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New indole oxygenase from the leaves of *Tecoma stans* L. Part I: Affinity purification and properties

SATYA P. KUNAPULI* AND C. S. VAIDYANATHAN
Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India.

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Abstract

Indole oxygenase from the leaves of *Tecoma stans* was purified to homogeneity using 5-hydroxyindole-coupled epoxy-activated Sepharose. The purity was checked by polyacrylamide gel electrophoresis and immunoelectrophoresis. This enzyme was shown to be identical to the one purified by conventional purification method. Indole oxidase, an enzyme catalyzing the conversion of indole to anthranil, was not co-purified during affinity chromatography. A model for the orientation of the substrate in the substrate–enzyme complex was proposed from the substrate specificity, inhibition studies, and the ability of the enzyme to bind to affinity matrices with different substrate orientation. Dissociation constants for 5-hydroxyindole, 5-bromoindole and 7-methylindole were determined by kinetic and fluorescence-quenching experiments and are compared. A mechanism is proposed for the conversion of indole to anthranilic acid, with the intermediary formation of N-formylaminobenzaldehyde and *o*-aminobenzaldehyde, by indole oxygenase from the trapping experiments.

Key words: Indole oxygenase, *Tecoma stans*.

1. Introduction

Indolic compounds and their metabolism have been attracting the attention of biochemists increasingly due to the presence of the indole ring in many biologically important compounds, such as tryptophan, indoleacetic acid, indole alkaloids, etc. So far, only a few indole-metabolising enzymes were isolated from higher plants[1-4] (and references therein). Of these, only two, one from the leaves of *Tecoma stans* and the other from the leaves of *Jasminum grandiflorum*, were purified to homogeneity[3,5]. The enzyme indole oxidase from *Tecoma stans*[1] converts indole to anthranil. To avoid any confusion, the *Tecoma* leaf enzyme[5] which converts indole to anthranilic acid has been named indole oxygenase. A rapid purification of

* Present address: 522 OMS Thrombosis Research Center, Temple University Medical School, 3400 N. Broad Street, Philadelphia, PA 19140, USA.

indole oxygenase from *Tecoma* leaves using affinity chromatography was reported in a preliminary communication[6]. In this paper, the details of purification and properties are reported.

2. Experimental details

2.1. *Assay of indole oxygenase*

The enzyme was assayed as described by Kunapuli and Vaidyanathan[5]. One unit of the enzyme is defined as the amount of the enzyme that causes the disappearance of 1 nmole of indole in 1 min at 30°C. Specific activity is expressed as units/mg protein. Proteins were precipitated with ethanol and centrifuged for 5 min at 3000 × *g*. The precipitate was redissolved in the buffer and the protein content of the various fractions was estimated by the method of Lowry *et al*[7] using bovine serum albumin as the standard.

2.2. *Estimation of anthranilic acid*

The unreacted indole was extracted from the reaction mixture (2·0 ml) with 2·5 ml toluene and anthranilic acid was estimated in a 0·5 ml aliquot of the aqueous phase according to the method of Venkataraman *et al*[8], using Ehrlich reagent.

2.3. *Immunochemical techniques*

Antibodies to the purified indole oxygenase were raised in albino rabbits. The protein dissolved in 10 mM sodium phosphate buffer (pH 7·2)/0·15M NaCl (1mg/ml) and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories) was injected subcutaneously at multiple points at weekly intervals. After 4 weeks, a booster dose of 0·5 mg protein in 10 mM sodium phosphate/0·15 M NaCl was administered. After 10 more days, the rabbits were bled through the ear vein and serum was prepared. γ-Globulin fraction of the normal serum and antisera of the rabbit were prepared by ammonium sulfate precipitation followed by negative adsorption on DE-52.

2.4. *Immunoelectrophoresis*

Immunoelectrophoresis of the enzyme on agar was performed as described by Clausen[9]. Agar (1·5% w/v) in 60 mM sodium veronol buffer, pH 8·6, was layered on a glass slide (3·5 × 9·0 cm) and allowed to form a uniform layer of gel. The enzyme (60–70 μg) was placed in the well, punched at one end of the plate, and electrophoresed at 4°C for 3 h at 150 V. After electrophoresis, troughs running through the entire length of the gel were cut on either side of the antigen well. Rabbit anti-enzyme IgG (5 mg/0·5 ml) was poured in each trough and allowed to diffuse overnight at room temperature.

2.5. *SDS–Polyacrylamide gel electrophoresis*

SDS gel electrophoresis was carried out according to the method of Weber and Osborn[10] on 7·5% (w/v) gels at 6 mA/gel and continued until the dye band reached the lower end of the gel. The proteins on the gels were stained with Coomassie Brilliant Blue

R-250 and destained with methanol/acetic acid/water (50:7:43 v/v). The following proteins of known molecular weight served as markers : Cytochrome *c* (12,270), Chymotrypsin (23,200), ovalbumin (46,000), BSA (68,000) and Hexokinase (102,000).

2.6. *Chemicals*

Indole, anthranilic acid, 5-hydroxyindole, 5-bromoindole, and 7-methylindole were purchased from Sigma, and *o*-aminobenzaldehyde was prepared from *o*-nitrobenzaldehyde by selective reduction according to the method of Smith and Opie[11]. All other chemicals used were of analytical grade available commercially.

3. Results

3.1. *Use of indole-coupled benzidyl Sepharose for affinity chromatography*

Indole oxygenase from *Tecoma* leaves was taken to 30–50% ammonium sulfate saturation as described earlier[5]. The precipitate was dissolved in various buffers (10 ml) listed below and this enzyme preparation was loaded on to the indole-coupled benzidyl Sepharose column (0·5 × 4·0 cm): 0·1 and 0·01 M acetate buffer, pH 4·0; 0·1 and 0·01 M phosphate buffer, pH 7·2; and 0·1 M/0·2 M and 0·01 M citric acid–0·02 M sodium phosphate buffer, pH 5·2. Fractions (2 ml) were collected and assayed for enzyme activity under standard conditions. The enzyme did not bind to the matrix under any of the above conditions as evidenced by the enzyme activity in the flow through.

3.2. *Purification of the enzyme using 5-hydroxyindole-coupled epoxy-activated Sepharose*

All operations were carried out at 0–5°C unless otherwise stated. All centrifugations were performed at 17,000 × *g* for 15 min.

Step I: Preparation of the crude extract: Fresh, mature leaves (50 g) of *Tecoma stans* L. were harvested at midnight and stored at −20°C till the next morning and used. The midribs from the leaves were detached and washed thoroughly with cold glass distilled water. The leaves were then ground to a paste in a chilled porcelain mortar with acid-washed glass powder (10 g) and slurry was extracted with 90 ml of 0·01 M citric acid–0·02 M sodium phosphate buffer, pH 5·2. The slurry was strained through a double cheese cloth and the greenish extract obtained was centrifuged. The clear supernatant obtained was designated as the crude extract.

Step II: Ammonium sulfate fractionation: The above enzyme preparation was taken to 20% saturation by the addition of solid ammonium sulfate with constant stirring and set aside for 15 min. The precipitate obtained by centrifugation was discarded. To the supernatant, solid ammonium sulfate was added to raise the saturation level to 50% and was set aside for 30 min. The precipitate obtained on centrifugation was dissolved in minimum amount of 0·01 M citric acid–0·02 M sodium phosphate buffer, pH 5·2 and was designated as 20–50% ammonium sulfate fraction.

Step III: Affinity chromatography: The solution obtained from the above step was loaded on to the 5-hydroxyindole-coupled epoxy-activated Sepharose column

Table I
Purification chart for indole oxygenase from *Tecoma* leaves by affinity chromatography

Preparation	*Total protein (mg)*	*Total activity (units)*	*Specific activity*	*Fold purification*	*% Yield*
Crude extract	760	91·0	0·12	1	100
Ammonium sulfate fractionation (20–50%)	25·25	75·75	3·0	25	83·2
Affinity chromatography	3·97	62·3	15·7	131·1	68·5

(0·5 × 4·0 cm), which was equilibrated with 0·01 M citric acid–0·02 M sodium phosphate buffer, pH 5·2. The affinity column was washed with 25 ml of the same buffer and the enzyme was eluted with 50 mM sodium phosphate buffer, pH 7·0. The results of the purification procedure are summarized in Table I.

3.3. *Purity of the enzyme*

Analysis of the purified enzyme by polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8·3, revealed a single protein band. On electrophoresis under acidic conditions in β-alanine/acetic acid buffer, pH 4·3, the protein did not enter the gel. The enzyme preparation gave a single precipitin line on immunoelectrophoresis (Fig. 1) confirming the homogeneous nature of the enzyme preparation.

3.4. *Properties of indole oxygenase from Tecoma stans*

The properties of indole oxygenase purified by affinity chromatography are identical to those of indole oxygenase purified by conventional methods[5]. The purified enzyme is optimally active at pH 5·2 in citric acid–sodium phosphate buffer and had an apparent K_m of 18·9 μM for indole. The enzymatic product was identified as anthranilic acid by comparing its chromatographic mobilities and spectral properties with those of an authentic sample. The procedure for isolation and identification of the enzymatic product is identical to the one described earlier[5]. No anthranil could be detected in the reaction mixture. The enzyme catalyzes the conversion of one mole of indole and two moles of oxygen into one mole of anthranilic acid. The approximate molecular weight of the enzyme as determined by gel filtration on Sephadex G-100 is 58,000. SDS Gel electrophoresis also has the same value for the molecular weight indicating that the protein consists of a single polypeptide chain.

FIG. 1. Immunoelectrophoresis of Tecoma leaf indole oxygenase.

3.5. *Inhibition by substrate analogs*

The effect of several substrate analogs such as 5-hydroxyindole, 5-bromoindole and 7-methylindole on the initial velocity of the reaction catalyzed by the enzyme was investigated. These compounds were found to inhibit the enzyme reaction and the nature of the inhibition was investigated. The initial velocity of the reaction was determined at various fixed substrate concentrations. The results are plotted according to the method of Dixon[12], where $1/v$ is plotted against the

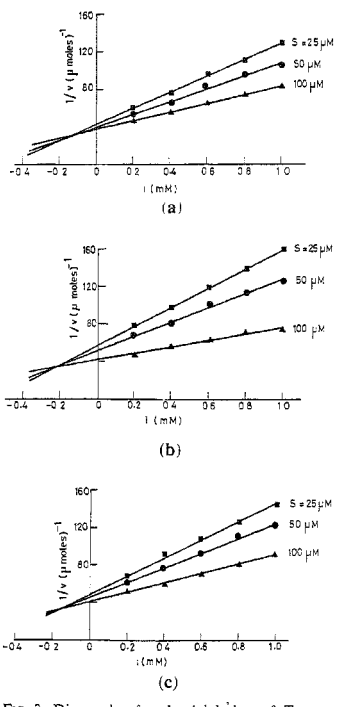

FIG. 2. Dixon plot for the inhibition of Tecoma leaf indole oxygenase by (a) 5-hydroxyindole, (b) 5-bromoindole, and (c) 7-methylindole.

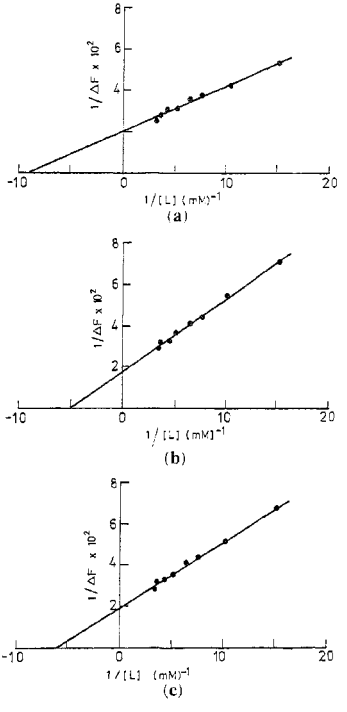

FIG. 3. Double-reciprocal plot for the fluorescence quenching of Techoma leaf indole oxygenase by (a) 5-hydroxyindole, (b) 5-bromoindole, and (c) 7-methylindole.

Table II
Binding constants of indole oxygenase for substrate and substrate analogs

Compound	*Kinetic*	*Fluorescence*
Indole	18·9 μM	47·6 μM
5-Hydroxyindole	0·12 mM	0·11 mM
5-Bromoindole	0·21 mM	0·20 mM
7-Methylindole	0·19 mM	0·16 mM

The binding constants from kinetic and fluorescence experiments were calculated from Figs 2a,b,c and 3a,b,c for each substrate analog.
The binding constant for the substrate was calculated from Fig. 4.

concentration of the inhibitor (Figs 2a,b,c) for each $[S]$ value, and a straight line was obtained. In each case, a set of converging lines well above the X-axis was obtained, indicating that the inhibition is competitive. The abscissa of the point of concurrence gives $-K_i$. From these, the dissociation constants of these substrate analogs (K_i) were determined (Table II).

3.6. *Effect of substrate analogs and substrate on the fluorescence of the enzyme*

The effect of various substrate analogs and substrate itself on the intrinsic fluorescence of indole oxygenase was studied. The purified enzyme at a concentration of 150 μg/ml in 0·02 M citric acid–0·04 M sodium phosphate buffer, pH 5·2, was taken in a 3 ml quartz fluorescence cuvette. The enzyme was excited at 285 nm and the emission at 335 nm was measured. The fluorescence in the absence of the added ligand was measured and normalized to 100. The ligand solution was added in 10 μl increments with a Hamilton microsyringe (100 μl) to both sample and control cuvettes, mixed well and the change in the fluorescence intensity at 335 nm followed with excitation at 285 nm. The emission after each addition was measured and expressed as per cent of normalized protein fluorescence. Both the substrate and the substrate analogs quenched protein fluorescence indicating ligand-induced conformational change in the protein.

The fluorescence-quenching data were analyzed by plotting in double-reciprocal form, where $1/\Delta F$ is plotted against $1/[L]$. Straight lines of positive slope were obtained for

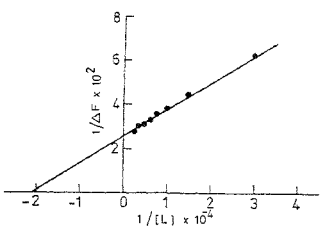

FIG. 4. Double-reciprocal plot for the fluorescence quenching of Tecoma leaf indole oxygenase by indole (see Fig. 3 for details).

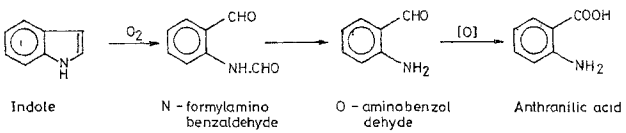

FIG. 5. Scheme for oxidation of indole by indole oxygenase.

each of the substrate analogs (Figs 3a,b,c) and for indole (Fig. 4). The intercept on the X-axis equals $1/K_D$, while the intercept on the Y-axis equals $1/\Delta F_{max}$. From this, the K_D values were calculated for each of the substrate analogs studied (Table II).

3.7. *Plausible sequence of the reaction*

The first step in the reaction sequence is cleavage of the indole ring as the expected product might be a dialdehyde. When the reaction was carried out in the presence of phenylhydrazine oxalate (1 mM), a trapping agent for aldehyde, no anthranilic acid was formed as determined by the method of Venkataraman *et al*[8]. There is no effect of this compound on indole disappearance as expected. But the phenylhydrazone of formyl aminobenzaldehyde could not be detected by the following method. The reaction mixture was scaled up ten times in the presence of 1 mM phenylhydrazine oxalate, extracted twice with 50 ml of ethylacetate and concentrated. The concentrate was chromatographed on Whatman No. 3 filter paper in butanol/acetic acid/water (4:1:5 v/v) and sprayed with 0·2% alcoholic NaOH. No brown spots could be detected at R_f 0·91. *o*-Aminobenzaldehyde was tested for its conversion to anthranilic acid by indole oxygenase. The reaction mixture containing 1 mg of the enzyme, 15 ml of 0·1M citric acid–0·2M sodium phosphate buffer, pH 5·2 and 1 μ mole of *o*-aminobenzaldehyde were incubated for 30 min and the pH of the reaction was adjusted to 1·0 with 2 N HCl. The contents were extracted thrice with 50 ml of diethylether each time, dried over anhydrous sodium sulfate and concentrated *in vacuo*. The concentrate was chromatographed on Whatman No. 3 filter paper in ethanol/ammonia/water (18:1:1, v/v) and anthranilic acid was detected on the chromatograms by its blue fluorescence and its color with Ehrlich reagent, and its chromatographic mobility comparison with authentic anthranilic acid. Depending on these observations the probable reaction sequence is given in Fig. 5.

4. Discussion

The purification of indole oxygenase was first attempted using indole-coupled benzidyl Sepharose matrix. In this matrix, benzidyl Sepharose is linked to indole at C-3. Under different ionic strength and pH conditions indole oxygenase did not bind to the matrix. Indole oxygenase from Tecoma leaves, apart from indole, attacks 5-hydroxyindole, 5-bromoindole and 5-methylindole. It fails to oxidize 2-methylindole, 3-methylindole, indoleacetic acid or tryptophan[5]. This suggested that free pyrrole ring

might be essential for the enzyme substrate complex formation. Hence, a different orientation of substrate in the affinity matrix was achieved by coupling 5-hydroxyindole to epoxy-activated Sepharose. This results in a stable ether linkage at C-5 of the indole ring and projects the pyrrole ring from the matrix. Indole oxygenase strongly binds to the matrix at pH 5·2 and can either be eluted with 1 mM indole in citrate phosphate buffer, pH 5·2 or by specifically changing the pH to 7·0. The purification steps developed in this investigation represent a simple and specific procedure for obtaining indole oxygenase in a homogeneous form as judged by polyacrylamide gel electrophoresis and immunoelectrophoresis. The yield is improved more than three fold, compared to conventional purification procedure[5]. However, specific activity remains the same. Strong binding of the enzyme to 5-hydroxyindole-coupled epoxy-activated Sepharose and the inability of the enzyme to bind to indole-coupled benzidyl Sepharose matrix suggests the possible orientation of the substrate in the enzyme substrate complex (Fig. 6). The ability of the enzyme to attack 2-methylindole, 3-methylindole, tryptophan and indoleacetic acid, and its ability to cause disappearance of 5-hydroxyindole, 5-bromoindole and 5-methylindole is also consistent with this model.

The approximate molecular weight of the enzyme was 58,000 as judged by SDS gel electrophoresis and Sephadex G-100 gel filtration. When the proteins isolated by affinity chromatography and conventional purification method[5] were co-electrophoressed either on polyacrylamide gel or SDS polyacrylamide gel, a single stainable band was observed confirming their identity in their mobility and molecular weight. The optimum pH of the enzymatic reaction is 5·2, which is essentially the same for conventionally purified indole oxygenase. Anthranilic acid is the only detectable product and no anthranil could be detected in the reaction catalyzed by enzymes purified by conventional and affinity techniques, confirming the absence of any contamination of indole oxidase[1].

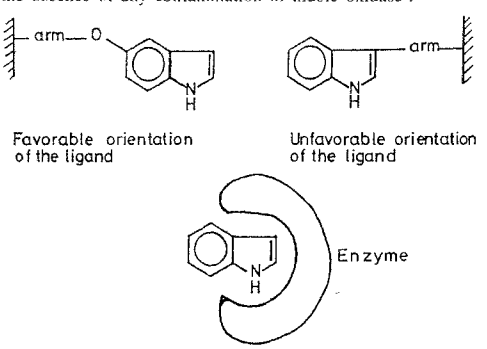

FIG. 6. Orientation of indole in enzyme–substrate complex.

Nair and Vaidyanathan[1] reported indole oxidase from the leaves of the same plant. This enzyme catalyzes the oxidation of indole to anthranil. The homogeneous nature of indole oxygenase purified by affinity chromatography and its identity with indole oxygenase purified by conventional purification methods suggest that indole oxidase is not (co-) purified by affinity procedure. This may be due to the seasonal variation of indole oxidase in Tecoma leaves. In this case, both indole oxygenase[5,6] and indole oxidase[1] may be operating in the same plant depending on the needs of the plant in different seasons.

Changes in the intrinsic fluorescence of a protein with increasing concentrations of a ligand may be used to obtain dissociation constants (K_D). In kinetic parlance, these K_D values are termed K_i values for competitive inhibitors. The values of the dissociation constants for the three compounds obtained by fluorescence-quenching studies are compared with those obtained by kinetic studies (Table II). They are in good agreement. The dissociation constant for the substrate is slightly higher (47·6 μM) compared to kinetic constant (18·9 μM). Similar studies have been carried out on the binding of the appropriate substrates to salicylate hydroxylase[13], anthranilate hydroxylase[14] and glyceraldehyde-3-phosphate dehydrogenase[15].

The sequence of indole oxidation reported by Fujioka and Wada[16] includes the hydroxylation of indole ring to indoxyl. Isatin formed from indoxyl on cleavage of the pyrrole ring gives rise to formylanthranilic acid which on further oxidation gives catechol. Many oxidizing agents like ozone and perbenzoic acid lead to the formation of formylaminobenzaldehyde[17-20]. This type of cleavage is favored by the electron density of the indole ring at carbons 2 and 3. In the reaction catalyzed by indole oxygenase form *T. stans* the product was unambiguously shown to be anthranilic acid and anthranil could not be detected. Attempts were made to detect the intermediates in the reaction. By analogy with the chemical oxidation of indole, the initial cleavage product would be formylaminobenzaldehyde. When the oxidation of indole was carried out in the presence of phenylhydrazine oxalate, a trapping agent for aldehyde, the formation of anthranilic acid was inhibited. But the phenylhydrazone of formylaminobenzaldehyde could not be detected. *o*-Aminobenzaldehyde was converted to anthranilic acid by indole oxygenase. Thus it is plausible that intermediates are strongly bound to the enzyme. However, the enzyme might be able to accept the intermediates as substrates. We have not tested N-formylaminobenzaldehyde or N-formylanthranilic acid as substrates. The possible sequence of oxidation of indole to anthranilic acid has been shown in Fig. 5. In the case of indole oxidase from *T. stans*, the oxidation of indole to anthranil proceeds *via* N-formylaminobenzaldehyde and *o*-aminobenzaldehyde. These two intermediates are converted to anthranil[1]. Similar results were obtained in the case of crude indole oxidase from *Zea mays*[2]. Indole oxygenase from *Jasminum grandiflorum* does not convert formylaminobenzaldehyde and *o*-aminobenzaldehyde to anthranilic acid. Though the enzyme activity is inhibited by phenylhydrazine oxalate, formylaminobenzaldehyde phenylhydrazone could not be detected[3].

Thus the first step of the reaction sequence is formation of N-formylaminobenzaldehyde from indole. This seems to be a dioxygenase type of reaction. N-formylaminobenzaldehyde is converted to *o*-aminobenzaldehyde by hydrolytic cleavage of formylase resulting in the formation of formic acid. The *o*-aminobenzaldehyde may be oxidized to anthranilic acid by an aldehyde oxidase type of reaction requiring one mole of oxygen. Thus, the plausible sequence of reaction catalyzed by indole oxygenase from *Tecoma stans* involves, in addition to dioxygenase type of reaction, two other enzymatic activities, *viz.*, formylase and aldehyde oxidase, all carried out by a single enzyme at the same active site. However, more confirmatory evidence is required to establish this sequence of reaction.

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References

1. NAIR, P.M. AND VAIDYANATHAN, C.S. — *Biochim. Biophys. Acta*, 1964, **81**, 496–506.
2. CHAUHAN, Y.S., RATHORE, V.S., GARG, G.K. AND ARUN BHARGAVA — *Biochem. Biophys. Res. Commun.*, 1978, **83**, 1237–1245.
3. DIVAKAR, N.G., SUBRAMANIAN, V., SUGUMARAN, M. AND VAIDYANATHAN, C.S. — *Pl. Sci. Lett.*, 1979, **15**, 177–181.
4. KUNAPULI, S.P. AND VAIDYANATHAN, C.S. — *Pl. Sci. Lett.*, 1982, **24**, 183–187.
5. KUNAPULI, S.P. AND VAIDYANATHAN, C.S. — *Pl. Physiol.*, 1983, **71**, 19–23.
6. KUNAPULI, S.P. AND VAIDYANATHAN, C.S. — *Biochem. Int.*, 1983, **7**, 647–653.
7. LOWRY, O.E., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J. — *J. Biol. Chem.*, 1951, **193**, 265–275.
8. VENKATARAMAN, A., VENKATARAMAN, P.R. AND LEWIS, H.B. — *J. Biol. Chem.*, 1948, **173**, 641–651.
9. CLAUSEN, J. — In *The laboratory techniques in biochemistrty and molecular biology* (Work, T.S. and Work, E., eds), 1970, Vol. 1, Part III, p. 454, North-Holland.
10. WEBER, K. AND OSBORN, M. — *J. Biol. Chem.*, 1969, **244**, 4406–4412.
11. SMITH, L.J. AND OPIE, J.W. — In *Organic syntheses* (E.C. Horning, ed.), Vol. 3, p. 56, Wiley.
12. DIXON, M. — *Biochem. J.*, 1953, **55**, 170–171.
13. SUZUKI, M., TAKEMORI, S. AND KATAGIRI, M. — *Biochim. Biophys. Acta*, 1969, **191**, 77–85.

14. SUBRAMANIAN, V. *Studies on anthranilic acid hydroxylase from Aspergillus niger*, Ph.D. Dissertation, Indian Institute of Science, Bangalore, India, 1979.

15. VELICK, S.F. *J. Biol. Chem.*, 1958, **233**, 1455–1467.

16. FUZIOKA, M. AND WADA, H. *Biochim. Biophys. Acta*, 1968, **158**, 70–78.

17. WITKOP, B. *Ann. Chem.* 1944, **556**, 103–114.

18. WITKOP, B. AND PATRICK, J.B. *J. Am. Chem. Soc.*, 1951, **73**, 713–718.

19. WITKOP, B. AND PATRICK, J.B. *J. Am. Chem. Soc.*, 1951, **73**, 2196–2200.

20. WITKOP, B. *J. Am. Chem. Soc.*, 1950, **72**, 1428–1429.