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PYRROLOOXYGENASES: ISOLATION, PROPERTIES, AND PRODUCTS FORMED*

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SUMMARY

1. A new group of enzymes was isolated from wheat germ and rat liver which oxidized the pyrrole ring of indoles affording *o*-formamidophenacyl derivatives. They behaved as mixed-function oxidases and were named pyrrolooxygenases. Tryptophan, ethyl *N*-acetyltryptophan, skatole, 3-indoleacetic acid, 3-indolepropionic acid and indole were substrates of the pyrrolooxygenases. The enzymatic oxidations were catalyzed by at least two enzymes within the group; one acting on tryptophan and its derivatives, and the other one acting on skatole and the other indoles.

2. The pyrrolooxygenases had an absolute requirement for oxygen and an exogenous reducing agent. The reducing agents were illuminated chloroplasts for the plant enzymes, and NADPH and a microsomal transport system for the mammalian enzymes. Both could be replaced by sodium dithionite.

3. Chelating agents, such as α,α'-dipyridyl and EDTA inhibited the enzymatic activity, while sodium cyanide had no effect. The enzymes were also inactivated by dithiothreitol and mercaptoethanol.

4. A natural heat-labile macromolecular inhibitor of the pyrrolooxygenases was present in the crude extracts and was separated during the succesive purification steps.

5. Formamidase (EC 3.5.1.9) activity was present in the extracts together with the pyrrolooxygenases and transformed the *o*-formamidophenacyl derivatives into the corresponding *o*-amino derivatives. During the purification steps the formylase activity was partly removed.

INTRODUCTION

The oxygenases are a group of ubiquituous enzymes, which oxidize aromatic, aliphatic and heterocyclic substrates[1]. Many of them are induced by substrates, and play a major role in drug metabolism[2]. We describe in this paper the existence in plants and animals of a new group of oxygenases, for which we proposed the name of

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

* Dedicated to Professor V. Deulofeu on his seventieth birthday.

pyrrolooxygenases[3], which oxidize the pyrrolic ring of indoles with formation of *o*-formamidophenacyl derivatives.

Among the indoles which were substrates of the pyrrolooxygenases were skatole (I), 3-indoleacetic acid (II), L- and D-tryptophan (III), ethyl *N*-acetyl-L-tryptophan (IV) and 3-indolepropionic acid (V). The extracts which contained the pyrrolooxygenases also contained the formamidases which hydrolyzed the *o*-formamido compounds to the *o*-aminophenacyl derivatives. The oxidation of these indolic substrates was catalyzed by at least two different enzymes; one specific for L- and D-tryptophan and ethyl *N*-acetyl-L-tryptophan, and the other specific for skatole, 3-indoleacetic acid, 3-indolepropionic acid and indole. Although these enzymes were not completely separated, their ratios of specific activity toward skatole and tryptophan differed in the purified preparations to that found in the crude extracts. Their different properties also confirmed the existence of two enzymes.

The products formed by oxidation of L-tryptophan were kynurenine, formylkynurenine and 2-oxotryptophan, the same products as those obtained from L-tryptophan by the action of tryptophan pyrrolase (EC 1.13.1.12)[4]. Tryptophan pyrrolooxygenase was, however, entirely different from tryptophan pyrrolase in its substrate specificity, its distribution in nature, and its intrinsic enzymatic properties. It was isolated without any induction by substrate, and it showed a greater affinity toward substituted tryptophan than toward L- and D-tryptophan itself. Skatole pyrrolooxygenase formed mainly 2-formamidoacetophenone, which was hydrolyzed enzymatically to 2-aminoacetophenone. The same products were formed from 3-indoleacetic acid.

Pyrrolooxygenases had the properties of mixed-function oxidases[1] since they had a specific requirement for a reducing agent, in addition to oxygen. The natural reducing systems were efficiently replaced by sodium dithionite. The enzymes were purified from wheat germ and from rat liver, and their presence was also detected in spinach and Swiss chard chloroplasts and in rat brain.

MATERIALS AND METHODS

Materials

NADH, NAD^+, $NADP^+$ and NADPH, L-tryptophan and D-tryptophan were purchased from Sigma Chemical Co. Skatole, 3-indoleacetic acid, 3-indolepropionic acid and 2,6-dichlorophenolindophenol (DCIP) were obtained from Eastman Kodak Company. DL-Kynurenine and 2-aminoacetophenone were purchased from Fluka. Sodium dithionite was a product of Amend Drug and Chemicals. L-[3-^{14}C]tryptophan (54.5 mCi/mM) was purchased from Amersham-Searle Corp. *N*-Acetylskatole, 2,3-dimethylindole, ethyl *N*-acetyl-L-tryptophan, 2-formamidoacetophenone, ethyl 2-acetamido-3-(2′-formamidobenzoyl)-propionate and ethyl *N*-acetyl-2-oxotryptophan were obtained by synthesis. All other reagents used were of analytical grade. Wheat germ was a gift of Molinos Rio de la Plata (Buenos Aires). Spinach was purchased in a local market. Spinach ferredoxin was prepared by the method of Tagawa and Arnon[5]. Spinach chloroplasts were prepared according to the method of Kalberer *et al.*[6], except for the omission of EDTA and isoascorbate. Heated chloroplasts were obtained after heating the whole chloroplasts for 10 min at 55 °C. Wistar albino rats weighing 150–200 g were used. All the solvents had been previously distilled.

TABLE I

PURIFICATION OF SKATOLE AND TRYPTOPHAN PYRROLOOXYGENASES FROM WHEAT GERM

One unit was defined as the amount of enzyme which catalyzed the consumption of 1 μmole of indolic substrate per ml of enzyme per h, under the conditions described in Materials and Methods.

Step	*Vol. (ml)*	*Total (mg)*	*Pyrrolooxygenase activity*							
			Skatole				*Tryptophan*			
			Total units	*Spec. act. (units/mg protein)*	*Yield (%)*	*Purification factor*	*Total units*	*Spec. act. (units/mg protein)*	*Yield (%)*	*Purification factor*
1. Crude extract	210	5460	156	0.028	100	—	471	0.086	100	—
2. pH 5 supernatant	210	4410	206	0.047	132	1.6	683	0.154	145	1.7
3. Ammonium sulphate 30–50%	40	1200	108	0.09	70	3.2	200	0.16	42	1.8
4. DEAE pooled fractions	168	122.4	715	5.84	458	208.5	1351	11	287	130
5. Sephadex G-100*	25	6	73	12.1	520	428				

* 5 ml of a DEAE pooled fraction were used in this step.

Preparation and partial purification of pyrrolooxygenases

The total purification of wheat germ pyrrolooxygenases is shown in Table I. All operations were performed at 0–4 °C. 100 g of wheat germ were extracted with 400 ml of water. The slurry was filtered through a nylon cloth and centrifuged at 20 000 × *g* for 15 min. The supernatant was adjusted to pH 5 with 1 M acetic acid and allowed to stand for 1 to 2 h. This suspension was centrifuged for 15 min at 20 000 × *g* and the supernatant fluid fractionated with solid ammonium sulphate. The fraction precipitating between 30 and 50% was dissolved in a minimum volume of 0.01 M Tris–HCl buffer (pH 7.6) and dialyzed overnight against 4 l of either the same buffer or water. After dialysis the fraction was centrifuged again in order to remove a precipitate which appeared during the dialysis, and then applied to a 3 cm × 20 cm DEAE-cellulose column equilibrated with 0.01 M Tris–HCl buffer (pH 7.6). Almost all the activity was eluted with 430 ml of the above mentioned buffer. Fractions of 40 ml were collected. Fig. 1 shows the activity of the different fractions toward skatole and tryptophan. Further purification of the enzymes was obtained by gel filtration through Sephadex G-100. 5 ml of the pooled fractions from DEAE-cellulose were filtered through a Sephadex G-100 column (3 cm × 40 cm) equilibrated with 0.05 M phosphate buffer, pH 7.4. Fractions of 5 ml were collected.

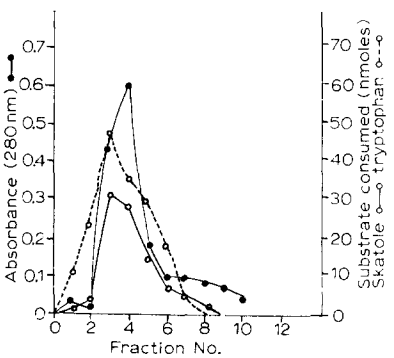


Fig. 1. Elution profile of wheat germ pyrrolooxygenases from a DEAE-cellulose column. The absorbtion and elution conditions were described in the text. Activities were assayed as in Materials and Methods.

When rat liver was used as a source of pyrrolooxygenases, 38 g (wet wt) were homogenized in a Sorvall blendor with 0.01 M Tris–HCl buffer, pH 7.6. The homogenate was centrifuged for 20 min at 20 000 × *g* (Crude Extract) and the supernatant solution was fractionated with solid ammonium sulphate. The fraction precipitating at 30–70% saturation was dissolved in a small volume of 0.01 M Tris–HCl buffer (pH 7.6) and dialyzed overnight against 4 l of water. The dialyzed fraction (300 mg of protein) was applied to a DEAE-cellulose column (3 cm × 20 cm) equilibrated with 0.003 M phosphate buffer (pH 7.4) and most of the activity was eluted with the same buffer. The active fractions were concentrated to one tenth of the original volume with Carbowax and dialyzed. The dialyzed fraction (2 mg) was applied to a second DEAE-cellulose column (1 cm × 15 cm) equilibrated with 0.003 M phosphate buffer, pH 7.4, and eluted with the same buffer. The active fractions were concentrated and used for

TABLE II

PURIFICATION OF SKATOLE PYRROLOOXYGENASE FROM RAT LIVER

The incubations were carried out as described in Materials and Methods.

Step	*Spec. act. (units/mg protein)*	*Activity recovered (%)*	*Purification factor*	*Total units*
1. Crude extract	0.0018	100	—	2.7
2. Ammonium sulphate 30–70%	0.02	330	—	9
3. First DEAE-cellulose	1	330	50*	9
4. Second DEAE-cellulose	5.4	430	2700*	11.7

* With respect to Step 2.

subsequent work. The purification steps for skatole pyrrolooxygenase from rat liver are summarized in Table II and similar results were obtained with tryptophan pyrrolooxygenase.

Liver microsomes were prepared by homogenizing 8 g of rat liver in a buffer containing 0.05 M phosphate, 0.3 M sucrose and 0.15 M KCl, pH 7.4. The homogenate was centrifuged at 5000 × *g* for 15 min, followed by a 20 000 × *g* centrifugation for 30 min. The supernatant was then centrifuged at 105 000 × *g* for 60 min: the pellet resuspended in the described buffer was used as a microsomes suspension.

Assay of pyrrolooxygenases

Unless otherwise indicated the standard reaction mixture contained, in a final volume of 100 μl: 10 μmoles of phosphate buffer (pH 7.4), 0.05 μmole of sodium dithionite, enzyme (5–20 μg of protein) and the indicated substrate, (skatole 50 nmoles; tryptophan or ethyl *N*-acetyl-L-tryptophan, 65 nmoles). Blanks were run simultaneously omitting either enzyme or dithionite. Incubations were usually run for 15–30 min at 37 °C. In the case of skatole substrate consumption was assayed 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+}. When the disappearance of either tryptophan or ethyl *N*-acetyl-L-tryptophan was assayed, the method of Udenfriend and Peterson[7] was used, reduced to one third of its volume. The amount of substrate consumed was calculated from standard concentration curves. In the case of tryptophan the standard curve was determined for each run. Protein was estimated by the method of Lowry *et al.*[8]. Alternatively, the protein concentration was estimated from the absorption at 260–280 nm, using a Beckman DU Spectrophotometer. The stoichiometric consumption of sodium dithionite was determined after 5 min incubation, by adding an excess of a 1% iodine solution and titrating the excess of iodine with a 0.01 M valorated solution of sodium thiosulfate. Blanks omitting either substrate or enzyme were run simultaneously. Oxygen consumption was measured using an Oxygraph Model K (Gilson Medical Electronics).

Large scale incubations with skatole or ethyl *N*-acetyl-L-tryptophan were carried out for isolation of the products formed during the enzymatic reaction. The preparative incubation mixture contained in a final volume of 4 ml, 4 mg of ethyl *N*-acetyl-L-tryptophan or skatole, 1 ml of 0.5 M phosphate buffer (pH 7.5), 4 ml of the

DEAE-cellulose enzyme and 20 mg of sodium dithionite. The incubations were kept at 37 °C for 3 h, by which time 50% of the substrate was consumed. Six of these incubations were pooled, diluted with 25 ml of water and extracted with methylene chloride (3 × 5 ml). The pooled extracts were dried (Na_2SO_4), evaporated to dryness and the residues obtained were separated by preparative thin-layer chromatography on silica-gel plates (E. Merck DC-Fertigplatten Kiesegel F254, Schichtdicke 2 mm were used). Benzene–methanol (98.5:1.5, v/v) was used as a developing solvent for separating the products obtained from skatole, and chloroform–methanol (97.5:2.5, v/v) was used for separating the products obtained from ethyl *N*-acetyl-L-tryptophan. The products were located by their fluorescence under ultraviolet light and were revealed by heating with Ehrlich's reagent, or when necessary were revealed by spraying first with Folin and Ciocalteu's reagent (E. Merck Folin–Ciocalteu's Phenol reagent diluted with 2 vol. of water) followed by a 16% sodium carbonate solution. For isolation purposes the fluorescent bands were cut out and the products eluted from the silica with ethyl acetate. On evaporation of the solvent to dryness, the products were obtained as pure substances.

NMR spectra were run on a Perkin Elmer R-12. Infrared spectra were determined in KBr wafers in a Perkin Elmer IR 21 and several mass spectra were determined by Morgan and Schaffer Comp. (Montreal, Canada). The products formed from [^{14}C]tryptophan were separated by paper chromatography on Whatman No. 1, using as solvents the upper layer of butanol–acetic acid–water (4:1:5, v/v/v) or *m*-cresol saturated with water and added with 8-oxyquinoline and KCN, as described[9].

RESULTS

Purification of wheat germ pyrrolooxygenases

The purification scheme for the pyrrolooxygenases (Table I) shows an increase of the total activity. The lower yields in the 30–50% ammonium sulphate step were due to the loss of enzymatic activity which remained in the supernatant and precipitated with 50–70% ammonium sulphate. An increase in activity was also obtained by storing the enzyme for a week or more at 0–4 °C. The increase in activity was due to the separation or inactivation (by cold) of an inhibitor present in the extracts. This inhibitor could be partly separated from the enzymes by adsorption and subsequent elution from C-γ alumina. Its presence in the crude extract or in the C-γ alumina eluate was evident when the extract or the eluate was added to a DEAE purified enzyme which resulted in a strong inhibition of the pyrrolooxygenase activity (Table III). The inhibitor was labile to cold and heat and is very likely a protein.

Filtration through Sephadex G-75 (Fig. 2) showed that skatole pyrrolooxygenase was present in two peaks of different molecular weights. Similar results were obtained when skatole pyrrolooxygenase (DEAE enzyme) was examined by analytical polyacrylamide gel electrophoresis, where several different bands with pyrrolooxygenase activity were detected.

Purification of rat liver pyrrolooxygenases

Crude extracts from rat liver showed almost no enzymatic activity (Table II). However, the subsequent purification steps markedly increased the activity. This was again due to the presence of a powerful proteic inhibitor in the crude extract which

TABLE III

PRESENCE OF A LABILE PYRROLOOXYGENASE INHIBITOR IN WHEAT GERM

The enzyme was assayed as described in Materials and Methods except for the indicated additions. 1 ml of the DEAE purified enzyme (2.7 mg/ml) was adsorbed on 1 ml alumina C-γ (dry weight: 5 mg alumina/ml) and eluted consecutively with 0.1, 0.2, 0.3, and 0.5 M of phosphate buffer, pH 7.4. The eluates were assayed for pyrrolooxygenase activity and for inhibiting activity.

Enzyme	*Addition*	*Skatole consumed (nmoles)*
DEAE enzyme	None	18
	Crude extract (10 μl)	5.6
	Heated extract (100 °C for 5 min)	19
	0.3 M phosphate eluate (25 μl)	9
	Heated 0.3 M phosphate eluate (55 °C for 15 min)	13.5
	Heated 0.3 M phosphate eluate (100 °C for 5 min)	18
	Aged 0.3 M phosphate eluate (3 days at 0–4 °C)	17
0.3 M phosphate eluate (25 μl)		1.8

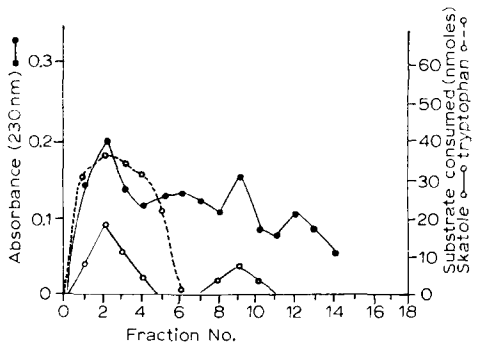


Fig. 2. Gel filtration chromatography of pyrrolooxygenases on Sephadex G-75. 100 μl of the DEAE purified wheat germ enzyme (containing 100 to 150 μg of protein) was applied to a Sephadex G-75 column (1 cm × 25 cm) and eluted with 0.05 M phosphate buffer, pH 7.4. 1 ml fractions were collected after discarding the excluded volume. Aliquots (50 μl) from each fraction were assayed for tryptophan and skatole pyrrolooxygenase activity. When higher enzyme concentrations were applied to this column, the enzyme elution profile differed from that presented in this figure.

was partially removed during the purification procedure. As was the case with the plant enzymes it was heat and cold labile and its addition to the active pyrrolooxygenase preparations resulted in an inhibition of the latter.

Pyrrolooxygenases as mixed-function oxidases

Mixed-function oxidases require both oxygen and a reducing agent for their activity. This was also the case with pyrrolooxygenases. They were inactive when measured anaerobically, or aerobically without the addition of an external reducing agent. The best was sodium dithionite, while ascorbate, cysteine, reduced glutathione, NADH or NADPH (in the case of the plant enzyme), methylene blue, and methylene blue *plus* ascorbic acid did not act as reducing agents. Since sodium dithionite is de-

composed in aqueous solution to form mainly sodium sulphite and bisulphite, the latter were also assayed as reducing agents during the enzymatic reaction. The results closely paralleled those obtained with sodium dithionite.

When the stoichiometry of the reaction was measured using L-tryptophan as a substrate, it was found (average values) that 13 nmoles of oxygen and 11 nmoles of dithionite were used up when 13 nmoles of substrate were consumed in 5 min. Similar results were obtained using skatole as a substrate, and they are consistent with a mixed-function oxidase mechanism, requiring that an equivalent amount of a reducing agent should be used up for each mole of oxygen consumed in the reaction.

The natural substitute in plants for sodium dithionite was found when intact illuminated spinach chloroplasts were used as a reducing agent (Table IV). With heated chloroplasts which lost the ability of carrying out water photolysis, an external electron donor (reduced 2,6-dichlorophenolindophenol) had to be supplied in order to carry out the enzymatic reaction. Spinach ferredoxin, although the usual reducing agent formed during photosynthesis, did not act by itself. On the contrary, it seemed to compete with the pyrrolooxygenase for the reducing capacity of the chloroplasts. Similar results were obtained with tryptophan pyrrolooxygenase.

TABLE IV

THE NATURAL REDUCING SYSTEM OF PYRROLOOXYGENASES IN PLANTS

The incubation mixture was as indicated in Materials and Methods. DEAE enzyme from wheat germ was used, except for the addition of 20 μl of a suspension of spinach chloroplasts or a mixture of heated chloroplasts, reduced DCIP (1 mM *plus* 50 mM of ascorbic acid) and ferredoxin. The incubations were run for 40 min at 25 °C, with illumination from a 100 W lamp.

Reducing system	*Skatole consumed (nmoles)*
Dithionite	24
Chloroplasts	0
Chloroplasts + *hv*	20
Heated chloroplasts + *hv*	0
Heated chloroplasts + DCIP red + *hv*	19
Heated chloroplasts + DCIP red + ferredoxin + *hv*	14
DCIP red	0

When rat liver enzyme was tested, it was found that sodium dithionite was the only artificial reducing agent capable of carrying out the enzymatic reaction. However, pyrrolooxygenase activity was also detected when NADPH and a 105 000 $\times$ *g* supernatant was added to a DEAE purified enzyme, although the activities were lower than with sodium dithionite (Table V). NADPH and the 105 000 $\times$ *g* supernatant were excellent reducing agents when liver microsomes were used as a source for the enzymes (Table V). This indicated the presence of pyrrolooxygenases in liver microsomes, and also that NADPH together with heat-stable factors present in the supernatant were the natural reducing agents.

Properties of the enzymes

The enzymes were very stable either at −15 °C or at 0–4 °C. The most purified

TABLE V

PYRROLOOXYGENASES AS MICROSOMAL ENZYMES IN RAT LIVER

The incubation mixture contained in a final volume of 100 μl: phosphate buffer, skatole and sodium dithionite as indicated in Materials and Methods, enzyme (first DEAE enzyme, (Table II)) 50 μl; or 25 μl of a microsomes suspension with or without addition of 25 μl of the 105 000 $\times$ g supernatant. When NADPH was used as reducing agent, the mixture contained: NADPH (0.04 μmole) nicotinamide (5 μmoles), glucose 6-phosphate (0.5 μmole), glucose 6-phosphate dehydrogenase (4 μl) and $MgCl_2$ (1 μmole). When heated microsomes or 105 000 $\times$ g supernatant were used, they were heated for 5 min at 90 °C. Incubations were run for 30 min at 37 °C.

System	*Reducing agent*	*Skatole consumed* (nmoles)*
DEAE enzyme	Sodium dithionite	18
	NADPH	0
	NADPH + 105 000 $\times$ g supernatant	8
Microsomes (25 μl)	NADPH	4.5
Microsomes + 105 000 $\times$ g supernatant	NADPH	16.2
Microsomes + heated 105 000 $\times$ g supernatant	NADPH	15.7
Heated Microsomes + 105 000 $\times$ g supernatant	NADPH	0

* Similar results were obtained using tryptophan as a substrate.

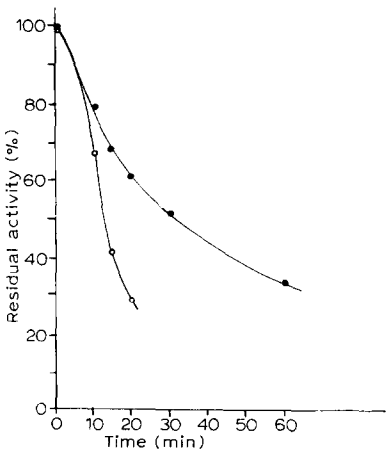


Fig. 3. Time course of temperature inactivation of wheat germ pyrrolooxygenases. The DEAE-cellulose purified enzyme (4.5 μg of protein) in 50 μl of 0.1 M phosphate buffer, pH 7.4, was preincubated for the time indicated at 60 °C. Measurements of residual activity were done by adding dithionite and either skatole (57 nmoles) or tryptophan (84 nmoles). Incubations were carried out in a final volume of 100 μl at 37 °C for 30 min. ●—●, skatole consumed; ○—○, tryptophan consumed.

fractions (Sephadex G-100) were less stable at −15 °C than at 0–4 °C. They could be kept for more than a year at 0–4 °C without any loss of activity.

Heat stability. The enzymes were quite resistent to temperature increases. Skatole pyrrolooxygenase lost only 20% of its activity when preincubated during 15 min at 54 °C. Tryptophan pyrrolooxygenase lost about 50% of its activity under the

same conditions. Thermal inactivation studies with the DEAE-cellulose purified skatole and tryptophan pyrrolooxygenases are shown in Fig. 3. Both enzymes were found to have different labilities. The optimum temperature for both pyrrolooxygenases was found to be 37 °C.

pH optimum. The pH curve profile determined for skatole pyrrolooxygenase was different from the one determined for tryptophan or ethyl *N*-acetyl-L-tryptophan pyrrolooxygenase (Figs 4a and 4b).

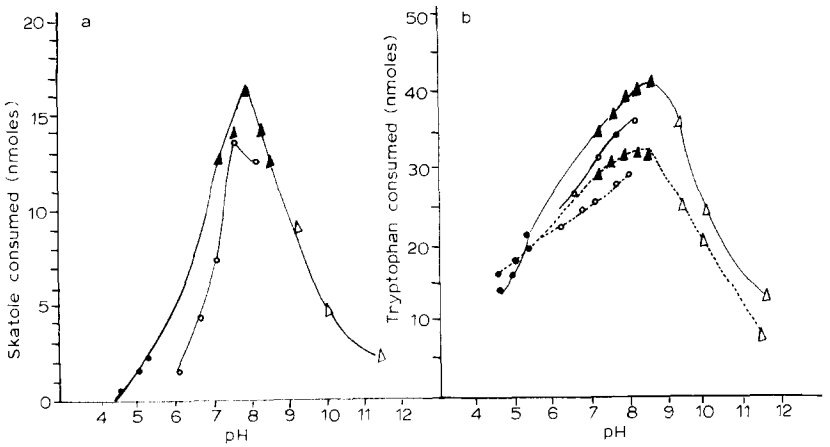


Fig. 4. Effect of pH on the reaction rates of wheat germ pyrrolooxygenases. The incubation was carried out with 10 μmoles of the indicated buffers at the indicated pH values. The incubation mixtures, except for the buffer, were indicated in Materials and Methods. Incubations were carried out in a final volume of 100 μl for 30 min at 37 °C. Citrate buffer (●); phosphate buffer (○); Tris-HCl buffer (▲) and glycine buffer (△). (a) skatole, (b) tryptophan (full lines), ethyl *N*-acetyl-L-tryptophan (dashed lines).

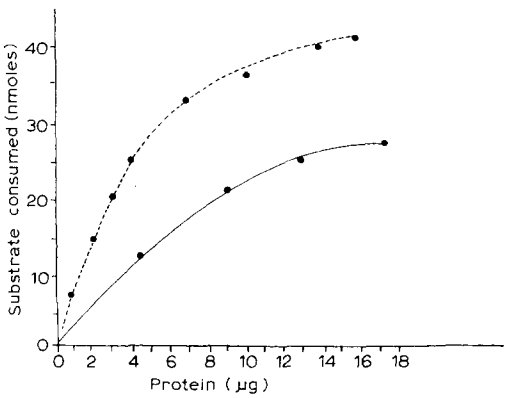


Fig. 5. Effect of protein concentration on pyrrolooxygenase activities. The incubation mixtures were the described except for the indicated wheat germ enzyme concentrations. The reaction was run for 15 min at 37 °C. Skatole (●—●); tryptophan (●---●).

Kinetics. The effect of enzyme and substrate concentration can be seen in Figs 5 and 6. The K_m for skatole was found to be 0.7 mM. The K_m for tryptophan was 0.8 mM, while for ethyl *N*-acetyl-L-tryptophan it was 0.2 mM, showing a greater affinity for the latter. The V values were 1 μmole/min per mg protein for skatole, 1.3 μmoles/min per mg protein for tryptophan and 0.67 μmole/min per mg protein for ethyl *N*-acetyl-L-tryptophan. The time course of the reactions can be seen in Fig. 7.

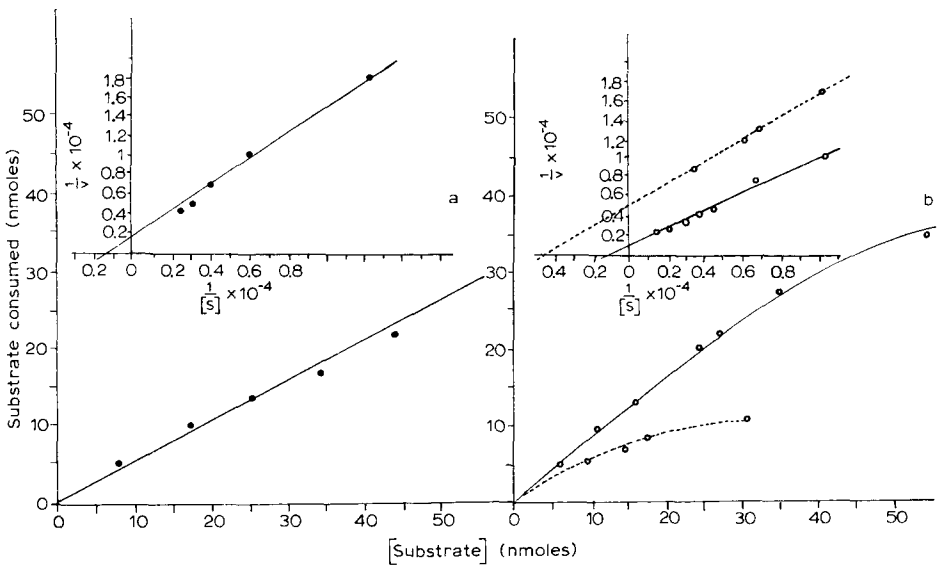


Fig. 6. Effect of substrate concentrations. Conditions were as described in Materials and Methods, except for the different substrate concentrations used. The Sephadex G-100 purified enzyme from wheat germ (2 μg) was used. The inserts show the Lineweaver–Burk plots for the different substrates used. Incubations were run at 37 °C for 30 min for skatole (●—●) and tryptophan (○—○) and 15 min for ethyl *N*-acetyl-tryptophan (○---○).

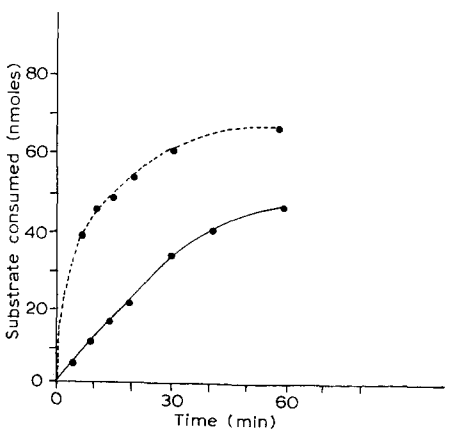


Fig. 7. Time course of the reaction. Standard incubation mixtures for wheat germ enzyme were run at 37 °C. Skatole (●—●); tryptophan (●---●).

Substrate specificity. The substrate specificity of the pyrrolooxygenases is shown in Table VI. The pyrrolooxygenases seem to have a structural requirement for an indole with an available free electron pair on the nitrogen and an α-unsubstituted carbon. *N*-Acetylskatole and 2,3-dimethylindole were other indolic compounds tested and found not to be substrates. Also histidine, phenylalanine, tyrosine and proline were not substrates.

TABLE VI

SUBSTRATE SPECIFICITY OF PYRROLOOXYGENASES

The incubation mixtures and conditions were described in Materials and Methods. Incubations were run for 30 min at 37 °C. The enzyme used was the DEAE purified pyrrolooxygenase from wheat germ.

Substrate	*Concentration (nmoles)*	*Activity (nmoles consumed)*	*Consumption (%)*
Skatole	50	26	52
Indole-3-propionic acid	50	27	54
Indole-3-acetic acid	48	16	33
Indole	41.5	3.6	9
L-Tryptophan	42	26	62
D-Tryptophan	42	21	50
Ethyl *N*-acetyl-L-tryptophan	34	26.3	77

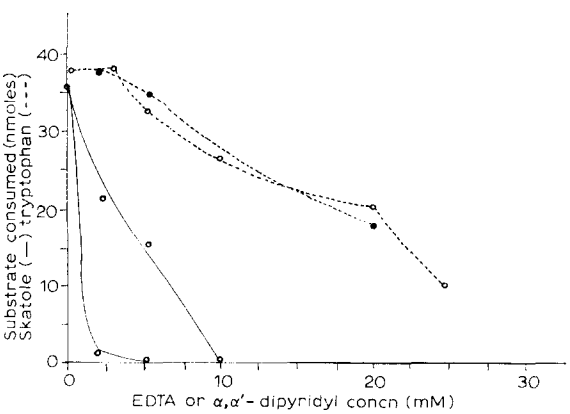


Fig. 8. Rate of skatole and tryptophan pyrrolooxygenases inactivation by chelating agents. The incubation mixtures were those indicated in Materials and Methods, except for the addition of EDTA (●) or α,α'-dipyridyl (○) at the indicated concentrations. The DEAE-wheat germ enzyme (5 μg) was used. Incubations were run for 15 min at 37 °C.

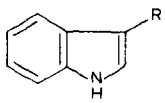
Effect of chelating agents. Metal chelating agents, such as EDTA, α,α'-dipyridyl and to a lesser extent *o*-phenanthroline, inhibited pyrrolooxygenase activities (Fig. 8). This indicated that pyrrolooxygenases were metalloproteins and that the most likely metal in view of the specificity of the inhibiting agents is Fe^{2+}. The Fe^{2+} should not be bound to haem since there was no inhibition by CN^- and no haem spectra could be observed in the most purified fractions. The inhibition of skatole pyrrolooxygenase was achieved at much lower concentrations of α,α'-dipyridyl and EDTA than those needed for tryptophan pyrrolooxygenase inhibition.

Both pyrrolooxygenases were totally inhibited by 1 mM dithiothreitol or mercaptoethanol, while lower concentrations (0.5 mM) of the thiols inhibited 25% of skatole pyrrolooxygenase and 60% of tryptophan pyrrolooxygenase. Different divalent cations such as Cu^{2+}, Ca^{2+}, Sr^{2+} and Cd^{2+} at 10 mM concentrations strongly inhibited the pyrrolooxygenases.

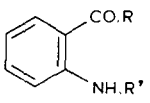
PRODUCTS FORMED

Enzymatic oxidation of skatole

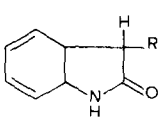
2-Formamidoacetophenone (VI). From 24 mg of skatole incubated as described with DEAE-cellulose enzyme, 10 mg (33% yield) of 2-formamidoacetophenone (Fig. 9) were obtained; m.p. 78–79 °C (literature[10] m.p. 78–79 °C); R_F, 0.50, (thin-layer chromatography); infrared spectroscopy, 3330 cm^{-1} (NH amide), 1695 (CO amide), 1655 (CO ketone); NMR (C^2HCl_3, tetramethylsilane, $\delta = 0$), 2.6 (s, 3, CH_3), 7.5 (m, 4, Ar–H), 8.5 (b, 1, NH), 8.65 (d, 1, CHO); mass spectroscopy, M^+: 163 ($C_9H_9NO_2$), 148 ($C_8H_6NO_2$), 135 (C_8H_9ON), 120 (C_7H_6NO), 92 (C_6H_6N), 77 (C_6H_5). These data secured structure (VI) for the product. It was identical with a synthetic sample of 2-formamidoacetophenone when compared by mixed m.p., thin-layer chromatography, and infrared spectroscopy. Synthetic 2-formamidoacetophenone was obtained either as described[10] or better by oxidizing skatole dissolved in methylene chloride with 3 equiv. of *p*-nitrobenzoic acid at 4 °C for 1 h. The mixture was then extracted with a 5% sodium bicarbonate solution, washed with water to neutrality, dried (Na_2SO_4) and evaporated to dryness *in vacuo*, when it afforded pure 2-formamidoacetophenone in 80% yield.



R: CH_3 (I)
R: CH_2CO_2H (II)
R: $CH_2-CH(NH_2)-CO_2H$ (III)
R: $CH_2.CH(NHOCCH_3).CO_2C_2H_5$ (IV)
R: $CH_2.CH_2.CO_2H$ (V)



R: CH_3; R': CHO (VI)
R: CH_3; R': H (VII)
R: $CH_2.CH(NHOCCH_3).CO_2C_2H_5$; R': CHO (IX)
R: $CH_2.CH(NHOCCH_3).CO_2C_2H_5$; R': H (X)
R: $CH_2.CH(NH_2).CO_2H$; R': H (XII)
R: $CH_2.CH(NH_2).CO_2H$; R': CHO (XIII)



R: CH_3 (VIII)
R: $CH_2.CH(NHOCCH_3).CO_2C_2H_5$ (XI)
R: $CH_2.CH(NH_2).CO_2H$ (XIV)

Fig. 9.

2-Aminoacetophenone (VII). From 24 mg of skatole incubated as described with DEAE-cellulose enzyme from wheat germ, 2.8 mg (12% yield) of 2-aminoacetophenone were obtained; R_F, 0.70 (thin-layer chromatography); NMR (C^2HCl_3, tetramethylsilane, $\delta = 0$), 2.5 (s, 3, CH_3), 6.2 (m, 2, NH_2), 6.6, 7.5 (m, 4, Ar–H); mass spectroscopy, M^+: 135 (C_8H_9ON), 120 (C_7H_6NO), 92 (C_6H_6N), 77 (C_6H_5). It was identical with an authentic sample of 2-aminoacetophenone when compared by thin-layer chromatography and NMR. When it was heated under reflux for 15 min and dissolved in concentrated formic acid it was promptly transformed in 2-formamidoacetophenone (VI). Saponification of (VI) back to 2-aminoacetophenone (VII) was achieved by dissolving it in a 2 M sodium hydroxide solution at 20 °C for 1 h.

3-Methyloxindole (VIII). From the described incubation mixture 1.3 mg (5% yield) of 3-methyloxindole was obtained, m.p. 120–122 °C (water), R_F, 0.17 (thin-layer chromatography), infrared spectroscopy 1720, 1680 cm^{-1} (CO amide, bands I and II); mass spectroscopy, M^+: 147 (C_9H_9ON), 132 (C_8H_6ON), 119 (C_8H_9N), 104 (C_8H_8), 91 (C_6H_5N), 89 (C_7H_5), 77 (C_6H_5). It was identical with a synthetic sample of 3-methyloxindole[11] when compared by mixed m.p., infrared spectroscopy and thin-layer chromatography.

Oxidation of 3-indoleacetic acid afforded 2-formamidoacetophenone and 2-aminoacetophenone in approximately 30% total yield. They were separated and identified as described above.

Enzymatic oxidation of ethyl N-acetyl-L-tryptophan (IV)

Ethyl 2-acetamido-3-(2'-formamidobenzoyl) propionate (IX). From 27 mg of ethyl *N*-acetyl-L-tryptophan incubated as described with DEAE-cellulose enzyme, 9.2 mg (30% yield) of (IX) were obtained; R_F, 0.37 (thin-layer chromatography); NMR (C^2HCl_3, tetramethylsilane, $\delta = 0$), 1.2 (t, 3, $\underline{CH_3}\cdot CH_2$, $J = 7$ cycles/s), 2.0 (s, 3, CH_3CO), 3.66 (d, 2, CH_2CO, $J = 4$ cycles/s), 4.16 (q, 2, $\underline{CH_2}\cdot CH_3$, $J = 7$ cycles/s), 4.97 (m, 1, $\underline{CH}$–$CO_2C_2H_5$), 6.62 (d, 1, $\underline{NH}$AC, $J = 8$ cycles/s), 7.5 (m, 4, Ar–H), 8.4 (b, 1, $\underline{CHO}$), 11.20 (b, 1, $\underline{NH}CHO$); infrared spectroscopy, 3300 cm^{-1} (NH amide), 1750 (CO ester), 1690 (CO amide), 1665 (CO ketone); mass spectroscopy, M^+: 306 ($C_{15}H_{18}N_2O_5$), 278 ($C_{14}H_{18}N_2O_4$), 263 ($C_{13}H_{15}N_2O_4$), 162 ($C_9H_8NO_2$), 158 ($C_7H_{12}NO_3$), 120 (C_7H_6NO), 148 ($C_8H_6O_2$), 92 (C_6H_6N). The NMR and mass spectroscopy data secured structure (IX) for the product. Ion *m/e* 158 is due to the ethyl *N*-acetylalanine side chain produced by fragmentation of the phenacyl structure. Fragment *m/e* 148 is the other half of the molecule. The isolated compound was identical, when compared by thin-layer chromatography, infrared spectroscopy and mass spectroscopy, with a synthetic sample prepared by chemical oxidation of ethyl *N*-acetyltryptophan (IV) with *p*-nitroperbenzoic acid as described above. Oxidation of (IV) was complete after 20 h at 25 °C (75% yield).

Ethyl 2-amino-3-(2'-formamidobenzoyl) propionate (ethyl N-acetylkynurenine) (X). From 27 mg of ethyl *N*-acetyltryptophan incubated as described, 4 mg (15% yield) of (X) obtained; R_F, 0.32 (thin-layer chromatography); mass spectroscopy, M^+: 278 ($C_{14}H_{18}N_2O_4$), 235 ($C_{12}H_{15}N_2O_3$), 134 (C_8H_8NO), 158 ($C_7H_{12}NO_3$), 120 (C_7H_6NO), 92 (C_6H_6N). Identification was secured by dissolving (X) in 0.2 ml of a 2 M sodium hydroxide solution at 25 °C during 2 h, after which the solution was adjusted to pH 4 with acetic acid, evaporated to dryness and the residue extracted with ethanol. On evaporation of the solvent a residue was obtained which was identical with

kynurenine when compared by paper chromatography using the described solvents[9].

Ethyl N-acetyl-2-oxotryptophan (XI). From 27 mg of ethyl *N*-acetyltryptophan, incubated as described, 1,4 mg (5% yield) of (XI) were obtained, R_F, 0.10 (thin-layer chromatography; infrared spectroscopy, 1750 cm^{-1} (CO ester), 1720 (CO lactam), 1690 (CO amide). It gave a blue colour characteristic of a 2-oxotryptophan[9] when sprayed with Folin's reagent as described in Materials and Methods. It was identical with a synthetic sample of ethyl *N*-acetyl-2-oxotryptophan[12] when compared by thin-layer chromatography in different solvents.

Enzymatic oxidation of L-*tryptophan*

The oxidations were carried out using L- [3-^{14}C]tryptophan as substrate (50 000 cpm). The products formed were separated by paper chromatography using the solvents described in Materials and Methods and identified by co-chromatography with authentic samples. Kynurenine (XII) and formylkynurenine (XIII) were eluted together and amounted for 90% of consumed tryptophan while 2-oxotryptophan (XIV) amounted to 10% of the consumed substrate.

Formylases

The transformation of the 2-formamido derivatives into the corresponding amino derivatives was due to formylases present in the extracts together with the pyrrolooxygenases. This was demonstrated by incubating the 2-formamido derivatives (VI) and (IX) under the same conditions as described in Materials and Methods, except for the addition of sodium dithionite, when the corresponding 2-amino derivatives were obtained.

The relative proportions of both products, formylated and deformylated, obtained from skatole with the different enzymatic preparations varied with the fractionation method. Similar results were obtained when ethyl *N*-acetyl-L-tryptophan was used as a substrate. The rate of deformylation for 2-formamidoacetophenone (VI) was lower than for ethyl 2-acetamido-3-(2'-formamidobenzoyl) propionate (IX).

DISCUSSION

Pyrrolooxygenases appear to be a new type of mixed-function oxidases. They may belong to the classical mixed-function oxidases which require the presence of both a reducing agent and oxygen for the enzymatic reaction to take place, or else, the reducing agent (dithionite) reduces the Fe^{3+} at the start of the reaction to give the active Fe^{2+} form. The enzymatic oxidation of indolic substrates afforded the same products that were obtained when the indoles were oxidized with peracids (see Results) or with the model hydroxylating system of Udenfriend (see ref. 13). If it is accepted that the formation of an epoxide is the first step in the oxidation processes where mixed-function oxidases are involved[14,15], it seems reasonable to consider 2,3-indoleepoxides (A) as the first intermediate in the enzymatic reaction (Fig. 10). This type of epoxide has never been isolated in indole chemistry, since the push of the nitrogen free electron pair will always favour the β-hydroxyindolenine forms (B). Hydration of the indolenine double bond will then give the diols (C), which on further oxidation will ring open to the *o*-formamidophenacyl derivatives (D). Dehydration of the diols will afford the 2-oxoindole (E) structures. A second oxidation of (B) could also afford the open ring

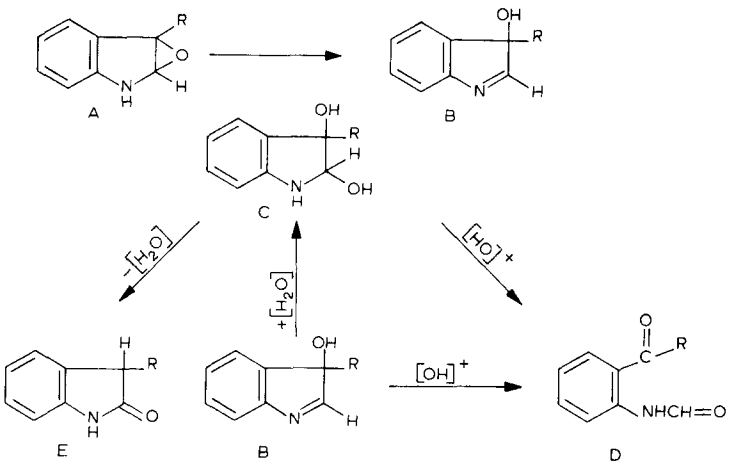


Fig. 10

structures (D), and a decision between the two mechanisms may be facilitated by studies with isotopic oxygen.

It has already been mentioned that *N*-acetylskatole was not a substrate, very likely due to its inability to form the β-hydroxyindolenine structure (B).

From the data presented in this paper it can be concluded that there are two pyrrolooxygenases, one that used skatole and related indoles as substrates, and a second one that consumed tryptophan (L or D) and its side-chain substituted derivatives. They differed in their purification factors (Table I), their optimum pH, heat stabilities, and sensitivity toward chelating agents. Skatole pyrrolooxygenase appeared under different aggregation forms (Fig. 2), while this was not the case with tryptophan pyrrolooxygenase.

Both enzymes are widely distributed. We described their isolation from wheat germ and rat liver, but they were also isolated from rat brain and from spinach and Swiss chard leaves where they are localized in the chloroplasts. The natural reducing system which triggers the pyrrolooxygenases in plants was also localized in the chloroplasts.

Since the products formed from L-tryptophan by the pyrrolooxygenase were the same as those formed by tryptophan pyrrolase, it is difficult to assess the relative importance of both enzymes in L-tryptophan metabolism. Tryptophan pyrrolase was not detected in plants, is induced (or activated) by substrate in animals, and is absolutely specific for L-tryptophan[4]. An enzyme which oxidizes D-tryptophan and resembles tryptophan pyrrolase was isolated from rabbit ileum and intestine[16,17], but it differs widely in its properties from tryptophan pyrrolooxygenase: in its requirements for reducing agents, its substrate specificity toward D- and L-tryptophan and its haemoproteic nature. The D-tryptophan oxidizing enzyme had an absolute requirement for methylene blue and was strongly inhibited by a 0.1 mM concentration of cyanide, while this was not the case for tryptophan pyrrolooxygenase, which remained active even in the presence of a 50 mM concentration of cyanide. The main metabolic function of tryptophan pyrrolooxygenase could then be the oxidation of either free tryptophan in plants, or tryptophanyl residues in proteins[3].

Pyrrolooxygenase activities were very low in the crude extracts due to the pres-

ence of a labile macromolecular inhibitor(s), whose removal strongly increased the enzymatic activity (Tables I and II). This could be the way oxygenases are regulated in the metabolism.

2-Aminoacetophenone was found to be the ultimate product of the oxidation of skatole and 3-indoleacetic acid by pyrrolooxygenase. Its biosynthesis has always been a matter of speculation, since the sulphate of 2-amino-3-hydroxyacetophenone was a well-known constituent of normal urines[18] and is suspected to be a carcinogenic compound in the bladder[19]. 2-Amino-3-hydroxyacetophenone has been found in the urine of rats and hens fed on L-tryptophan[20] and it has recently been found that a microsomal fraction from rat liver hydroxylated 2-aminoacetophenone to 2-amino-3-hydroxyacetophenone[21]. Since skatole is a well-known tryptophan metabolite in the gut, the role of skatole pyrrolooxygenase can be of importance in controlling an increased metabolic production of skatole. The observations of Dalgliesh[18], that the excretion of 2-amino-3-hydroxyacetophenone is strongly increased in certain pathological cases (leukaemias and anaemias), can be due to an increased pyrrolooxygenase activity *in vivo*, or to a lower content in the specific inhibitor.

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REFERENCES

1 O. Hayaishi, *Annu. Rev. Biochem.*, 38 (1969) 21.
2 S. Udenfriend, *Ann. N.Y. Acad. Sci.*, 179 (1971) 295.
3 R. B. Frydman, M. L. Tomaro and B. Frydman, *FEBS Lett.*, 15 (1971) 305.
4 E. Knox, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 242.
5 K. Tagawa and D. I. Arnon, *Nature*, 195 (1962) 537.
6 P. P. Kalberer, B. B. Buchanan and D. I. Arnon, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 1542.
7 S. Udenfriend and R. E. Peterson, in S. P. Colowick and N. P. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 613.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 256.
9 H. Hellman, *Hoppe Seyler's Z. Physiol. Chem.*, 287 (1951) 205.
10 Ll. J. Dolby and D. L. Both, *J. Am. Chem. Soc.*, 88 (1966) 1049.
11 A. S. Endler and E. J. Becker, *Org. Synth.*, 37 (1957) 60.
12 J. W. Cornforth, R. H. Cornforth, C. E. Dalgliesh and A. Neuberger, *Biochem. J.*, 48 (1951) 591.
13 R. A. Heacock and M. G. Mahon, *Can. J. Biochem. Physiol.*, 41 (1963) 2381.
14 G. A. Hamilton, *J. Am. Chem. Soc.*, 86 (1964) 3391.
15 D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman Nirenberg and S. Udenfriend, *Biochemistry*, 9 (1970) 147.
16 K. Higuchi and O. Hayaishi, *Arch. Biochem. Biophys.*, 120 (1967) 397.
17 S. Yamamoto and O. Hayaishi, *J. Biol. Chem.*, 242 (1967) 5260.
18 C. E. Dalgliesh, *Biochem. J.*, 61 (1955) 334.
19 M. J. Allen, E. Boyland, C. E. Dukes, E. S. Horning and J. G. Watson, *Br. J. Cancer*, 11 (1957) 212.
20 H. Kaseda, T. Noguchi, R. Kido and Y. Matsumura, *Experientia*, 26 (1970) 828.
21 H. Kaseda, T. Noguchi, N. Konishi and R. Kido, *Experientia*, 27 (1971) 368.