Biochem. J. (1984) **220**, 767–772
Printed in Great Britain

The kinetics of ammonia release during the catalytic cycle of pig plasma amine oxidase

F. Xavier RIUS,*‡ Peter F. KNOWLES† and Gösta PETTERSSON*
**Biokemi 1, Kemicentrum, Box 740, 22007 Lund 7, Sweden, and †Astbury Department of Biophysics, University of Leeds, Leeds LS2 9JT, U.K.*

(*Received 18 November 1983/Accepted 6 March 1984*)

The time course of NH_3 release during the catalytic cycle of pig plasma amine oxidase was followed by using the quenched-flow technique in conjunction with a sensitive assay for NH_3. These studies were made under both air and O_2-saturating conditions. The results establish unequivocally that NH_3 is released in the step whereby a reduced enzyme species is re-oxidized by molecular O_2 rather than in the step leading to the reduced enzyme. It is concluded that the catalytic cycle of the enzyme conforms to an aminotransferase mechanism rather than one in which an imine is an intermediate.

Amine oxidases catalyse the oxidative deamination of amines with stoichiometric release of aldehyde, NH_3 and H_2O_2:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RHCO + NH_3 + H_2O_2$$

Steady-state kinetic studies (see review by Yasunobu *et al.*, 1976) on copper-containing amine oxidases (EC 1.4.3.6) have provided strong evidence for a double-displacement mechanism where the amine substrate binds to the enzyme in the first step and product (or products) are released before O_2 binding. Two fundamentally different mechanisms for the action of these amine oxidases have been proposed. The first, advocated most strongly by Pettersson and co-workers (see Olsson *et al.*, 1978, and references cited therein), can be termed an aminotransferase mechanism and is consistent with the involvement of an enzyme-bound carbonyl grouping in the catalytic process (Scheme 1). According to this mechanism, aldehyde would be the only product liberated under anaerobic conditions, and evidence in favour of this has been presented (Lindström & Pettersson, 1978*b*).

An alternative mechanism has been proposed by Abeles and his colleagues (Suva & Abeles, 1978).

(i) $E\text{–}CHO + RCH_2NH_2 \rightarrow E\text{–}CH_2NH_2 + RC\dot{H}O$

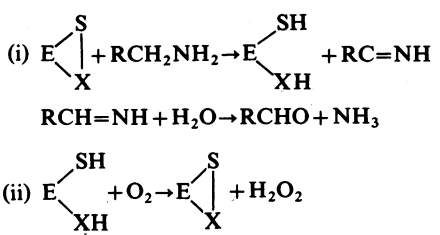
(ii) $E\text{–}CH_2NH_2 + O_2 + H_2O \rightarrow E\text{–}CHO + H_2O_2 + NH_3$

Scheme 1

‡ Present address: Facultat de Química, Universitat de Barcelona, Plaça Imperial Tarraco, Tarragona, Spain.

This can be described as an imine mechanism, where the imine HN=CH–R is hydrolysed under anaerobic conditions to liberate NH_3 and aldehyde products (Scheme 2). Evidence has been presented that these products are indeed released anaerobically (Berg & Abeles, 1980).

The time course of NH_3 release should provide unambiguous evidence to resolve these opposed views. Both mechanisms agree in postulating that the interaction of enzyme with amine substrate (step i) leads to formation of a reduced enzyme intermediate that is subsequently re-oxidized by O_2 in step (ii). The processes of enzyme reduction and re-oxidation have been characterized by stopped-flow studies in the case of amine oxidase from pig plasma (Lindström *et al.*, 1976; Olsson *et al.*, 1977), and are known to occur at readily distinguishable rates. Thus discriminating between the imine mechanism and the aminotransferase mechanism is a matter of testing whether the kinetics of transient NH_3 release conform to the rate of the reduction process or the rate of the re-oxidation process.



Scheme 2

Clearly we could also distinguish between the mechanisms by following the time course of NH_3 release under anaerobic conditions. We have chosen to study NH_3 release at different fixed O_2 concentrations, firstly because strictly anaerobic conditions are difficult to attain under the conditions of our experiment, and secondly because any demonstration that NH_3 release is dependent on the O_2 concentration is itself diagnostic.

The quench-flow studies described in the present paper provide unequivocal evidence in favour of the aminotransferase mechanism.

Materials and methods

2-Oxoglutarate, ADP, NADH, L-glutamate dehydrogenase (bovine liver, type II) and *p*-nitrophenyl acetate were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Mes (4-morpholine-ethanesulphonic acid) was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Other chemicals (reagent grade) were from BDH Chemicals, Poole, Dorset, U.K. 2-Oxo[^{14}C]-glutarate (10μCi/mg) was from New England Nuclear, Boston, MA, U.S.A.

Benzylamine was converted into the hydrochloride and recrystallized three times from water. All reagents were prepared with glass-distilled water, and those for the NH_3 assays from double-glass-distilled water.

Pig plasma benzylamine oxidase was purified by a procedure based on that described by Taylor *et al.* (1972). The modifications introduced were as follows. After step II of the purification procedure, the whole batch of enzyme was applied to a DEAE-cellulose column (4 litres bed volume) equilibrated with 5mM-sodium phosphate buffer, pH7.0. A linear salt gradient was applied (4 litres of 0.20mM-NaCl in 5mM-phosphate buffer, pH7.0, against 4 litres of the buffer alone), and 50ml fractions were collected. The elution profile indicated a major peak activity with a shoulder towards the higher salt concentrations. All fractions with specific activity greater than after step II were combined, precipitated by addition of $(NH_4)_2SO_4$ to 55% saturation and left at 4°C for at least 2 days; a significant increase in specific activity results from leaving the protein at this stage. The precipitate was subject to batchwise fractionation with hydroxyapatite as described by Taylor *et al.* (1972). The highest-specific-activity fractions were concentrated by addition of $(NH_4)_2SO_4$ to 55% saturation, and the precipitate was dissolved in 0.10M-potassium phosphate buffer, pH7.0, and applied to a column of concanavalin A–Sepharose (Pharmacia, Hounslow, Middx., U.K.) at a loading of 1g of protein/40ml bed volume. Typically, the column (40ml bed volume) was eluted with 0.10M-potassium phosphate buffer, pH7.0, containing 0.10M-NaCl until no further protein emerged; elution was continued with a linear gradient of 0.10M-potassium phosphate buffer, pH7.0, containing 0.10M-NaCl (125ml) against this same saline buffer containing 0.15M-α-methyl mannoside (125ml). The elution was conducted at room temperature with a flow rate of 20–30ml/h, 5ml fractions being collected. The elution profile showed a broad spread in specific activity; peak fractions were combined and the enzyme was crystallized as described by Blaschko & Buffoni (1965). Fractions either side of the main activity peak fractions from the concanavalin A–Sepharose column were combined and chromatographed on a column (1.1 litres bed volume) of Ultrogel ACA-34 (LKB, South Croydon, Surrey, U.K.) equilibrated with 0.05M-photassium phosphate buffer, pH7.0; up to 1.5g of protein could be loaded on the column described. The elution profile showed a major peak in specific activity with a shoulder towards smaller-M_r species. The major peak fractions were combined and crystallized as described above. All crystalline enzyme had specific activity in excess of 0.097 unit/mg; 1 unit is defined as the amount of enzyme required to catalyse the production of 1μmol of benzaldehyde/min under the standard assay conditions described by Tabor *et al.* (1954). This specific activity is equal to that reported previously (Barker *et al.*, 1979) and for which the enzyme was shown to be homogeneous according to analytical ultracentrifugation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Protein concentrations were determined by using the A_{260}/A_{280} method of Warburg & Christian (1941) corrected by a factor of 0.75 on the basis of gravimetric calibrations (Taylor *et al.*, 1972).

The rapid-quench apparatus used is described in Grant *et al.* (1978), and details on the stopped flow apparatus are contained in Yadav & Knowles (1981). The reliability of the quenched-flow apparatus was assessed against stopped-flow measurements by following the alkaline hydrolysis of *p*-nitrophenyl acetate by the procedure described by Barman & Gutfreund (1964).

Time course of NH_3 release with the use of the rapid-quench technique

Crystalline amine oxidase was dialysed at 4°C against ten 1000-volume-excess changes of 0.10M-sodium phosphate buffer, pH9.0, over a 60h period to remove $(NH_4)_2SO_4$. The relatively high pH would favour the presence of NH_3 rather than NH_4^+, and hence would facilitate removal of this species.

The dialysed enzyme (approx. 10mg/ml), con-

tained in a tube fitted with a rubber serum cap, was saturated with O_2 by directing a stream of the pure gas on to the solution surface and gentle shaking of the solution over a period of 15min. A solution of benzylamine hydrochloride (10.0mM) in this same buffer was re-adjusted to pH9.0, then saturated with O_2 by slowly bubbling the gas through the solution over a 10min period. Previous studies (Taylor *et al.*, 1972) have demonstrated that O_2 saturation requires passage of the gas for only 60s.

The syringes of the rapid-quench apparatus were filled with the O_2-saturated enzyme and benzylamine solutions and equilibrated at 20.0°C for 20min before the experiment. Similar temperature equilibration was followed in the case of the air-saturated enzyme and substrate solutions. After different reaction times (10ms–20s), samples of reaction mixture (0.36mls) were extruded into weighed Eppendorf vials containing 0.19ml of 1.0M-HCl; control experiments had shown that these conditions irreversibly inactivated the enzyme. Allowance was made for the slight differences in volume of the extruded samples by reweighing the Eppendorf tubes. The quenched reaction mixture was centrifuged [300*g* for 20min in a Hämofuge centrifuge (Heraeus, Österode, Germany)], the supernatant was removed and adjusted to pH6.5 by addition of 0.167ml of 2.0M-$KHCO_3$, and then re-centrifuged as above, and six 0.1ml samples of the supernatant were taken. NH_3 in these samples was determined by the radiochemical assay described by Kalb *et al.* (1978). A calibration curve for the assay was constructed by the addition of different amounts of NH_4^+ to the full reaction mixture but in the presence throughout of the HCl.

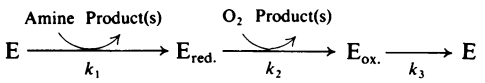
Results

Calibration of the quenched-flow method against stopped-flow measurements

The second-order rate constant (k_2) for alkaline hydrolysis of *p*-nitrophenyl acetate at 25.0°C was found to be $7.5 \pm 0.1\,M^{-1}\cdot s^{-1}$ by quenched flow and $6.8 \pm 0.1\,M^{-1}\cdot s^{-1}$ by stopped flow; the errors were assessed by using a linear-regression program. These values are in reasonable agreement with each other and are consistent with the k_2 value of $49.4\,M^{-1}\cdot s^{-1}$ reported by Barman & Gutfreund (1964) for alkaline hydrolysis of 2,4-dinitrophenyl acetate.

Time course of NH_3 release

The catalytic action of the amine oxidase from pig plasma can be described in terms of the simplified kinetic scheme shown in Scheme 3, where E, $E_{red.}$ and $E_{ox.}$ refer to the free, reduced and oxidized enzyme species characterized by



Scheme 3

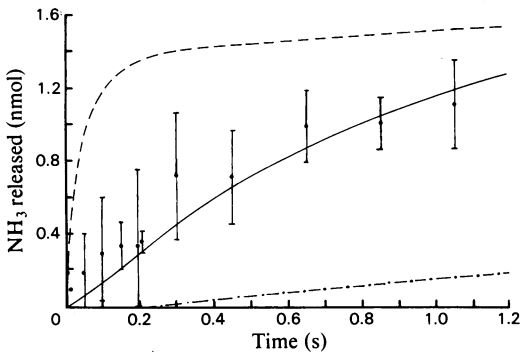


Fig. 1. *Transient-state kinetics for NH_3 release from benzylamine catalysed by pig plasma amine oxidase under air-saturating conditions at pH9.0 at 25.0°C*

The error bars represent limits of the standard deviations from assays on six samples at each time point. Reaction solutions contained 1.38nmol of enzyme. ----, Simulation for release of NH_3 in step 1 of Scheme 3; ——, simulation for release of NH_3 in step 2 of Scheme 3; -·-·-, simulation for release of NH_3 in step 3 of Scheme 3.

previous kinetic and spectroscopic studies of the system (Knowles *et al.*, 1983).

In the present study, the enzyme was treated at pH9.0 with benzylamine (final concn. 5.0mM) in solutions saturated either with air (0.25mM-O_2) or with O_2 gas (1.25mM-O_2). Transient and steady-state release of NH_3 during the reactions was followed by quenched-flow techniques combined with a radiochemical assay for NH_3 detection. Data thus obtained are given in Figs. 1–4 and are compared with simulated curves describing the expected time course for NH_3 release in the different reaction steps of Scheme 3. These curves were calculated by analytical methods (Pettersson, 1978), with the use of previously reported estimates of the apparent first-order rate constants defined in Scheme 3 (Table 1).

The experimental results in Figs. 1–4 establish that there is a transient burst of NH_3 release before the attainment of a steady-state rate of NH_3 production. The amount of NH_3 liberated in the burst agrees closely with the amount of enzyme used, and the rate of the transient process appears to be affected strongly by variation of the O_2 concentration. This was confirmed by regression analysis of the experimentally obtained data, which showed that NH_3 release occurs in an exponential transient burst with a rate constant of

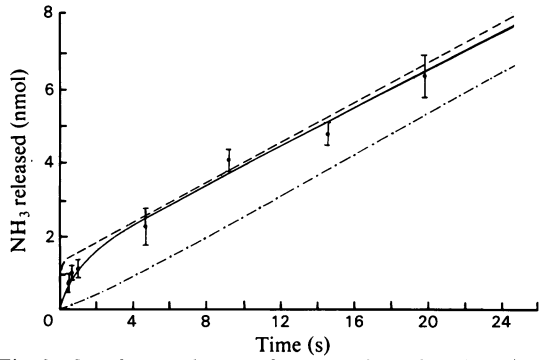


Fig. 2. *Steady-state kinetics for NH_3 release from benzylamine catalysed by pig plasma amine oxidase under air-saturating conditions at pH9.0 at 25.0°C*

The error bars represent limits of the standard deviations from assays on six samples at each time point. ----, Simulation for release of NH_3 in step 1 of Scheme 3; ——, simulation for release of NH_3 in step 2 of Scheme 3; -·-·-, simulation for release of NH_3 in step 3 of Scheme 3.

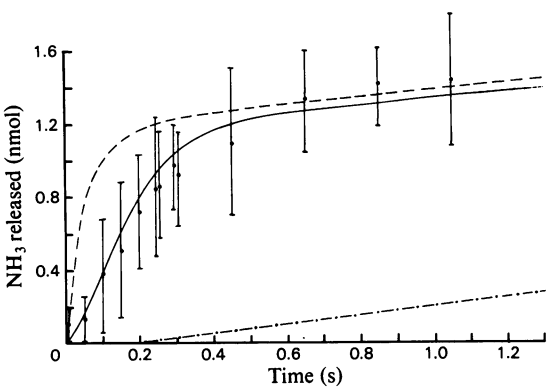


Fig. 3. *Transient-state kinetics for NH_3 release from benzylamine catalysed by pig plasma amine oxidase under O_2-saturating conditions*

Reaction solutions contained 1.19nmol of enzyme. For further details see the legend to Fig. 1.

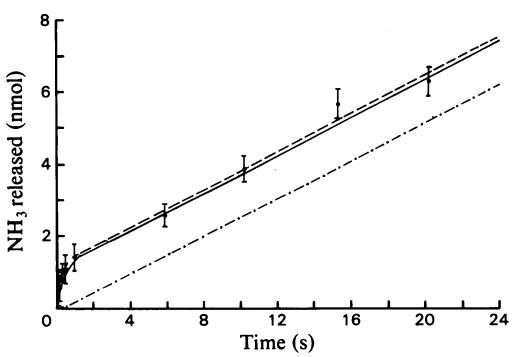


Fig. 4. *Steady-state kinetics for NH_3 release from benzylamine catalysed by pig plasma amine oxidase under O_2-saturating conditions*

For further details see the legend to Fig. 2.

Table 1. *Kinetic data for pig plasma amine oxidase*

Apparent first-order rate constants based on Scheme 3 for full reaction of enzyme with benzylamine (final concn. 5mM) at pH9.0 in solutions equilibrated with air or O_2 are shown.

	Rate constant (s^{-1})		
	Air	O_2	Reference
k_1	20	20	Lindström *et al.* (1976)
k_2	1.6	8.0	Olsson *et al.* (1977)
k_3	0.23	0.23	Olsson *et al.* (1977)

$1.0(\pm 0.3)s^{-1}$ and $5.1(\pm 1.9)s^{-1}$ respectively in air- and O_2-saturated solutions; the difference between these values is statistically significant at the 99.9% level of confidence. Estimates of the burst amplitude obtained correspond to transient release of 0.9–1.0mol of NH_3/mol of enzyme, consistent with other published data showing that the enzyme contains a single active site (Lindström & Pettersson, 1978*a*).

The observation that NH_3 is produced at an O_2-dependent transient rate provides qualitative evidence that catalytic NH_3 release is preceded by O_2 binding to the enzyme. This can be inferred also from the actual rate of the transient, which is far too low (particularly under air-saturating conditions) to reflect the rapid process of enzyme reduction by substrate (Table 1). The fact that transient-burst kinetics obtain excludes the possibility of NH_3 release in (or after) the rate-limiting step of the catalytic reaction. These implications of the present results lead to the conclusion that NH_3 must be released in the second step of Scheme 3, i.e. in the O_2-dependent step of the enzyme re-oxidation process. Confirmatively, rate constants now reported for transient NH_3 release agree well with those determined for transient H_2O_2 release in step 2 [$1.9s^{-1}$ (air); $5.5s^{-1}$ (O_2); Lindström & Pettersson, 1978*b*] as well as with the k_2 values given in Table 1; the latter values are based on stopped-flow studies of the 470nm-absorption changes associated with re-oxidation of the reduced enzyme species (Olsson *et al.*, 1977).

The excellent consistency between previously reported kinetic data and the present results is borne out by the simulated data in Figs. 1–4. It is immediately apparent from the Figures that release of NH_3 in the second step of Scheme 3 provides the best fit to the experimentally obtained data. *F* tests were applied to obtain an objective view of these observations (Table 2) (Sterling & Pollack, 1968). Considering, first, O_2-saturating conditions, there are 101 observations covering the transient and steady-state data. The *F*-test parameter of 2.06 excludes NH_3 release in step 1 relative to step 2 at the highest level of significance

Table 2. *F tests on the NH_3-release data*
Q_i denotes the sum of squares of deviations between observations and values predicted for NH_3 release in step i of Scheme 3 according to the data in Table 1. The units of Q_i are nmol^2. F denotes the F-test parameter.

	Air-saturating	O_2-saturating
Q_1	41.49	22.59
Q_2	10.81	10.97
Q_3	64.4	93.3
$F = Q_1/Q_2$	3.84	2.06

(99.9%). In the case of data for air-saturating conditions (87 observations), the F-test parameter of 3.84 is even more convincingly in favour of NH_3 release in step 2.

It may be noted that all simulations are based on the assumption of one active site per enzyme molecule. The quality of the fit confirms the validity of this assumption.

Discussion

The agreement between the rate constants for alkaline hydrolysis of *p*-nitrophenyl acetate as determined by quenched-flow and stopped-flow methods give confidence that the quenched-flow data for NH_3 release during the catalytic cycle of pig plasma amine oxidase are reliable.

The data presented for NH_3 release have been assessed rigorously by statistical methods and provide unambiguous evidence in favour of an aminotransference mechanism for the pig plasma enzyme. Thus, considering the results with respect to Scheme 3, it has been shown that NH_3 is released at a rate corresponding to literature values for k_2, and moreover that the rate is O_2-dependent. These facts establish that NH_3 release occurs in the step whereby reduced enzyme is oxidized, as predicted by the aminotransferase mechanism (Scheme 1). This step has previously been identified as the one leading to catalytic H_2O_2 formation (Lindström & Pettersson, 1978*b*), and the data now reported indicate that NH_3 and H_2O_2 are released at indistinguishable rates. Formation and dissociation of these products therefore appear to be rate-limited by the interaction of reduced enzyme with O_2. No precise information is yet available as to the nature of the third reaction step in Scheme 3, which is rate-limiting for the overall catalytic reaction at pH9. The present finding that NH_3 is released in step 2 provides the interesting inference that step 3, which is kinetically first-order, represents a slow step of enzyme isomerization, suggesting that structural re-arrangements of the enzyme may play an important role in the catalytic process. Alternatively, step 3 could involve water consumption or production.

Release of NH_3 in the first step of Scheme 3 (reduction of enzyme by amine substrate) as predicted by the imine mechanism (Suva & Abeles, 1978) is definitely excluded by the qualitative and quantitative information provided by the results in Figs. 1–4. Berg & Abeles (1980) presented evidence that stoichiometric amounts of NH_3 are released when amine oxidase from bovine plasma is reduced by amine substrate under anaerobic conditions. A similar claim for anaerobic NH_3 release in the case of the pig plasma enzyme was made by Taylor *et al.* (1972), but a later study by Lindström & Pettersson (1978*b*) established that only aldehyde product was formed under these conditions; Lindström & Pettersson (1978*b*) showed that blank reactions involving enzymic and/or non-enzymic reactants lead to apparent release of NH_3 that was not associated with substrate consumption and that occurred also when benzylamine was incubated under anaerobic conditions with the phenylhydrazine-inactivated derivative of the pig plasma enzyme. Until similar control experiments for NH_3 release are reported for the bovine plasma enzyme, therefore, the evidence presented by Berg & Abeles (1980) cannot be accepted as convincing. It should be noted that NH_3-release data reported in the present paper were obtained by using a calibration curve that allows for blank reactions deriving from the initial presence of trace amounts of NH_3 in the reactant solutions.

It would be speculative to draw more detailed conclusions on the catalytic mechanism of pig plasma amine oxidase from the present data. However, the presence of pyridoxal phosphate (Blaschko & Buffoni, 1965) or some other carbonyl-group cofactor (Hamilton, 1981) in the enzyme is supported by the main conclusion that an aminotransferase mechanism is operative. Detailed chemical proposals for the catalytic process based on such a mechanism have been made by Yadav & Knowles (1981) and Hamilton (1981).

F. X. R. was supported by a fellowship awarded by the CIRIT of Catalonia, Spain. The research was funded by the Science and Engineering Research Council (to P. F. K.) Preliminary studies (Knowles *et al.*, 1983) on this project were carried out in collaboration with Dr. K. D. S. Yadav, Dr. J. Peters, Dr. D. J. Lowe and Dr. R. N. F. Thorneley at the Agricultural Research Council Unit for Nitrogen Fixation, University of Sussex, Brighton, U.K.; many helpful discussions with these colleagues are acknowledged.

References

Barker, R., Boden, N., Cayley, G., Charlton, S. C., Henson, R., Holmes, M. C., Kelly, I. D. & Knowles, P. F. (1979) *Biochem. J.* **177**, 289–302

Barman, T. E. & Gutfreund, H. (1964) in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Eisenhardt, R. H., Gibson, Q. H. & Longerg-Holm, K. K., eds.), pp. 339–343, Academic Press, London and New York

Berg, K. A. & Abeles, R. H. (1980) *Biochemistry* **19**, 3186–3189

Blaschko, H. & Buffoni, F. (1965) *Proc. R. Soc. London Ser. B* **163**, 45–60

Grant, J., Kelly, I. D., Knowles, P. F., Olsson, J. & Pettersson, G. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1216–1224

Hamilton, G. A. (1981) in *Copper Proteins* (Spiro, T. G., ed.), pp. 205–218, John Wiley and Sons, New York

Kalb, V. F., Donohue, T. J., Corrigna, M. G. & Beruldur, R. W. (1978) *Anal. Biochem.* **90**, 47–57

Knowles, P. F., Lowe, D. J., Peters, J., Thorneley, R. N. F. & Yadav, K. D. S. (1983) in *The Co-ordination Chemistry of Metalloenzymes* (Bertini, I., Drago, R. S. & Luchinat, C., eds.), pp. 159–176, Reidel, Dordrecht

Lindström, A. & Pettersson, G. (1978*a*) *Eur. J. Biochem.* **83**, 131–135

Lindström, A. & Pettersson, G. (1978*b*) *Eur. J. Biochem.* **84**, 479–485

Lindström, A., Olsson, B., Olsson, J. & Pettersson, G. (1976) *Eur. J. Biochem.* **64**, 321–326

Olsson, B., Olsson, J. & Pettersson, G. (1977) *Eur. J. Biochem.* **74**, 329–335

Olsson, B., Olsson, J. & Pettersson, G. (1978) *Eur. J. Biochem.* **87**, 1–8

Pettersson, G. (1978) *Acta Chem. Scand. Ser. B* **32**, 437–476

Sterling, T. D. & Pollack, S. V. (1968) *Introduction to Statistical Data Processing*, Prentice-Hall, Englewood Cliffs

Suva, R. A. & Abeles, R. H. (1978) *Biochemistry* **17**, 3538–3545

Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954) *J. Biol. Chem.* **208**, 654–661

Taylor, C. E., Taylor, R. S., Rasmussen, C. & Knowles, P. F. (1972) *Biochem. J.* **130**, 713–728

Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421

Yadav, K. D. S. & Knowles, P. F. (1981) *Eur. J. Biochem.* **114**, 139–144

Yasunobu, K. T., Ishizaki, H. & Minamiura, N. (1976) *Mol. Cell. Biochem.* **13**, 3–29