frydman *et al.*, 1972b). When a

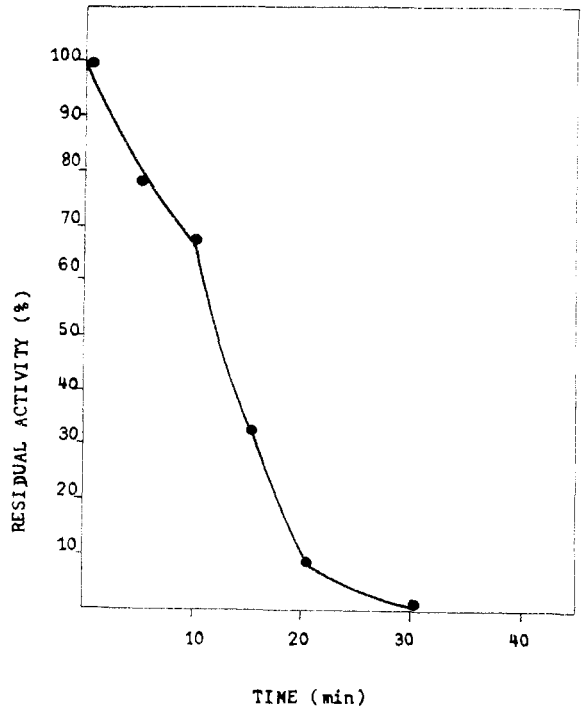


FIGURE 3: Time course of temperature inactivation. The DEAE-cellulose purified enzyme (4.5 μg of protein) was preincubated in the presence of 0.1 M phosphate buffer (pH 7.4) at 65° for the indicated time. Measurements of residual activity were done by adding dithionite and porphobilinogen. Incubations were carried out as described in Methods, during 30 min.

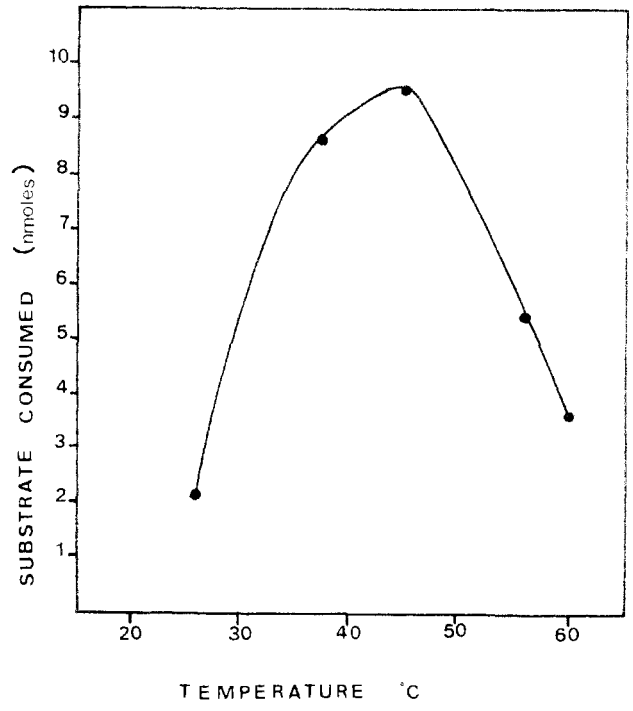


FIGURE 4: Effect of temperature on the reaction rates. The incubation mixture described in Assay was incubated during 30 min at the indicated temperatures.

DEAE-cellulose purified enzyme was filtered through a Sephadex G-100 column three active peaks of different molecular weights were eluted. When the molecular weights of the different fractions were measured by the method of Andrews (Andrews, 1965), using as internal markers RNase, bovine growth hormone, ovoalbumin, and bovine seroalbumin, it was found that the higher molecular form had a molecular weight of 100,000, the main fraction of molecular weight of 25,000, and a very minor fraction with a molecular weight of less than 13,500 existed. When the main fraction was subjected to a second filtration through a Sephadex G-100 column, again a multiple weight elution pattern was obtained, containing the 100,000 molecular weight fraction and the 25,000 molecular weight fraction. Intermediate forms were also occasionally observed. The presence of the several isozymes of the oxygenase is of considerable interest since the association-dissociation phenomena did not allow to establish which form was really active in the incubation mixture.

TABLE II: Effect of Different Treatments on the Inhibitor of Porphobilinogen Oxygenase.[a]

Treatment of the Inhibitor	Inhibition (%)
	63
Preincubated at 37°	67
Preincubated at 54°	53
Preincubated at 60°	0
Kept 4 days at 0–4°	0
Preincubated with trypsin	0
Preincubated with trypsin + STI[b]	55
Preincubated with STI	62

[a] The inhibitor (fraction 3, Figure 1) was used either as such or preincubated at different temperatures for 30 min. When the inhibitor was treated with trypsin, 0.2 mg of the proteolitic enzyme was added and the preincubation was carried out at 37° for 15 min. The remaining inhibitory activity was assayed by adding STI (0.2 mg) to the preincubated mixture and then measuring porphobilinogen oxygenase activity. Porphobilinogen oxygenase in the control consumed 8.1 nmol of porphobilinogen under the standard assay conditions. The activity of the inhibitor is expressed as per cent of inhibition of the control oxygenase activity. [b] STI = soybean trypsin inhibitor.

Properties of Porphobilinogen Oxygenase. In our preliminary report (Frydman *et al.*, 1972a), we indicated that the enzyme was an oxygenase, requiring both oxygen and a reducing agent for its activity. The best reducing agent was sodium dithionite which could not be replaced by other reducing agents (Methylene Blue plus ascorbic acid, glutathione, cysteine, reduced ascorbate, NADH, and NADPH) and was only partially replaced by sodium bisulfite. The stoichiometry of the reaction indicated that equimolar amounts of porphobilinogen, oxygen, and sodium dithionite were consumed (Frydman *et al.*, 1972a). The enzyme had a very low absorption at 280 nm as compared with its absorption at 230 nm (amide band), suggesting that the protein was devoid of tryptophan. Oxidation attempts of possible tryptophanyl residues of the enzyme, essential for its activity, caused no inhibition of the enzyme.

STABILITY OF THE ENZYME. The 30–50 AS fraction was very stable at −15° and could be kept for several months. The more purified fractions must be kept at 0° and were more easily inactivated at −15°. When the enzyme was heated during 15 min at 60° it lost only 20% of its original activity. The time course of the enzyme inactivation at 65° can be seen in Figure 3.

The enzyme was entirely destroyed by preincubation with trypsin during 30 min at 37°. The optimum temperature of

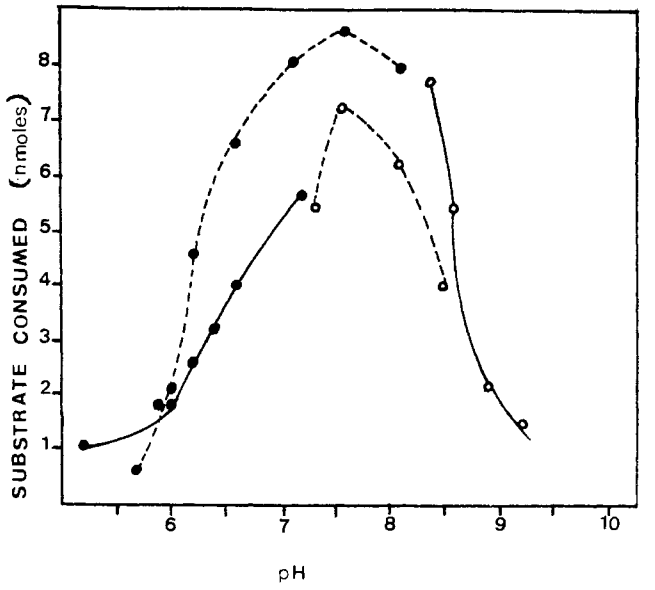


FIGURE 5: Effect of pH on the reaction rates. The incubations were carried out with 10 μmol of the indicated buffers at the indicated pH values. The incubation mixtures and conditions were the indicated in Methods: (—●-●—) citrate–phosphate buffer; (- -●- -●- -) phosphate buffer; (- -○- -○- -) Tris-HCl buffer; (—○-○—) glycine–sodium hydroxide buffer. Incubations were carried out during 30 min.

porphobilinogen oxygenase was found between 37 and 45°. At 60° only 50% of the optimum activity remained, while at 25° the remaining activity was of only 30% (Figure 4).

pH OPTIMUM. The pH profile for the Sephadex G-100 purified wheat germ porphobilinogen oxygenase is shown in Figure 5. The optimum pH was found between pH 7 and 8, with a sharp decrease at either alkaline or acid pH. Citrate and maleate were found to inhibit the enzyme's activity. At high concentrations of phosphate buffer (0.2 M or higher) the phosphate was found to inhibit the enzymatic activity.

SUBSTRATE SPECIFICITY. Porphobilinogen oxygenase oxidized porphobilinogen (**1**) (Chart I) as well as a number of closely related pyrroles. 2-Aminomethyl-4-methyl-3-pyrroleacetic acid (**5**) had a higher V_{max} than porphobilinogen itself. Very good substrates were also 2-aminomethyl-4-ethyl-3-pyrroleacetic acid (**7**), 2-methyl-3-carboxymethyl-4-carboxyethylpyrrole (**8**), and its dimethyl ester **9**. The lactams of the 2-aminomethyl-3-pyrroleacetic acids were better substrates than the pyrroles from which they were derived.

All those substrates had in common a free α position and a 2,3,4-trialkylpyrrole substitution pattern. The only exception was **3** and **4** which had a 2,3-dialkylpyrrole substitution pattern. When pyrroles substituted at C-5 (*e.g.*, 5-carboxyporphobilinogen) were assayed as substrates, they were found not to be consumed by the enzyme. The pyrroles which had a substituent with a strong positive inductive effect at C-4 were better substrates of the enzyme, as could be expected from a substance undergoing an electrophilic substitution reaction such as an oxidation. The oxidation rates of 2-aminomethyl-4-methyl-3-pyrroleacetic acid (**5**) and its lactam **6** were higher than those of porphobilinogen (**1**) and its lactam **2** (Figure 6), while 2-aminomethyl-3-pyrroleacetic acid (**3**) its lactam **4** had the slowest oxidation rates.

Kinetics. The effect of enzyme concentration using DEAE-Sephadex purified enzyme is shown in Figure 7. The time course of the reaction (DEAE-cellulose purified enzyme) as a function of different substrate concentrations is depicted in

CHART I

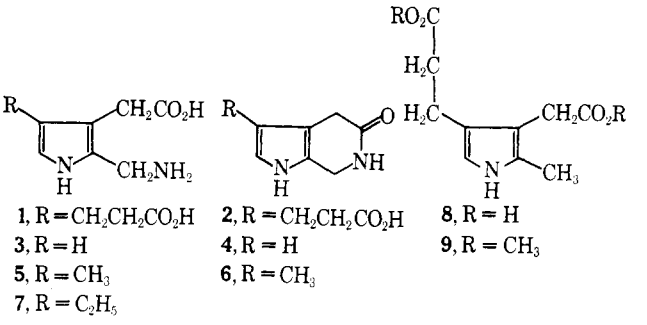


Figure 8. A value of V_{max} = 0.33 μmol/min per mg of protein was obtained by extrapolating the resulting concave upward curve on the double-reciprocal plot ot the ordinate (Figure 9). The interaction coefficient of the substrate derived from Hill plots was found to be n = 1.7. With more purified enzyme preparations (DEAE-Sephadex purified enzyme or Sephadex G-100 purified enzyme), the resulting kinetic was also found to be sigmoidal (Figure 10). By addition of either Co^{2+} or Ni^{2+} the sigmoidal kinetic was transformed into a Michaelis–Menten one. A similar desensibilization of the enzyme was obtained by freezing. The interaction coefficients dropped from values of n = 2.2 for the Sephadex G-100 purified enzyme, to n = 1.3 for the enzyme in the presence of Ni^{2+}, and to n = 1.1 for the frozen enzyme. The V_{max} increased from a value of V_{max} = 0.32 μmol/min per mg of protein for the Sephadex G-100 enzyme to a V_{max} = 0.42 μmol/min per mg of protein for the frozen enzyme, and V_{max} = 0.8 μmol/min per mg of protein for the enzyme in the presence of Ni^{2+}. The frozen enzyme had a $K_{m,app}$ of 0.28 mM, and a $K_{m,app}$ of 1.0 mM was obtained for the enzyme in the presence of Ni^{2+} and Co^{2+}. It is interesting to note that the sigmoidal kinetics were determined in glycine, phosphate, and Tris-HCl buffers.

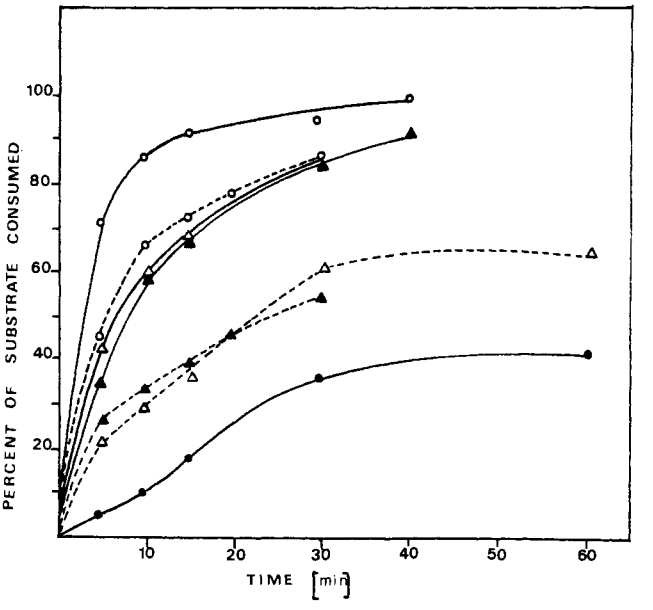


FIGURE 6: Oxidation rates of PBG, MPaa, PBGL, MPaaL, and PaaL and methyl ester of PBGL. The incubation conditions and assay were those described under Methods, except for substrate and incubation times which were the indicated in the figure: (—●-●—) PaaL **4** (17 nmol); (- -△- -△- -) PBG **1** (10.8 nmol); (- -▲- -▲- -) PBG **1** (19.2 nmol); (—▲—▲—) PBGL **2** (19 nmol); (—△—△—) methyl ester of PBGL (21.5 nmol); (- -○- -○- -) MPaa **5** (12.5 nmol); (—○—○—) MPaaL **6** (13.5 nmol).

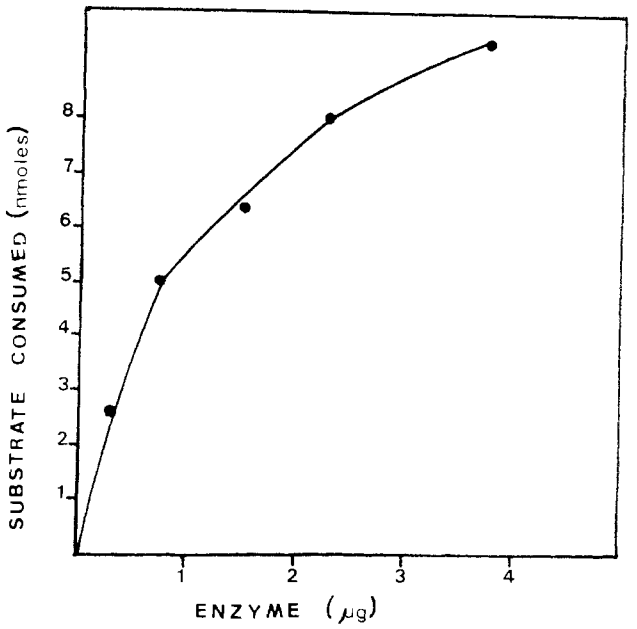


FIGURE 7: Effect of enzyme concentration. The incubations conditions and assay were described under Methods except for the amount of enzyme used. Incubations were carried out during 30 min.

In Bicine buffer at pH 7.4 a Michaelis–Menten kinetic was obtained. Cd^{2+} (10 mM) was found to be a negative effector of the oxygenase, decreasing its V_{max} to one-third of its original value.

When the oxidation of 2-aminomethyl-4-methyl-3-pyrroleacetic acid was examined, its kinetic was also found to be

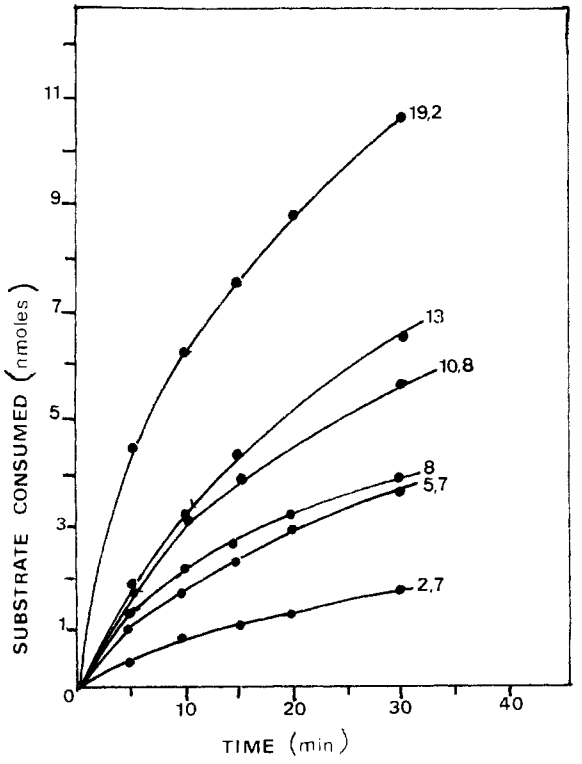


FIGURE 8: Time course of the reaction. The incubation mixtures were those described under Methods, except for the amounts of substrate, which were the indicated in the figure (values in micromoles). Incubation times were those given in the figure.

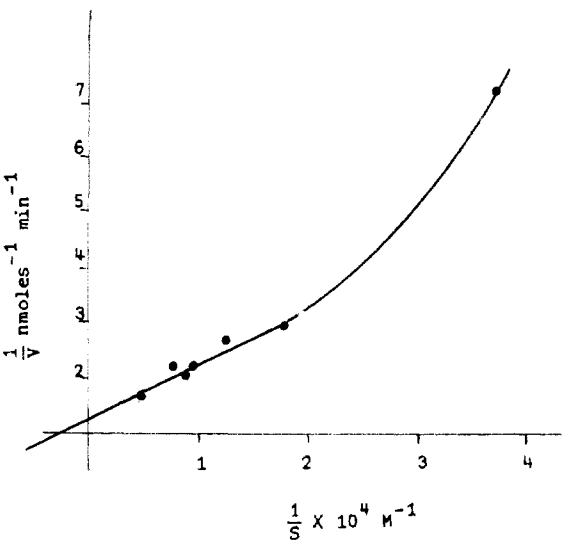


FIGURE 9: Double-reciprocal plot of the porphobilinogen oxygenase activity. Initial velocities were calculated from the data given in Figure 8. DEAE-cellulose enzyme was used.

sigmoidal (Figure 11). A V_{max} of 0.5 μmol/min per mg of protein was found, and a n of 1.4 was calculated.

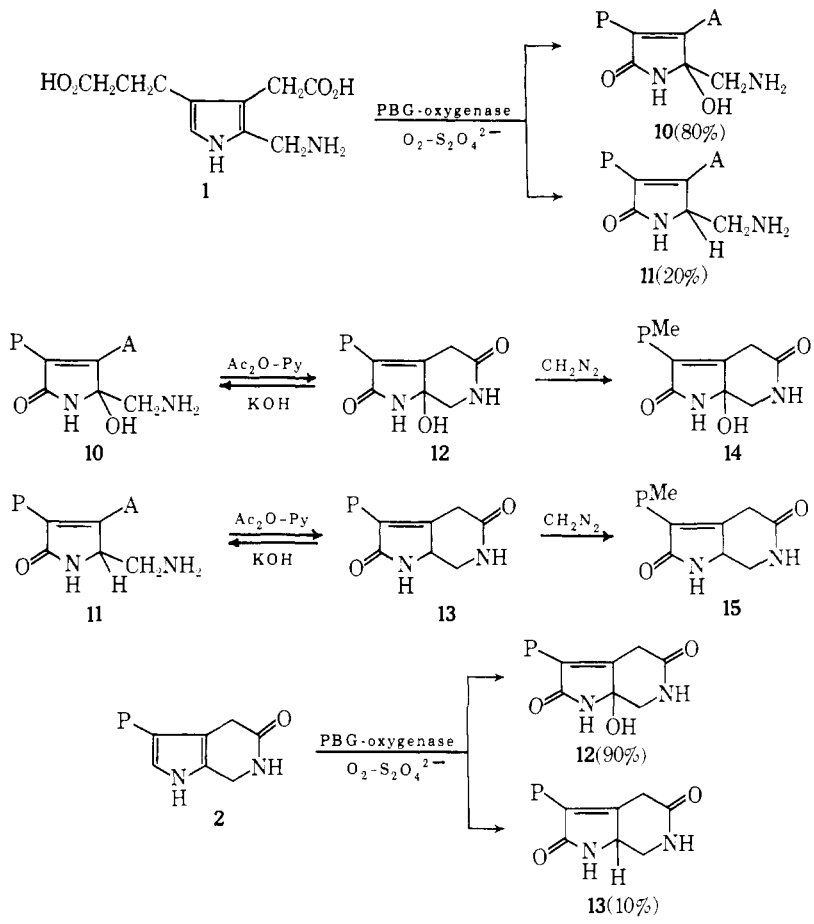
Effects of Inhibitors and Activators. Porphobilinogen oxygenase is a metalloenzyme. This was demonstrated by its total inactivation in the presence of EDTA (0.5 mM) and α,α′-dipyridyl (5 mM). The metal bound to the enzyme was found to be iron, identified by its atomic absorption spectrum. The inhibitory effect of α,α′-dipyridyl indicated that it was probably present as ferrous ion. The metal could be separated from the protein by a 24-hr dialysis against EDTA (1 mM). The dialyzed inactive enzyme was filtered through a Sephadex G-25 column (glycine–sodium hydroxide buffer, pH 8.2) and the eluted fraction with the highest absorption at 230 and 280 nm was assayed for enzyme activity and was found to be inactive. By addition to the incubation mixture of ferric chloride

TABLE III: Influence of Metal Ions on Porphobilinogen Oxygenase Activity.[a]

Metal Ions	Concn (mM)	Remanent Porphobilinogen Oxygenase Act. (%)
$CaCl_2$	2.5	90
	25	20
$CdCl_2$	2.5	7.1
	1.2	14
	0.5	67
$PbCl_2$	0.5	50
	2.5	0
$CuCl_2$	2.5	0
KCl	100	100
$MnSO_4$	10	25
$FeSO_4$	25	100
$FeCL_3$	25	100

[a] The DEAE purified enzyme dialyzed against water was used as enzyme source and its activity was considered 100%. The metals were added to the standard assay mixture at the indicated final concentrations. The buffer used was glycine–sodium hydroxide (pH 8.4). Incubations were performed during 30 min.

SCHEME I



A = CH_2CO_2H; P = $CH_2CH_2CO_2H$; P^{Me} = $CH_2CH_2CO_2CH_3$

or magnesium chloride (25 mM), 70% of the enzymatic activity was restored. Addition of Cd^{2+}, Ca^{2+}, and Cu^{2+} was without effect. The effect of several metal ions acting as positive and negative effectors on the kinetics of the enzyme has already been described. A number of metals had also an inhibitory effect on the oxygenase (Table III). Especially noteworthy is the inhibitory effect of lead salts. 2-Mercaptoethanol (5 mM) and dithiothreitol (5 mM) abolished entirely the porphobilinogen-consuming activity of the purified enzyme. *p*-Chloromercuribenzoate (5 mM) had no effect on the enzymatic activity. *N*-Ethylmaleimide (1 mM) inhibited 80% of the activity. Sodium azide (24 mM) and sodium cyanide (25 mM) had no effect on the DEAE-cellulose purified enzyme. The same concentration of sodium cyanide inhibited 50% of the activity of the Sephadex G-75 purified enzyme. The addition of tryptophan (2.5 mM) inhibited more than 50% of porphobilinogen oxygenase activity. The inhibition was of a noncompetitive nature.

Products Formed

Enzymatic Oxidation of Porphobilinogen (1). The enzymatic oxidation of porphobilinogen (1) afforded two products: 2-aminomethyl-2-hydroxy-3-carboxymethyl-4-carboxyethyl-3-pyrrolin-5-one (**10**) as the main product and 2-aminomethyl-3-carboxymethyl-4-carboxyethyl-3-pyrrolin-5-one (**11**) as the secondary product (Scheme I).

Both 3-pyrrolin-5-ones **10** and **11** had the same R_F when examined by tlc on cellulose-coated plates even when different solvents were used as developers. They can however be separated by converting them into the corresponding lactams with acetic anhydride in pyridine, separating the methyl esters of the latter, and saponifying them back to the starting 2-amino-

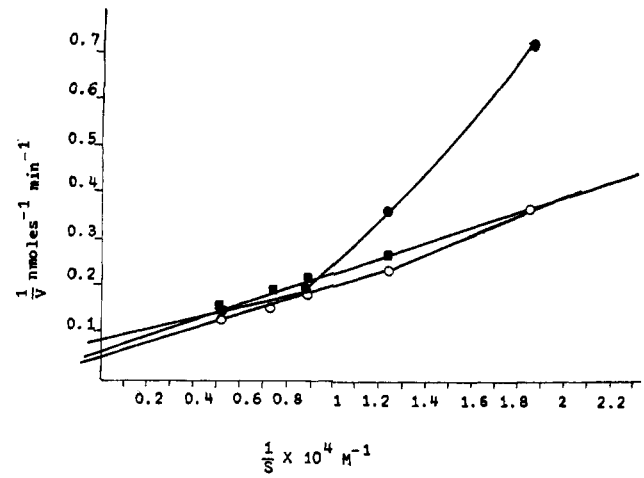


FIGURE 10: Double-reciprocal plots of Sephadex G-100 purified enzyme. The incubation mixtures were the indicated in Methods. Incubations were carried out at 37° during 10 min: (●) enzyme without additions; (○) with Ni^{2+} (10 mM) added to the standard incubation mixture; (■) enzyme frozen at −15° during 7 days.

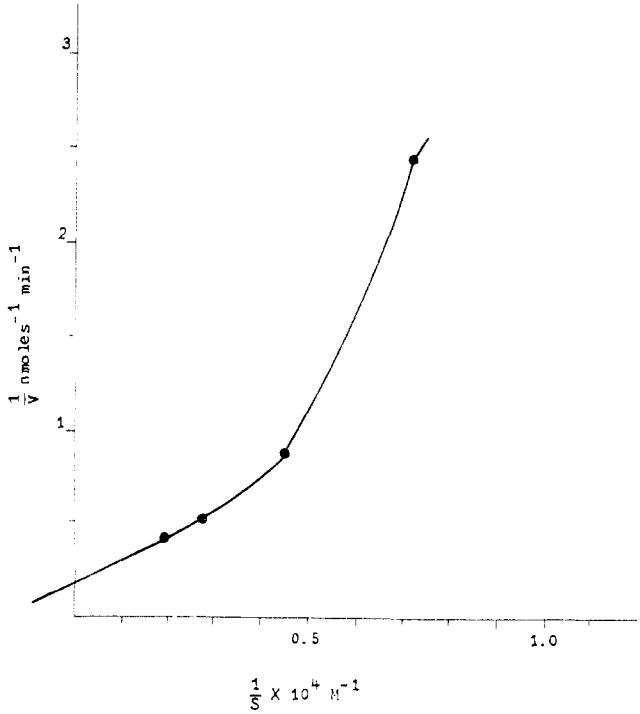
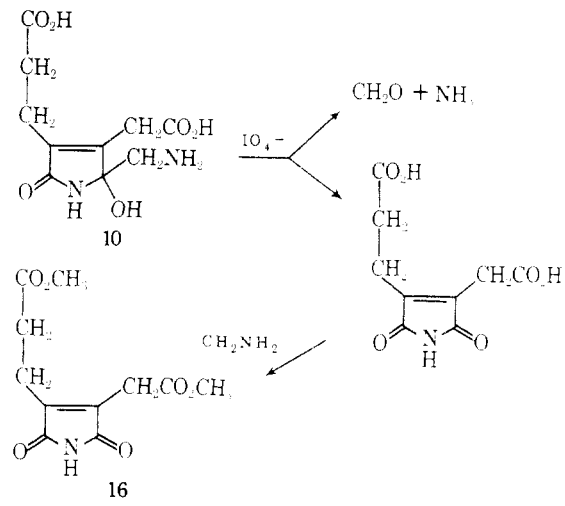


FIGURE 11: Effect of MPaa **5** concentration on the rate of the enzymatic activity. The incubation mixture was the described in Methods, except for the substrate, which was the indicated in the title. Incubation time was 5 min at 37°.

methyl-3-pyrrolin-5-ones **10** and **11** (Scheme I). The lactams **14** and **15** could be efficiently separated by column chromatography as described under Methods. The methyl esters **14** and **15** were also volatile enough to allow mass spectra determinations. In our preliminary report (Frydman *et al.*, 1972), we reported that the 2-aminomethyl-3-pyrrolin-5-one (**11**) afforded a carbobenzyloxy derivative which was useful for isolating and characterizing the compound. The 5-hydroxy-3-pyrrolin-2-one derivative **10**, however, did not afford an isolable carbobenzyloxy derivative and was not detected by this method.

2-Aminomethyl-2-hydroxy-3-carboxymethyl-4-carboxyethyl-3-pyrrolin-5-one (**10**). The mixture of oxidation products from porphobilinogen were isolated as described in Methods using preparative incubations. The obtained compounds had an R_F of 0.25 (tlc on cellulose). The product mixture (11.5 mg) was dissolved in dry pyridine (1 ml) and treated at 5° with 0.2 ml of acetic anhydride added in a small portions. The solution was kept at 5° during 1 hr, evaporated to dryness *in vacuo*, the residue dissolved in 1 ml of methanol, and treated with an excess of ethereal diazomethane during 15 min. The residue obtained after evaporation of the solution to dryness was examined by tlc (solvent I) and was found to consist of two compounds: R_F 0.57 and 0.39. They were separated by column chromatography on silica gel as described in Methods. The substance with an R_F of 0.57 (10 mg) was pyrrole lactam **14**: mass spectra (relative intensities, %) 254 (M^+, 10), 236 (254 − H_2O, 50), 205 (236 − OCH_3, 55), 204 (236 − $HOCH_3$, 43), 162 (236 − $CH_2CO_2CH_3$ base peak), 149 (254 − H_2O − $CH_2CH_2CO_2CH_3$, 45), 107 (254 − H_2O − CH_2CO − $CH_2CH_2CO_2CH_3$, 89); nmr (CF_3COOH) (δ = 0 for Me_4Si): δ 2.8 (m, 4), CH_2CH_2; 4.0 (s, 3), OCH_3; 3 8 (b, 4, CH_2CO; CH_2NH). The pyrrole lactam **14** (10 mg) was dissolved in 0.2 ml of 2 N KOH and kept at 25° during 72 hr. The solution was adjusted to pH 5 with acetic acid and purified through a Dowex 1-X4

SCHEME II



column as described in Methods. The product isolated after evaporation to dryness of the acidic eluates was the 5-aminomethyl-5-hydroxy-3-pyrrolin-2-one (**10**); R_F 0.25 (tlc on cellulose); nmr (D_2O; δ = 0 for sodium 4,4-dimethyl-4-silapentane-1-sulfonate), δ 2.7 (m, 4, CH_2CH_2CO), 3.4 (s, 2, CH_2CO), 3.5 (b, 2, CH_2NH_2). It had no detectable absorption in the ultraviolet or visible spectra, was very soluble in water and ethanol, and insoluble in organic solvents.

An independent chemical proof of structure was achieved by oxidizing the pyrrolinone **10** with sodium metaperiodate (Scheme II).

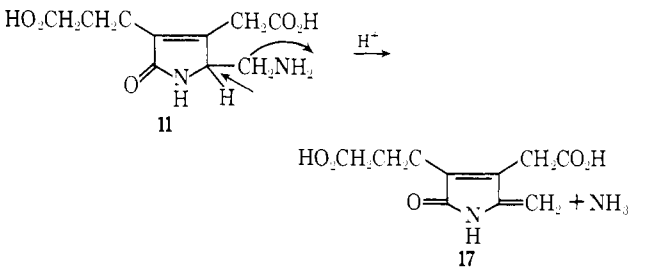
The pyrrolinone **10** (5 mg) was dissolved in 0.8 ml of water and 0.1 ml of a 0.037 M sodium metaperiodate solution was added. The solution was kept at 25° during 15 min, after which 0.1 ml of a 0.1 M solution of sodium arsenite was added. After 20 min at 25°, 1 ml of chromotropic acid solution (200 mg of reagent in 100 ml of 10 N sulfuric acid) was added and the formed formaldehyde was determined according to the usual method (Speck, 1962), using a previously prepared calibration curve. One molar equivalent of formaldehyde was liberated per mole of oxidized pyrrolinone.

In a second identical oxidation run, the oxidation mixture was adjusted to pH 2 with hydrochloric acid after the sodium arsenite step, evaporated to dryness, the residue was dissolved in methanol and treated with an excess of ethereal diazomethane for 15 min at 25°. The solution was evaporated to dryness and the residue was examined by tlc against a standard of the dimethyl ester of 3-carboxymethyl-4-carboxyethylmaleimide (**16**) (prepared by chromic acid oxidation at 25° of the octamethyl ester of uroporphyrin).[a] Using different solvents (carbon tetrachloride–ethyl acetate–cyclohexane, 5:3:1; carbon tetrachloride–cyclohexane, 6.5:3.5) it was found that the main component of the metaperiodate oxidation mixture was identical with the dimethyl ester **16**, in agreement with the expected sequence (Scheme II).

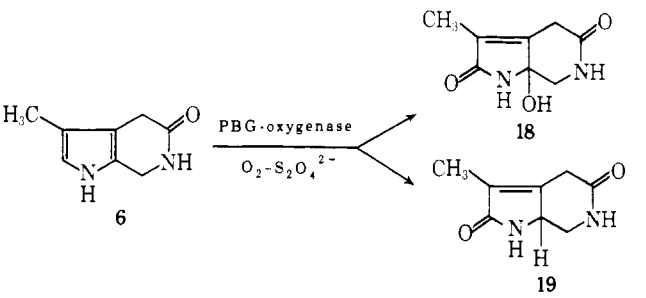
2-Aminomethyl-3-carboxymethyl-4-carboxyethyl-3-pyrrolin-5-one (**11**). The pyrrole lactam methyl ester with an R_F of 0.39 (2.6 mg), obtained as described in the preparation of **10**, had the structure **15**: mass spectra *m/e* (relative intensities, %) 238 (M^+, 90), 209 (238 − CH_2NH, 45), 207 (238 − OCH_3, 40), 181 (238 − CH_2NH − CO, 55); nmr (CF_3COOH, δ = 0 for Me_4Si), δ 2.75 (m, 4, CH_2CH_2), 3.8 (b, 2, CH_2CO), 3.97

[a] We are indebted to Professor W. Rudiger (München) for this technique.

SCHEME III



SCHEME IV



(s, 3, OCH_3), 4.3–3.8 (m, 3, $NHCH_2CH$); ir (KBr) 1675 cm^{-1} (CO lactam), 1750 cm^{-1} (CO ester); mp 156–158° (methanol–ether).

By saponification of the lactam with the technique used for the saponification of **14**, and isolation of the 2-aminomethylpyrrolone **11** through a Dowex 1-X4 resin, the latter compound was obtained as a slightly colored solid: R_F 0.25 (tlc on cellulose); nmr (D_2O; δ = 0 for sodium 4,4-dimethyl-4-silapentane-1-sulfonate) δ 2.65 (m, 4, CH_2CH_2CO), 3.32 (s, 2, CH_2CO), 3.5 (b, 2, CH_2NH_2), 4.15 (m, 1, C-2H); ir (KBr) 1690 cm^{-1}, (CO pyrrolinone), 1720 cm^{-1} (CO_2H).

By heating at reflux **11** (5 mg) dissolved in 1 ml of glacial acetic acid during 30 min, followed by evaporation of the solution *in vacuo*, 3-carboxyethyl-4-carboxymethyl-5-methylene-3-pyrrolin-2-one (**17**) was obtained (Scheme III): R_F 0.80 (tlc on cellulose located with Ehrlich's reagent at 100°); uv_{max}^{EtOH} 265 nm (ϵ 13,200); nmr (D_2O); δ 5.18 (b, 2, $=CH_2$), 3.40 (s, 2, CH_2CO), 2.70 (m, 4, CH_2CH_2CO). Its dimethyl ester **20** (obtained either with diazomethane or with methanol–hydrogen chloride) had R_F 0.59 (tlc on silica gel, solvent II); uv_{max}^{EtOH} 265 nm (ϵ 5300)); nmr (Cl_3CH, δ = 0, Me_4Si), δ 2.6 (b, 4, CH_2CH_2), 3.5 (s, 2, CH_2CO), 3.65, 3.7 (s, 6, OCH_3), 4.9 (m, 2, $=CH_2$), 8.1 (b, 1, NH). Both the acid **17** and its ester **20** were identical with synthetic samples (see below) when compared by tlc, uv, and nmr.

Enzymatic Oxidation of Porphobilinogen Lactam (**2**). The enzymatic oxidation of porphobilinogen lactam **2** afforded the 5-oxopyrrole lactams **12** and **13** (Scheme I). They were isolated by transforming them into the corresponding methyl esters **14** and **15** which were separated as above described. The relative proportions of both in the oxidation mixture depended of the enzyme preparation used and varied with its purification stage. The main component was always the 2-hydroxypyrrole lactam **12** and its concentration among the oxidation products oscillated between 85% (crude enzyme) and 100% (purified enzyme).

Enzymatic Oxidation of Pyrrole Lactam (**6**). The oxidation of the lactam of 2-aminomethyl-3-carboxymethyl-4-methylpyrrole (**6**) afforded two products; R_F 0.6 and 0.30 (tlc on silica; solvent I) (Scheme IV). They were separated by column chromatography on silica gel as described under Methods.

3-Methyl-2,5-dioxo-7a-hydroxy-2,4,5,6,7,7a-hexahydro-1H-pyrrolo[2,3-c]pyridine (**18**). It was identified as the product with an R_F of 0.6 and it made up 80% of the oxidation mixture. Its structure was secured from its spectral properties: mass spectra *m/e* (relative intensities, %), 182 (M^+, 10%), 164 (182 − H_2O, 60%), 153 (M − CH_2NH, 10%), 135 (M − H_2O − CH_2NH, 50%), 125 (153 − CO, 60%), 107 (125 − H_2O, 45%); nmr (CF_3COOH, δ = 0 Me_4Si) δ 2.5 (s, 3, CH_3), 4.0 (b, 2, CH_2CO), 4.5 (b, 2, CH_2NH); ir (KBr), 1645 cm^{-1} (CO, six-membered ring), 1675 cm^{-1} (CO, five-membered ring). The lactam **18** was devoid of uv and visible absorption spectra above 230 nm.

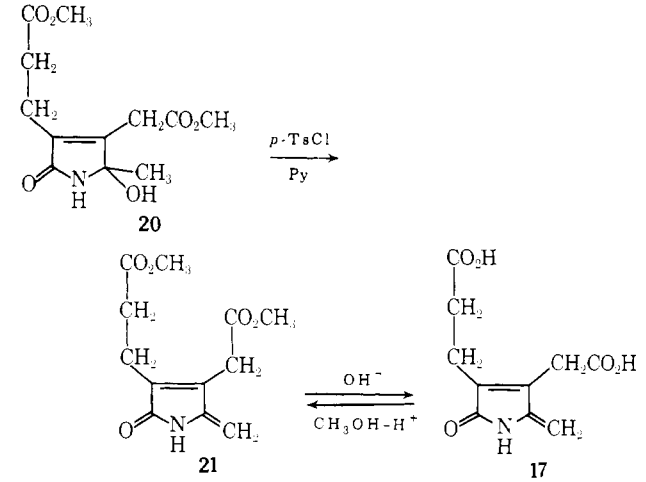
3-Methyl-2,5-dioxo-2,4,5,6,7,7a-hexahydro-1H-pyrrolo-[2,3-c]pyridine (**19**). The substance was identified as the product with an R_F of 0.30. It has mp 250–253° (methanol) and its spectral properties secured its structure: mass spectra *m/e* (relative intensities, %), 166 (M^+, 30%); 137 (M − CH_2NH, 60), 151 (M − CH_3, 15), 110 (M − CHNHCO, 50), 109 (M − CH_2NHCO, base peak); nmr (CF_3COOH) δ 2.5 (s, 3, CH_3), 3.95 (b, 2, CH_2CO), 4.4 (m, 2, CH_2NH), 3.2 (t, 1, >CH, J = 6 Hz); ir (KBr) 1675 cm^{-1} (CO, six-membered ring), 1695 cm^{-1} (CO, five-membered ring). The lactam was devoid of uv and visible absorption spectra above 230 nm.

Enzymatic Oxidation of 2-Methyl-3-methoxycarbonylmethyl-4-methoxycarbonylethylpyrrole (**9**). The enzymatic oxidation with porphobilinogen oxygenase of **9**, afforded as only oxidation product, 2-methyl-2-hydroxy-3-methoxycarbonylmethyl-4-methoxycarbonylethyl-3-pyrrolenin-5-one (**20**). Two-hundred incubation mixtures prepared as described in Methods were pooled (5 ml) and evaporated to dryness, and the residue was repeatedly extracted with hot methanol. The extracts were evaporated to dryness *in vacuo*, and the residue was filtered through a silica gel column as described under Methods (solvent II was used). The eluted fractions containing the pyrrolinone **20** were pooled and evaporated *in vacuo*. The pure oily residue, R_F 0.30, gave the following spectral data: mass spectra *m/e* (relative intensities, %), 271 (M^+, 36), 256 (M − CH_3, 74), 254 (M − OH, 42), 253 (M − H_2O, 96), 240 (M − OCH_3, base peak); nmr (Cl_3CH, δ = 0, Me_4Si), δ 1.5 (s, 3, CH_3), 2.5 (b, 4, CH_2CH_2), 3.6 (s, 2, CH_2), 3.68, 3.7 (s, 6, OCH_3), 3.41 (b, 1, OH), 6.9 (b, 1, NH); ir (Cl_3CH) 3460 cm^{-1} (OH), 1740 (CO). No uv absorption above 220 nm.

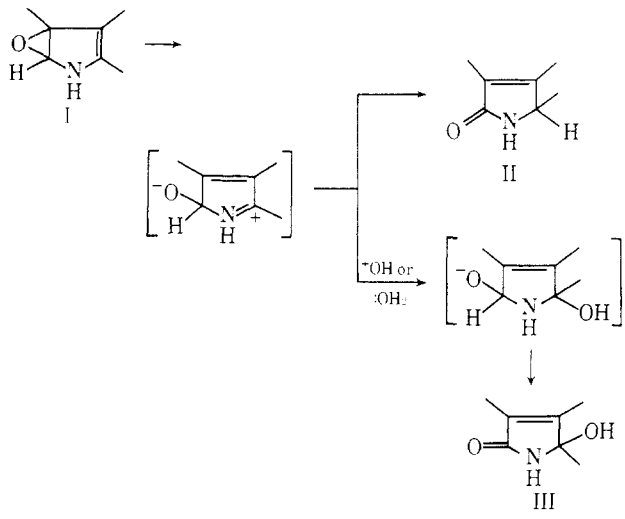
The pyrroleninone **20** was dehydrated by heating with *p*-toluenesulfonyl chloride in pyridine (Scheme V).

The reaction was carried out by dissolving 6 mg of **20** and

SCHEME V



SCHEME VI



14 mg of *p*-toluenesulfonyl chloride in 0.5 ml of dry pyridine, The mixture was heated during 90 min at 80°, the solvent was evaporated to dryness *in vacuo*, the residue was redissolved in water, the solution was adjusted to pH 7 with sodium carbonate, evaporated again to dryness, and the residue was extracted twice with hot ethanol. The extracts were evaporated to dryness and the obtained product was purified by filtration through a silica gel column (solvent II). The eluted product (3 mg) had an R_F of 0.59 and was identical (uv, nmr and tlc) with the diester obtained from the 2-methylene-3-pyrrolenin-5-one acid (**17**), derived from the deamination of **11** (Scheme III). The acid **17** was obtained from its diester **21**, by dissolving 5 mg of the latter in 0.2 ml of a 2 N sodium hydroxide solution, heating at reflux during 30 min, and acidifying with a Dowex 50W-X2 resin in its acid form.

Discussion

Porphobilinogen oxygenase isolated from wheat germ is the only known enzyme which oxidizes pyrrole derivatives. As part of the group of the pyrrolooxygenases (Frydman *et al.*, 1972b) it behaves as a mixed-function oxygenase, requiring the presence of both oxygen and a reducing agent for its activity. It oxidized not only porphobilinogen but also different synthetic alkylpyrroles with a free C-5 position. If the formation of an intermediate "oxene" is accepted as a mechanism for mixed-function oxidases (Jerina *et al.*, 1970), the oxidation of a 2,3,4-trialkylpyrrole can be visualized by the formation of a transient pyrrole epoxide (I) (Scheme VI).

Ring opening of the very unstable epoxide (I) will then afford a 5-oxo-3-pyrrolinone (II). Alternatively, hydration of the >C=NH$^+$ double bond will lead to a 2-oxo-3-pyrrolin-5-one (III). This last compound could also be formed during a second oxidation reaction, and the discrimination among both possibilities must be achieved by the use of $^{18}O_2$. No hydrase was yet detected in association with the oxygenase. The main oxidation product formed, the 2-oxo-3-pyrrolin-5-one (III), is very soluble in water and was not converted into porphyrin derivatives any more.

An important feature of the oxygenase is its presence in an inhibited form in the crude extracts due to its association with a proteic inhibitor. This property, as well as its allosteric nature, could prevent porphobilinogen consumption by the enzyme at low concentrations, and make it available as a substrate for porphobilinogen deaminase. It has already been mentioned that porphobilinogen is present in very low concentrations under physiological conditions and the higher $K_{m,app}$ values of porphobilinogen oxygenase as compared with that of porphobilinogen deaminase give the latter the lead during porphobilinogen consumption.

The enzyme was a metalloenzyme containing Fe^{2+}, but its noninhibition by sodium azide and cyanide and its lack of ultraviolet and visible absorption spectra discard an hemoproteic structure for the oxygenase. The influence of various divalent metals on its allosteric kinetics, acting as positive and negative effectors of the enzyme, is closely related to its existence in multiple molecular weight forms and is currently under study.

Porphobilinogen oxygenase was located in the chloroplasts when searched for in green leaves (Frydman *et al.*, 1972a). It has already been described how illuminated chloroplasts provide the necessary reducing power to trigger pyrrolooxygenase activity (Frydman *et al.*, 1972b), a feature which can be of relevance for regulating the concentration of porphobilinogen available during chlorophyll biosynthesis. The action of certain metals (Ni^{2+}, Co^{2+}) in activating the enzyme and of others (Pb^{2+}, Cd^{2+}) in inhibiting it, may be of physiological relevance in plants. As was discussed elsewhere (Frydman and Frydman, 1973), tryptophan pyrrolooxygenase from wheat germ has a powerful inhibitory action on porphobilinogen deaminase. This effect, together wiith the enzymatic oxidation of porphobilinogen by porphobilinogen oxygenase may afford a twofold check on the amount of porphyrins formed under normal metabolis conditions.

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